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The Structure of Myostatin:Follistatin 288: Insights into Receptor Utilization and Heparin Binding

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(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 09 March 2009

Thank you very much for submitting your research manuscript for consideration to The EMBO Journal editorial office. I received three rather encouraging reports from scientists working in the field that you will find attached below. All three referees indicate strong interest in your study, though depending on further modifications that will have to involve additional experimentation. As all the reports are rather self-explanatory, there is not much reason for me to repeat them here in very much detail. Most importantly, the pre-helix region defining type I receptor specificity is currently solely supported by the activin/myosin fusion. As demanded by both ref#2 and #3 further functional verification by mutating structurally identified key amino-acid residues would be essential to solidify the current conclusions. Conditioned on this, we are happy to invite submission of a revised version that also addresses the rather considerate and constructive comments from our referees.

I do also have to remind you that the decision on acceptance or rejection still depends on the content of the final version of your manuscript and that it is EMBO Journal policy to usually allow a single round of revisions only.

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

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Cash and coworkers provides the X-ray structure of myostatin in complex with a C-terminal truncated form of follistatin, Fst-288, at 2.15 Å. This structure is significant and worthwhile reporting in EMBO because it reveals unique features of myotstatin and its complex with its antagonist follistatin that should facilitate the design of novel myostatin antagonists for use in the treatment of muscle wasting disorders.

First, it represents the only structure thus reported for myostatin, a critical negative regulator of muscle growth. Though the overall fold is shown to be similar to that of other TGF-beta superfamily members, there are important unanticipated differences in the pre-helix region, shown previously to be an important specificity determinant for type I receptor binding. This is significant since the structure, shown to be most similar to that of TGF-b3, provides a rationale explanation for observation that myostatin signals through the TGF-b type I receptor, Alk5. To support this, the authors show that substitution of myostatin's pre-helix loop into activin enables it to signal through Alk-5; though not direct, this nevertheless provides a convincing gain-of-function argument in support of their model.

Second, their data shows that while the overall manner of antagonism observed with follistatin is similar to that observed with activinA, there are nevertheless important differences, namely a tightening of the interaction between follistatin's N-terminal domain (ND) and the N-terminus and likely type I receptor binding site of the ligand.

Third, it explains how myostatin enhances follistatin's propensity (particularly Fst-288 and Fst-315), to interact with heparin, hence providing an explanation for the lack of myostatin:Fst-288 (and myostatin:Fst-315) and complex in serum.

Overall, this study provides some interesting new information regarding specificity determinants for (type I) receptor binding in the TGF-beta superfamily, but as well how follistatin has evolved to specifically bind activin and myostatin, but not BMPs or TGF-betas. It also provides new insights as to some of the fine tuning that follistatin may have evolved to regulate ligand activity, such as electostatic repulsion to moduate its affinity for myostatin and differential affinities of the different Fst isoform:myostatin complexes for heparin.

One minor criticism of the manuscript is that it discusses extensively how myostatin might bind Alk5, but it says little or nothing about Alk4, myostatin's other reported type I receptor. Though to some extent understandable due to the lack of direct structural data for Alk4, some biochemical data has been published. In light of this, it would be worthwhile if the authors were to summarize how myostatin and activinA might be similar or different for binding this shared type I receptor.

One other minor point is that the authors suggest on page 13 that the myostatin:Fstl3 complex is likely to have a lower propensity to interact with heparin than the Fst315-myostatin or Fst288-myostatin complexes (based on prior results obtained with activin in complex with these different Fst isoforms). Though reasonable, why was the heparin binding affinity of the Fstl3-myostatin complex not tested and reported as part of Fig. 7?

One last minor point, it would be helpful if the authors were to color code their labels to match the structures to which they refer (such as in Fig. 2B and 3C).

Referee #2 (Remarks to the Author):

Reviewer report on EMBOJ-2009-70618 entitled „The Structure of Myostatin:Follistatin 288: Insights into Receptor Utilization and Heparin Binding" by J.N. Cash, A.C. McPherron and T.B. Thompson.

The manuscript describes the crystal structure analysis of the myostatin:follistatin complex. GDF-8 or myostatin, a member of the TGF-β superfamily, has been identified as a key element in the regulation of muscle growth. Animals with deactivating mutations in GDF-8 or mice having null mutations in the GDF-8 gene exhibit a muscle hypertrophy phenotype showing that GDF-

8/myostatin acts as a negative regulator of muscle growth. The increase in muscle is due to an elevated number as well as increased thickness of the muscle fibers. Knockout of myostatin in mice doesn't seem to have other deleterious effects. The very specific regulation of muscle growth by myostatin has fueled the idea of myostatin being an ideal target for therapeutic intervention in muscle disorder diseases, such as muscular dystrophies, e.g. Duchenne, or motor neuron diseases, e.g. amyotrophic lateral sclerosis.
Follistatin is a modulator protein, which is known to interact tightly with Activins thereby regulating Activin activity in vivo. Besides Activin it can also bind some BMPs, but weakly and thus its importance for BMP activity is not completely clear. Overexpression of follistatin in transgenic mice leads to a similar phenotype as observed for the myostatin-null mice and thus shows that follistatin is an important regulator of myostatin as well.
The study presented here now yields the first insight into the interaction of myostatin bound to its important antagonist follistatin. On the basis of the structure binding determinants in myostatin for the TGF-β type I receptor TβR-I (Alk5) could be identified. The prehelix region, which has been identified as region important for type I receptor specificity in BMPs before, has similarity to the TGF-β ligands and a chimera of this segment of myostatin and Activin-A showed indeed enhanced signaling through Alk5 in a cell-based assay. A detailed description of the differences between the complex structures of Activin and myostatin bound to follistatin provides insights into how follistatin recognizes both ligands and how binding specificity is obtained. The findings are important as their knowledge might facilitate the design of myostatin-specific inhibitors, which could be used for the treatment of muscle disorders. The overall technical quality is sound and the conclusions drawn seem justified, however a several minor and major points require revision.

Minor points:

1.) The introduction section contains several generalizations that are partly incorrect or unclear:
- In the description of the three TGF-β classes the subgroup of GDFs is missing.
- The authors refer to the ligands as homo- and heterodimers, there is only very limited evidence for naturally occurring heterodimers (mainly Dpp/Scw in Drosophila and bovine OP-1/BMP-2 heterodimer isolated by Sampath et al. 1990), most heterodimer studies were done with the heterodimer produced recombinantly. Thus heterodimers seem rather the exception than the rule as implicated here. In addition no references are given.
- The sentence "Receptor specificity exists for the three classes of TGF-β ligands" is incorrect when used in general. One of the features of the TGF-β family is that the ligands can utilize many receptors with rather limited specificity.
TGF-βs bind besides Alk5 also Alk1, BMPs and Activins bind Activin type II receptors, myostatin can bind Alk5 and Alk4. Thus only very few receptors are highly specific to one TGF-β ligand subclass or even specific for one particular ligand.
- Binding locations of type I and type II receptor are termed as concave and convex site, which is rather imprecise, as the binding of the TGF-β type I receptor on the concave site of TGF-β is different from that of the BMP type I receptors binding to BMPs. The differences should be noted here, especially since this is a paper presenting and comparing structural data.
- The authors refer to myostatin belonging to the Activin class (page 3 and 4). The authors should provide evidence why they classify myostatin as an Activin-like TGF-β member; the reference at the end of the sentence does not make this classification. Blast searches show myostatin to have the highest similarity to BMP-6, not Activin. The sequence identity to Activins is on the same level (between 38 to 42%) as for a number of TGF-β ligands.
- It's unclear why only the structure of Fst288 bound to Activin is described and referenced as the other complexes reveal the same principle how follistatin antagonizes Activin-A.
- The binding affinities of Fst288 to Activin-A and myostatin are stated as 1.72 and 12.3nM. The authors should be aware of accuracy provided by the SPR method and rather state reasonable numbers without two digits non-existing accuracy.
2.) Results section, the authors refer to their complex structure as to the first structure of myostatin. This is certainly correct, but especially with respect to the flexibility seen for Activin-A in structures of different complexes and on its own, the authors should not cause the misimpression that the myostatin structure is a structure of the free myostatin or that the structure of the follistatin-bound and free myostatin are unconditionally identical.
3.) Results, page 5. The authors compare myostatin to Activin-A and TGF-β3 stating that the rmsd for the Calpha atoms is 1.6 and 2.7Å respectively. A reference is given for the program used to

calculate the rmsd, the really important information, which regions were used for the superposition, how many atoms were used for the rmsd calculation, is however missing.
No information is provided which structures of Activin-A and TGF-β3 have been used, neither PDB entry codes nor literature references. Thus the rmsd numbers are completely useless for the reader. More details how the analysis was performed, which structures and what part of these structures have been used for fitting, must be provided. A figure showing the structural overlay of these structures should be given along with an rmsd plot of the fit, which allows to see what regions really deviate and which parts superimpose.
Since the structures of TGF-β ligands are sufficiently similar, one does not need to use CE and can do a structural comparison with a much simpler fitting program, such as Pymol, Insight, Coot, etc.
4.) Results, page 6: GDF-11 and GDF9 also utilize Alk5 but are not used in the sequence analysis here.
5.) Results, page 6: The authors state that the amino acid sequences of myostatin and Activin-A are very similar. I think a 40% sequence identity does not justify this statement especially considering the large sequence differences in the putative type I and type II receptor binding sites of both TGF-β ligands. GDF-11 and myostatin can be considered highly similar, similarity between all other ligands is on a much lower level.
6.) Results, page 6: Given the very clear sequence alignment (similar alignments have published earlier) I think it is not a big surprise that the prehelix region of myostatin resembles that of TGF-β. Myostatin and TGF-β have amino acid deletions in this region, whereas Activin-A has additional residues compared to the BMPs. The author should make their point without to much ballyhoo.
7.) Results, page 6: What is meant with elongated dimer conformation for TGF-βs?
8.) As above, the rmsd analysis (Figure 2) lacks information which residues were involved in the comparison, a plot rms deviation vs. residue would be more informative.
9.) Results, page 7: How do the authors explain binding of Alk5 to myostatin, if binding of Alk5 to TGF-β3 is strictly dependent on the presence of the TGF-β type II receptor. Thus binding of the Activin type II receptor onto myostatin could be similar to TβR-II, which is unlikely or Alk5 binding to myostatin differs from TGF-β3. Somehow an explanation how this conflicting binding data can be interpreted is missing.
10.) TGF-β should be used as a control in the reporter gene assay to test Alk5 signaling.
11.) Results, page 7: "In addition of being the first structure of myostatin, this is the highest resolution structure complete Fst structure to date. First it is Fst288, not the complete Fst315, second it is a single structure, not an independent structure of free myostatin and one of the complex.
12.) Results, page 8: The program to do the buried surface analysis is not referenced. This is necessary as different programs yield different solvent areas. Additionally no parameters are given to whether this is the solvent accessible area or the van der Waals area. It is not mentioned whether the buried area indicated is of both (myostatin/Activin and the Fst modules) or only of one molecule.
13.) Results, page 9: The authors state that Fst288 forms a more open conformation in the Fst288:myostatin complex. From reading the manuscript I got the impression that Fst288 itself is the cause of the different Fst288 conformation. However going back to the literature and structure data available for Activin, I find Activin adopting rather different dimer architectures (different inter-monomer angles). Since there is no comparison or figure provided (other than figure 4, which does not provide detailed information for that purpose) showing a structural overlay of myostatin and Activin-A, could it be that the dimer architecture of myostatin and Activin-A is different and this causes the difference in the Fst domain orientation?
14.) Results, page 10 and figure 4: In figure 4A the area on Fst ND next to the helix is colored in yellow, although in panel 4C the helix of myostatin (in green) seems much closer. How was that analysis done?
15.) Results, page 10: in figure 5A the transparent surfaces on both panels (left and right) do not cover the myostatin loop in grey. With respect to the figure legend, should not the loop be wrapped in the transparent surface in one of the two panels?
16.) Results, page 11: "bent rotamer" please specify.
17.) Results, page 12: No parameters provided for the electrostatic potential calculation, e.g. ionic strength, force field, protonation, etc.
18.) Discussion, page 15: "Type II receptor binding sites are conserved within classes of TGF-β family ligands..." should be clearer " Type II receptor binding sites are conserved within the three different classes of TGF-β family ligands..".
"The type I receptor binding sites are significantly more diverse due to the variability of the prehelix regions." I disagree, as the type II receptors either bind at the classical "knuckle" epitope (Activin and BMPs) or at the finger tips (TGF-βs), whereas the type I receptors seem always to bind at the or

close to the concave site also termed the "wrist" epitope, although the location and especially the orientation of the TGF-β type I receptor differs in the TGF-β ligand receptor complex from that of BMP type I receptor IA bound to BMP-2.
19.) Discussion, page 15: On-rates, reference missing.
20.) Discussion, page 16: "Conversely, it is possible that the types of binding experiments (surface plasmon resonance) done ion this subject are not sufficient to properly test the affinity of Fst for these ligands." In my opinion it is bad to discredit a technique, when results from the same method were used elsewhere in the manuscript to make the point.
21.) Materials and methods, page 18: The final crystallization conditions yielding the crystal used for data acquisition should be provided and not a range.
- Residues, which were observed in the electron density are mentioned and indicated by chain C/chain D without information what chain D and C are.
- The structure data is not deposited in the PDB! This is a requirement for publication in EMBO journal.

General minor points:
Referencing is poor throughout the manuscript, often statements are incompletely referenced, and reference style is not fully consistent. The lack of complete referencing becomes most apparent on structure data (but is not limited to!), several structures are not cited, or cited structures and "their" references do not match, e.g. figure legend of figure 5. Whereas this is matter of avoidable negligence when just older work is not cited properly, it severly impairs the comprehensibility if comparisons cannot be reproduced, since it is unclear which data the authors have used. If it is a matter of space limitations, there are several recurrences and "padding" sentences that can be removed.
There are also a few anthropomorphic fallacies, which should be rephrased. E.g. page 11: "The TGF-β antagonist is able to alter itself in order to act differently...", "the ND...sometimes remodeling itself to form alternate...".

Major points:
A major limitation of this study is the lack of functional data to support many of the conclusions made. The authors propose that type I receptor specificity is confined to the prehelix region but provide only an Activin/myostatin chimera to prove their suggestions (Page 7). However, no further analysis via single amino acid mutations for myostatin or Alk5 has been done. No direct binding measurement/analysis of myostatin to its type I or type II receptors is provided.
Different regions and residues are proposed on the basis of the structural comparison to explain the specificity of follistatin for myostatin and Activin (Page 10), but whether these differences seen in the structures really contribute to the specificity is not shown!
I can image that a mutagenesis study in combination with a binding/interaction analysis is not as easy as in other examples published, given the fact that a mammalian expression system is time-consuming for that particular purpose. Nevertheless, using their Activin/myostatin experimental setup or doing immunoprecipitations a functional study can be done also in this case yielding important data to support or refute their conclusions.

Referee #3 (Remarks to the Author):

Although the first crystal structures of a transforming growth factor-β superfamily ligand (TGF-β2) were reported nearly seventeen years ago, until now the structure of one of the most biologically significant members, myostatin or GDF-8, had remained undetermined. With this paper, the authors provide insight into the structural basis of the unusual selectivity of type I receptor binding observed for myostatin, as well as the differential interactions of myostatin and the related ligand activin with antagonists of the follistatin family. In addition, an unanticipated feature of the myostatin:follistatin complexes was revealed that sheds light on the distribution of the ligand and an unrecognized level of regulation of signaling. The senior authors are experts in the field, as McPherron initially identified myostatin (Nature, 1997) and Thompson was the first to determine the crystal structures of activin in complex with receptor extracellular domains and with follistatin (Embo J., 2003; Dev Cell, 2005). The lead author here (Cash) produced a high quality structure from a remarkably high

resolution data set for a protein:protein complex of this size and followed up with sound functional analyses. The only shortcoming of any consequence stems from the lack of a slightly more thorough analysis of the role of a determinant of type I receptor selectivity identified in the study.

Major criticism

1) Analysis of prehelix segment swap (Figure 2F). Much of the impact of the paper stems from the identification of this structural element, which should therefore be reasonably explored. At the very least, response from cells transfected with a wildtype myostatin construct (-/+ Alk5) should have been assayed in parallel to determine the extent of Alk5 binding activity conferred on the activin variant relative to myostatin, i.e. is the chimera as active as myostatin due to the swapped segment, or do other features unique to myostatin contribute to the broader selectivity? A parallel experiment with co-transfection of Alk4, as performed in the initial analyses from the Wrana and Attisano Laboratories, would have been insightful but is perhaps being reserved for a future paper on crystal structures of myostatin in complex with the extracellular domains of the two type I receptors.

Minor criticisms

1) Nomenclature: TBRII > T(beta)RII As in ligand, use of Greek symbol for beta would be advisable rather than capital letter B (employed initially by Massague, Wrana and others).

2) p.3 In reference to Rebbapragada et al. (2003), perhaps out of caution a clause could be inserted to point out that thus far signaling through Alk5 by myostatin has been observed in transiently transfected mammalian cells. Other evidence since has been confined to tissue culture or relied on small molecules that inhibit both Alk4 and Alk5 kinases. Although the broad selectivity is likely to be documented in other contexts, TGF-β superfamily ligand-receptor specificity was and to some extent still is rather misinterpreted due to cell-based assays and the task of rectifying the proper pairings has fallen on the shoulders of structural biologists.

3) p.4 Perhaps a sentence could be introduced to reference the recent paper from Kavita Arora: "Follistatin preferentially antagonizes activin rather than BMP signaling in Drosophila". Pentek J, Parker L, Wu A, Arora K. Genesis. 2009 Feb 23. [Epub ahead of print]. Along with the caveat that follistatin binds BMPs weakly, this reference would bolster the argument that myostatin and activin are the principle binding partners, thus identification of the structural features that lead to differential binding is an important goal (this paper).

4) p.5 cysteine knot motif > cystine knot motif. Cystine = disulfide linkages that create the knot in the secreted, oxidized ligand.

5) p.6 "There is actually significant structural variability in this region within activin A itself". Perhaps here and throughout, the perceived and real roles of the prehelix segment can be elaborated on and driven home. On the one hand, these segments also adopt variable comformations both within and between BMP dimers, as well as between complexes. Because of the apparent flexibility of these polypeptide segments as judged by high B-factor values, the conformations of these loops in models have all too often been over interpreted. On the other hand, this paper, and a recent Embo J. paper on the crystal structure of a GDF-5 complex (Kotzsch et al., 2009), have shown that this segment plays an important role in conferring type I receptor specificity. "Crystal structure analysis reveals a spring-loaded latch as a molecular mechanism for GDF-5-type I receptor specificity". Kotzsch A, Nickel J, Seher A, Sebald W, Müller TD. EMBO J. 2009 Feb 19. [Epub ahead of print]

6) p.6-7 "This led us to hypothesize that the prehelix loop of myostatin is a determinant for signaling through Alk5". Although true, the statement is somewhat misleading since the determinant acts passively through relief of steric hindrance, rather than actively as a component of a binding epitope. Perhaps clarification here and elsewhere would be beneficial to the ability of the reader to more fully grasp the concept.

7) p.7 and elsewhere. Alk5's: Use of apostrophes to denote possession would be appropriate in laboratory discussion, but perhaps not in a formal text description. May be at the discretion of the copy editor.

8) p.7 "This led us to conclude that the prehelix loop of myostatin is (at least in part) responsible for its ability to signal through Alk5, as we predicted". As pointed out in the single major criticism, without the wildtype myostatin control assayed in parallel, the issue of necessary or sufficient remains unresolved, hence the interpretation should be qualified, unless of course the control is eventually included and shows that the swapped segment is sufficient.

9) p.9 "...due to the flexibility of the ligand, which is likely to be lacking in myostatin". Because no other crystal structure of myostatin has been determined, no evidence supports this conjecture, which actually runs contrary to the documented behavior of the most closely related ligand activin. Perhaps advisable to simply speculate "may".

10) p.10 Here and subsequently in the discussion, perhaps the energetic cost of induced fit could be mentioned to account for the order of magnitude lower affinity of follistatin for myostatin relative to activin. Might this also contribute to the strongly diminished affinity for BMPs? Induced fit mechanisms have been shown to be as prevalent as rigid body, hence ought not be ignored here.

11) p.12 "...more suited to interact with myostatin than activin A due to this interface". Disconcerting if not qualified with "Nevertheless the affinity for myostatin is lower, hence despite the more suitable interaction, other aspects of binding appear to play an important role" or something to that effect.

12) p.12, bottom of page- on and off rates in support of role of electrostatics in binding affinity. Wouldn't these same rates be consistent with an induced fit mechanism or contribution thereof?

13) p.13 If myostatin enhances Fst288/Fst315 binding, doesn't myostatin alone also bind? If so, then wouldn't myostatin:Fstl3 be expected to bind? Perhaps the basic surfaces of the myostatin dimer are insufficient. Have myostatin:heparin interactions been described elsewhere and could this conundrum be discussed?
14) p.14 "...whether or not Alk5 actually interacts more strongly with myostatin itself than it does with TGF-β." Indeed, Alk5 may interact more strongly with myostatin than TGF-β, because abolishing TβRII: TβRI (Alk5) interaction abolishes assembly and signaling, indicating that TGF-β binding to Alk5 alone is negligible and requires cooperative recruitment, whereas in the myostatin signaling complex, the type II and type I receptors likely do not interact directly, hence the ligand-type receptor interaction may be greater. Along those lines, even weak interactions could be compensated for by a membrane-mediated mechanism, as postulated for BMP assembly (Greenwald et al., 2003; Sebald, Mueller Review, 2003), which may also be noteworthy here.

15) p.15 "Type II receptor sites are conserved within classes of TGF-β ligands,...". Although the point is understood, the statement is inaccurate because the TGF-β site is literally and figuratively an outlier.

16) p.15 "Fittingly, the ND of Fst288 uses this site as a specificity determinant...". Perhaps more conservative to describe as "appears to use", in keeping with the last sentence of the paragraph?

17) p.15 This paragraph may be an appropriate venue for mentioning broader concepts, such as the role of the prehelix segment as a switch (Kotzsch et al., 2009) and of the role of short loops or extensions, which evolutionarily are readily acquired, in conferring altered binding properties. In the TGF-β ternary complex (Groppe et al., 2008), these segments were introduced into the receptors, including Alk5 (and Alk4). On the ligand side, myostatin appears to have lost a segment (or activin gained an insertion, depending on which appeared first). With respect to the antagonists, Noggin acquired an N-terminal extension (Groppe et al., 2002) and Follistatin an N-terminal domain.

18) p.17 "It is also interesting to speculate that the combination of TGF-β family ligands with other antagonists may also create new cell surface binding properties, conferring similar forms of regulation." Although an attractive notion, unfortunately other family members are not good candidates. TGF-β ligands do not bind heparin, nor activin as shown here (and likely neither Nodal), and BMPs bind through a highly basic N-terminal extension that is highly flexible (protease sensitive, not modeled in crystal structures) that could not form a continuous interface, if the implication.

19) p.17 If indeed the implication, wouldn't non-contiguous interactions, for example those provided by the Noggin:BMP complex (both bind heparin with high affinity from opposite surfaces) enhance binding through avidity effects, not as a result of a composite electropositive surface? Maybe the above statement was in reference to such a situation. Regardless, a composite surface such as that in the myostatin:Fst complexes could conceivably yield a composite heparin binding site that, unlike the Noggin:BMP complex, required a contiguous surface for enhanced affinity. Once more, maybe this is indeed the whole point, but if so, wasn't laid out completely enough to be grasped. Moosa Mohammadi at NYU has published stellar structures showing the role of a composite site in the FGF dimer in assembly of the ligand receptor complex that might be worth mentioning in this context.

20) p.20-24 Unfortunately EndNote downloads references from PubMed without the original Greek symbols, thus throughout the references the Greek symbol for beta is corrupted and would require manual editing to restore to the published form.

21) Figure 1 A review panel described in the Introduction, that while helpful to a broad audience, may be more suitable for a Supplementary Figure in the interest of space reduction.

22) Figure 2E Perspective is somewhat misleading, in that the majority of the surface of Alk5 proximal to the prehelix loop is actually raised well above. Also, perhaps the ligands were aligned toward the ends of the fingers, but near the palm the strands do not appear to align at all. If so, the figure again is somewhat misleading, because the disposition of the prehelix loop relative to the superimposed Alk5 extracellular domain may be highly speculative, one of several possible arrangements. Maybe additional views are needed to fairly depict and convince the reader of this very important aspect of the paper.

1st Revision - authors' response 07 June 2009

Thank you for considering our manuscript and for the thoughtful comments from the reviewers. We are submitting a revised manuscript that provides more evidence to support claims made in the original document. If we were not able to provide unequivocal support, we have tried to alter the manuscript to reflect this limitation. We hope that the alterations made to the original document satisfy, at least in part, a significant number of concerns/criticisms of the reviewers, which have been outlined below.

Reviewer #2 Major points & Reviewer #3 Major criticism:

To address the major points of reviewer #2, we have tried to provide additional functional data in support of our claims, but experimental technical limitations hinder our ability to address all concerns. In cases where we cannot unequivocally support our conclusion, we have tried to modify the text to reflect this. This is most apparent with regards to type I receptor specificity. We have included additional supplementary data that clearly shows, through titration of each plasmid, that activin A WT does not effectively signal through Alk5, whereas the activin A prehelix-switch does. We have also done this in parallel with Alk4 and have shown that both activin A WT and activin A prehelix-switch signal robustly through Alk4. To probe this further, we tested single point mutations of individual residues in the prehelix-switch region. Point mutations did not disrupt signaling through Alk5, supporting the idea that the prehelix loop conformation is more important than individual interactions of side-chains with Alk5.

We were unable to perform similar experiments with myostatin WT (as suggested by reviewer #3) or a chimera of myostatin with the activin A prehelix loop because the myostatin plasmid transfection did not yield a strong enough signal even though significant protein was expressed (Reviewer Fig. 1). This is likely due to the fact that the propeptide of myostatin is inefficiently cleaved from the mature segment, and the propeptide remains tightly bound even after cleavage, thus keeping myostatin latent. Attempts to circumvent this by co-expression of furin protease (to process the mature from the propeptide) and several tolloid proteases (to cleave and remove the propeptide itself) to activate myostatin were not successful. This is also likely the reason we have had to use significantly more TGF- plasmid to get reasonable signaling in our luciferase assay, as

TGF- ligands are also subject to inhibition by their propeptide regions. In the paper, we attempt to clarify that we cannot unequivocally claim that the prehelix loop of myostatin participates in a direct interaction with Alk5. Just as reviewer #3 has pointed out, we do not know if this is a product of replacing a section of activin A that does not permit effective alk5 signaling or if this section truly promotes binding.

To resolve this, reviewer #2 is correct that the ideal experiments would include a direct assessment of the activin A prehelix-switch binding to Alk5. Because the type I receptors (Alk4 and Alk5) are the low affinity binding receptors for myostatin and activins, these kinds of experiments are challenging. The experiments typically done in the field include co-expressing type II and I receptors in heterologous cells and performing radio-ligand binding followed by cross-linking and co-IP with antibodies against the type I receptor (or an epitope tag contained therein). As a proof of concept, we co-expressed ActRIIB (a type II receptor for both activin A and myostatin) along with myc-tagged Alk4 or Alk5 in CHO cells. We then performed binding experiments with iodinated myostatin and activin A (the same ligands used in the membrane binding and degradation assays added to the revised manuscript, Reviewer Fig. 2). We confirmed data from the published literature that myostatin can bind complexes of ActRIIB and Alk4 or Alk5 (Rebbapragada et al., 2003, Mol Cell Biol 23(20):7230-42). Myostatin complexed to the type I receptors was confirmed by myc IP. We also observed complexes of activin A with ActRIIB and Alk4. Unexpectedly, we observed activin A in complex with ActRIIB and Alk5. This has not been reported previously or at least not consistently; however, many of the original studies used DSS as the cross-linker, and we typically use BS3. We prefer the latter as it is membrane-impermeable and therefore will only cross-link receptor/ligand interactions at the cell surface. Therefore, our assays were not sufficient to discriminate binding to Alk5. Indeed, our functional data in L17 cells are consistent with the lack of signaling of activin A WT via Alk5. For this reason, we emphasize throughout the manuscript the differential 'signaling' rather than 'binding' of myostatin and activin A to Alk5. Even if our binding assay system was effective (i.e., we did not observe activin A WT binding to Alk5), it has proven difficult to generate sufficient quantities of recombinant activin A prehelix-switch mutant to perform binding studies. Therefore, we have relied on the functional assays in L17 cells to address differences in signaling capacity via Alk5 for the different ligands. Because these cells lack Alk5 expression, we think this provides the best available model for examination of acquired signaling upon ectopic expression of Alk5.

We also agree with reviewer #2 that mutations of follistatin will need to be made and the protein purified to further address the questions of specificity. We would argue that this is not a simple 3-month task and may take an additional 1-2 years to complete and is planned for a future study. However, our structure does provide an explanation to the observation that a follistatin construct of ND-FSD1-FSD1 binds myostatin much more tightly than activin A (Nakatani et al., 2007, FASEB J 22(2):477-487).

To strengthen the conclusions we made about myostatin:follistatin complexes having stronger heparin binding affinity than activin A or unbound follistatin, we have followed up the column chromatography results with biological assays providing evidence that myostatin:follistatin complexes bind more readily to cell surfaces than activin A:follistatin complexes, especially with respect to Fst315. The enhanced myostatin:Fst315 membrane-binding is associated with increased ligand degradation, which is not observed with activin A:Fst315 complex.

We hope that the additional data is sufficient for the reviewers to accept the manuscript for publication. Furthermore, we would like to address each reviewerís comments and suggestions in a systematic fashion.

Reviewer #1:

One minor criticism of the manuscript is that it discusses extensively how myostatin might bind Alk5, but it says little or nothing about Alk4, myostatin's other reported type I receptor. Though to some extent understandable due to the lack of direct structural data for Alk4, some biochemical data has been published. In light of this, it would be worthwhile if the authors were to summarize how myostatin and activinA might be similar or different for binding this shared type I receptor.

This is a great point, and a detailed discussion on how myostatin and activin A may be similar or different for binding Alk4 and Alk5 has been added to the discussion.

One other minor point is that the authors suggest on page 13 that the myostatin:Fstl3 complex is likely to have a lower propensity to interact with heparin than the Fst315-myostatin or Fst288-myostatin complexes (based on prior results obtained with activin in complex with these different Fst isoforms). Though reasonable, why was the heparin binding affinity of the Fstl3-myostatin complex not tested and reported as part of Fig. 7?

This information was discussed on pg 13, and results are shown in Sup. Fig. 3.

One last minor point, it would be helpful if the authors were to color code their labels to match the structures to which they refer (such as in Fig. 2B and 3C).

Labels have been color-coded.

Reviewer #2:

Minor points:

1.) The introduction section contains several generalizations that are partly incorrect or unclear:
- In the description of the three TGF- ; classes the subgroup of GDFs is missing.

GDF has been added.

- The authors refer to the ligands as homo- and heterodimers, there is only very limited evidence for naturally occurring heterodimers (mainly Dpp/Scw in Drosophila and bovine OP-1/BMP-2 heterodimer isolated by Sampath et al. 1990), most heterodimer studies were done with the heterodimer produced recombinantly. Thus heterodimers seem rather the exception than the rule as implicated here. In addition no references are given.

References, including those for in vivo studies, on heterodimers have been included.

- The sentence "Receptor specificity exists for the three classes of TGF- ligands" is incorrect when used in general. One of the features of the TGF- family is that the ligands can utilize many receptors with rather limited specificity.
TGF- s bind besides Alk5 also Alk1, BMPs and Activins bind Activin type II receptors, myostatin can bind Alk5 and Alk4. Thus only very few receptors are highly specific to one TGF- ligand subclass or even specific for one particular ligand.

The authors agree with this. What was meant by our statement is that each ligand/class of ligands utilizes a specific set of receptors for signaling. The text has been altered to clarify this point.

- Binding locations of type I and type II receptor are termed as concave and convex site, which is rather imprecise, as the binding of the TGF- type I receptor on the concave site of TGF- is different from that of the BMP type I receptors binding to BMPs. The differences should be noted here, especially since this is a paper presenting and comparing structural data.

This is a good point about the type I receptor differences, and we have added a more in-depth description of the differences in receptor binding.

- The authors refer to myostatin belonging to the Activin class (page 3 and 4). The authors should provide evidence why they classify myostatin as an Activin-like TGF- member; the reference at the end of the sentence does not make this classification. Blast searches show myostatin to have the highest similarity to BMP-6, not Activin. The sequence identity to Activins is on the same level (between 38 to 42%) as for a number of TGF- ligands.

Our initial classification of myostatin as an activin class member is based more on shared receptor utilization and inhibitors than on sequence identity. The manuscript text has been changed to clarify this.

- It's unclear why only the structure of Fst288 bound to Activin is described and referenced as the

other complexes reveal the same principle how follistatin antagonizes Activin-A.

The authors primarily focus on the activin A:Fst288 structure because it is the most directly comparable available Fst-type complex structure for the myostatin:Fst288 structure. However, in the overall description of how Fst-type molecules antagonize activin A, the other relevant structure references have now been added.

- The binding affinities of Fst288 to Activin-A and myostatin are stated as 1.72 and 12.3nM. The authors should be aware of accuracy provided by the SPR method and rather state reasonable numbers without two digits non-existing accuracy.

Although these are the actual numbers stated in the reference, we have truncated cited SPR values to the tenths place. This is how others appear to report most SPR data.

2.) Results section, the authors refer to their complex structure as to the first structure of myostatin. This is certainly correct, but especially with respect to the flexibility seen for Activin-A in structures of different complexes and on its own, the authors should not cause the misimpression that the myostatin structure is a structure of the free myostatin or that the structure of the follistatin-bound and free myostatin are unconditionally identical.

A clause has been inserted in the results section recognizing the possibility that the structures of free and bound myostatin may not be the same.

3.) Results, page 5. The authors compare myostatin to Activin-A and TGF- 3 stating that the rmsd for the Calpha atoms is 1.6 and 2.7Å respectively. A reference is given for the program used to calculate the rmsd, the really important information, which regions were used for the superposition, how many atoms were used for the rmsd calculation, is however missing. No information is provided which structures of Activin-A and TGF- 3 have been used, neither PDB entry codes nor literature references. Thus the rmsd numbers are completely useless for the reader. More details how the analysis was performed, which structures and what part of these structures have been used for fitting, must be provided. A figure showing the structural overlay of these structures should be given along with an rmsd plot of the fit, which allows to see what regions really deviate and which parts superimpose. Since the structures of TGF- ligands are sufficiently similar, one does not need to use CE and can do a structural comparison with a much simpler fitting program, such as Pymol, Insight, Coot, etc.

More detailed information on how the analyses were done has been included, along with PDB codes. The authors do not see a problem with using CE to do these comparisons and, in fact, believe that this is the best program to use, as structural alignments are primarily what this program was designed to do, unlike Coot and Pymol. However, this analysis was also done in Pymol. For the various alignments Pymol, typically matched only ~85 C , whereas CE was able to match ~105 C . Similar trends were observed for both programs. Structural overlays have been included as Supplementary Figure 1, showing alignments of myostatin with activin A, BMP2, and TGF- 3.

4.) Results, page 6: GDF-11 and GDF9 also utilize Alk5 but are not used in the sequence analysis here.

This sequence alignment includes only a selection of TGF- family ligands, and only those for which structures are available. This is a structure-based sequence alignment, as is stated in the figure legend.

5.) Results, page 6: The authors state that the amino acid sequences of myostatin and Activin-A are very similar. I think a 40% sequence identity does not justify this statement especially considering the large sequence differences in the putative type I and type II receptor binding sites of both TGF- ligands. GDF-11 and myostatin can be considered highly similar, similarity between all other ligands is on a much lower level.

"very similar" has been replaced by "similar"

6.) Results, page 6: Given the very clear sequence alignment (similar alignments have published

earlier) I think it is not a big surprise that the prehelix region of myostatin resembles that of TGF- . Myostatin and TGF- have amino acid deletions in this region, whereas Activin-A has additional residues compared to the BMPs. The author should make their point without to much ballyhoo.

The authors respectfully disagree with these statements, especially in light of the fact that the reviewersí comments suggest that they view this is the major finding of the manuscript. To our knowledge, this comparison has not been previously highlighted, and this has not been previously offered as an explanation for the ability of myostatin to signal through Alk5, even with the ternary TGF- 3 structure (2PJY) in hand. In fact, in looking at non structure-based sequence alignments (which would have to be done in the absence of a myostatin structure) it could be argued that the prehelix region of myostatin would be expected to be most similar to that of BMPs. The authors believe that our prehelix region finding is reasonably emphasized and discussed in the manuscript.

7.) Results, page 6: What is meant with elongated dimer conformation for TGF- s?

A reference to Supplementary Figure 1 (structure overlays) has now been made.

8.) As above, the rmsd analysis (Figure 2) lacks information which residues were involved in the comparison, a plot rms deviation vs. residue would be more informative.

All but the RMSD values for the prehelix regions of TGF- 3 and myostatin have been removed. The residues involved in the analysis were included in the figure legend.

9.) Results, page 7: How do the authors explain binding of Alk5 to myostatin, if binding of Alk5 to TGF- 3 is strictly dependent on the presence of the TGF- type II receptor. Thus binding of the Activin type II receptor onto myostatin could be similar to T R-II, which is unlikely or Alk5 binding to myostatin differs from TGF- 3. Somehow an explanation how this conflicting binding data can be interpreted is missing.

This dilemma was discussed in the discussion section.

10.) TGF- should be used as a control in the reporter gene assay to test Alk5 signaling.

The TGF- WT control has been included in the assay.

11.) Results, page 7: "In addition of being the first structure of myostatin, this is the highest resolution structure complete Fst structure to date. First it is Fst288, not the complete Fst315, second it is a single structure, not an independent structure of free myostatin and one of the complex.

This statement in the manuscript is incorrectly quoted. The authors do not infer that we have multiple structures, we say that within one structure, we have the first structure of a myostatin molecule in addition to the highest resolution complete Fst-type molecule structure to date, also considering Fstl3. Wording has been changed to remove this ambiguity. Fst288 should not be considered an incomplete protein, as it is one of multiple protein isoforms from the same gene, each with distinct biophysical characteristics and hence biological effects.

12.) Results, page 8: The program to do the buried surface analysis is not referenced. This is necessary as different programs yield different solvent areas. Additionally no parameters are given to whether this is the solvent accessible area or the van der Waals area. It is not mentioned whether the buried area indicated is of both (myostatin/Activin and the Fst modules) or only of one molecule.

This information has now been included in the Materials and Methods section.

13.) Results, page 9: The authors state that Fst288 forms a more open conformation in the Fst288:myostatin complex. From reading the manuscript I got the impression that Fst288 itself is the cause of the different Fst288 conformation. However going back to the literature and structure data available for Activin, I find Activin adopting rather different dimer architectures (different inter-monomer angles). Since there is no comparison or figure provided (other than figure 4, which does not provide detailed information for that purpose) showing a structural overlay of myostatin

and Activin-A, could it be that the dimer architecture of myostatin and Activin-A is different and this causes the difference in the Fst domain orientation?

This could certainly be the case. The authors suggested this at the end of the paragraph. We have added an additional statement to summarize and clarify the point: "Therefore, whether Fst288 itself or differences between myostatin and activin A, or possibly both, are the cause of the altered conformation is unclear." We have also added the structure overlay as Supplementary Figure 1A.

14.) Results, page 10 and figure 4: In figure 4A the area on Fst ND next to the helix is colored in yellow, although in panel 4C the helix of myostatin (in green) seems much closer. How was that analysis done?

The area on Fst ND in question is actually buried by the prehelix region of activin, not the major wrist helix of either of the ligands, which is why the area is colored yellow. The figure legend has been clarified as such: "The Fst288 surface is colored according to which ligand buries more surface on Fst288 in specific areas. Fst288 residues that are buried more in the activin A:Fst288 complex are shaded brighter yellow, while those that are buried more in the myostatin:Fst288 complex are shaded brighter green. The strongest shades represent buried surface area differences of up to 45≈2. Areas that are either not buried or buried equally by both ligands are tinted light purple. Differences in buried surface areas were calculated between the two complexes on a per residue basis."

15.) Results, page 10: in figure 5A the transparent surfaces on both panels (left and right) do not cover the myostatin loop in grey. With respect to the figure legend, should not the loop be wrapped in the transparent surface in one of the two panels?

For clarity, only part of the ligand structure is represented as a surface. The fingertip regions are carved out. In both cases, only the transparent residues and not the rest of the surface shown for the corresponding structures. In the left panel of figure 5A, the activin A loop is transparent. In the right panel, the myostatin loop is transparent. Surface representation is shown only for background purposes, and is shown as such in order to make the image less complicated and easier to assess. This has been clarified in the figure legend as such: "Superimposed on myostatin and activin A are the fingertip regions of the other ligand (transparent). The ND helix is shifted closer to myostatinA as compared to activin AA, allowing additional hydrophobic interactions and three hydrogen bonds to be formed at the ND helix:fingertip interface (in both panels shown in cartoon and stick)."

16.) Results, page 11: "bent rotamer" please specify.

The description of the residue to which this refers has been clarified in the text.

17.) Results, page 12: No parameters provided for the electrostatic potential calculation, e.g. ionic strength, force field, protonation, etc.

We have included that default parameters were used for this calculation, and additional information can be found in the figure legend.

18.) Discussion, page 15: "Type II receptor binding sites are conserved within classes of TGF- family ligands..." should be clearer " Type II receptor binding sites are conserved within the three different classes of TGF- family ligands..".
"The type I receptor binding sites are significantly more diverse due to the variability of the prehelix regions." I disagree, as the type II receptors either bind at the classical "knuckle" epitope (Activin and BMPs) or at the finger tips (TGF- s), whereas the type I receptors seem always to bind at the or close to the concave site also termed the "wrist" epitope, although the location and especially the orientation of the TGF- type I receptor differs in the TGF- ligand receptor complex from that of BMP type I receptor IA bound to BMP-2.

The authors agree with this point. However, we were referring more to the residues and conformations of the sites on the ligands rather than to the positions of the sites themselves and how the receptors bind. We have altered the text and made it more specific: "Residues contained within type II receptor binding sites are conserved between myostatin, activins, and several BMPs, and it

seems that Fst288 uses this site similarly between ligands, likely with high affinity. The type I receptor binding sites on the ligands are significantly more diverse due to the variability of the prehelix regions."

19.) Discussion, page 15: On-rates, reference missing.

Reference has been added.

20.) Discussion, page 16: "Conversely, it is possible that the types of binding experiments (surface plasmon resonance) done ion this subject are not sufficient to properly test the affinity of Fst for these ligands." In my opinion it is bad to discredit a technique, when results from the same method were used elsewhere in the manuscript to make the point.

This statement has been removed from the manuscript.

21.) Materials and methods, page 18: The final crystallization conditions yielding the crystal used for data acquisition should be provided and not a range.

This has been corrected.

- Residues, which were observed in the electron density are mentioned and indicated by chain C/chain D without information what chain D and C are.

This has been removed.

- The structure data is not deposited in the PDB! This is a requirement for publication in EMBO journal.

Coordinates have been deposited with the PDB code 3HH2.

General minor points:
Referencing is poor throughout the manuscript, often statements are incompletely referenced, and reference style is not fully consistent. The lack of complete referencing becomes most apparent on structure data (but is not limited to!), several structures are not cited, or cited structures and "their" references do not match, e.g. figure legend of figure 5. Whereas this is matter of avoidable negligence when just older work is not cited properly, it severly impairs the comprehensibility if comparisons cannot be reproduced, since it is unclear which data the authors have used. If it is a matter of space limitations, there are several recurrences and "padding" sentences that can be removed.

The authors have made a concerted effort towards including additional relevant references and making our references more complete and accurate. We have also made our referencing style more consistent. Structures, in particular, have been correctly cited.

There are also a few anthropomorphic fallacies, which should be rephrased. E.g. page 11: "The TGF- antagonist is able to alter itself in order to act differently...", "the ND...sometimes remodeling itself to form alternate...".

Anthropomorphic fallacies have been removed.

Major points:
A major limitation of this study is the lack of functional data to support many of the conclusions made. The authors propose that type I receptor specificity is confined to the prehelix region but provide only an Activin/myostatin chimera to prove their suggestions (Page 7). However, no further analysis via single amino acid mutations for myostatin or Alk5 has been done. No direct binding measurement/analysis of myostatin to its type I or type II receptors is provided.
Different regions and residues are proposed on the basis of the structural comparison to explain the specificity of follistatin for myostatin and Activin (Page 10), but whether these differences seen in the structures really contribute to the specificity is not shown!
I can image that a mutagenesis study in combination with a binding/interaction analysis is not as

easy as in other examples published, given the fact that a mammalian expression system is time-consuming for that particular purpose. Nevertheless, using their Activin/myostatin experimental setup or doing immunoprecipitations a functional study can be done also in this case yielding important data to support or refute their conclusions.

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Reviewer #3:

Major criticism

1) Analysis of prehelix segment swap (Figure 2F). Much of the impact of the paper stems from the identification of this structural element, which should therefore be reasonably explored. At the very least, response from cells transfected with a wildtype myostatin construct (-/+ Alk5) should have been assayed in parallel to determine the extent of Alk5 binding activity conferred on the activin variant relative to myostatin, i.e. is the chimera as active as myostatin due to the swapped segment, or do other features unique to myostatin contribute to the broader selectivity? A parallel experiment with co-transfection of Alk4, as performed in the initial analyses from the Wrana and Attisano Laboratories, would have been insightful but is perhaps being reserved for a future paper on crystal structures of myostatin in complex with the extracellular domains of the two type I receptors.

Please see top of document.

Minor criticisms

1) Nomenclature: TBRII > T(beta)RII As in ligand, use of Greek symbol for beta would be advisable rather than capital letter B (employed initially by Massague, Wrana and others).

This has been changed as per reviewer suggestion.

2) p.3 In reference to Rebbapragada et al. (2003), perhaps out of caution a clause could be inserted to point out that thus far signaling through Alk5 by myostatin has been observed in transiently transfected mammalian cells. Other evidence since has been confined to tissue culture or relied on small molecules that inhibit both Alk4 and Alk5 kinases. Although the broad selectivity is likely to be documented in other contexts, TGF- superfamily ligand-receptor specificity was and to some extent still is rather misinterpreted due to cell-based assays and the task of rectifying the proper pairings has fallen on the shoulders of structural biologists.

The following clause has been inserted: "GDF11, which is 89% identical at the amino acid level to myostatin, also has been shown to signal through Alk5 in vivo (Andersson et al., 2006)."

3) p.4 Perhaps a sentence could be introduced to reference the recent paper from Kavita Arora: "Follistatin preferentially antagonizes activin rather than BMP signaling in Drosophila". Pentek J, Parker L, Wu A, Arora K. Genesis. 2009 Feb 23. [Epub ahead of print]. Along with the caveat that follistatin binds BMPs weakly, this reference would bolster the argument that myostatin and activin are the principle binding partners, thus identification of the structural features that lead to differential binding is an important goal (this paper).

Although true, the Drosophila form of follistatin contains a significantly large insert in FSD1, which could have a large impact on its binding to different ligands. We feel more comfortable keeping these arguments separate until further information is available.

4) p.5 cysteine knot motif > cystine knot motif. Cystine = disulfide linkages that create the knot in the secreted, oxidized ligand.

This has been changed as per reviewer suggestion.

5) p.6 "There is actually significant structural variability in this region within activin A itself". Perhaps here and throughout, the perceived and real roles of the prehelix segment can be

elaborated on and driven home. On the one hand, these segments also adopt variable comformations both within and between BMP dimers, as well as between complexes. Because of the apparent flexibility of these polypeptide segments as judged by high B-factor values, the conformations of these loops in models have all too often been over interpreted. On the other hand, this paper, and a recent Embo J. paper on the crystal structure of a GDF-5 complex (Kotzsch et al., 2009), have shown that this segment plays an important role in conferring type I receptor specificity. "Crystal structure analysis reveals a spring-loaded latch as a molecular mechanism for GDF-5-type I receptor specificity". Kotzsch A, Nickel J, Seher A, Sebald W, Müller TD. EMBO J. 2009 Feb 19. [Epub ahead of print]

We have tried to modify the text some to reflect the fact that the prehelix is important for type I receptor specificity and can adopt multiple conformations. We have also included additional references. Additionally, we have added a disclaimer that myostatin may adopt different conformations in the unbound or receptor bound state.

6) p.6-7 "This led us to hypothesize that the prehelix loop of myostatin is a determinant for signaling through Alk5". Although true, the statement is somewhat misleading since the determinant acts passively through relief of steric hindrance, rather than actively as a component of a binding epitope. Perhaps clarification here and elsewhere would be beneficial to the ability of the reader to more fully grasp the concept.

We agree with the reviewer and have removed the assertion that the prehelix loop is a "determinant" for binding. Instead, we have emphasized throughout that the conformation of this region is an important feature for Alk5 signaling.

7) p.7 and elsewhere. Alk5's: Use of apostrophes to denote possession would be appropriate in laboratory discussion, but perhaps not in a formal text description. May be at the discretion of the copy editor.

Use of apostrophes to denote possession has been removed.

8) p.7 "This led us to conclude that the prehelix loop of myostatin is (at least in part) responsible for its ability to signal through Alk5, as we predicted". As pointed out in the single major criticism, without the wildtype myostatin control assayed in parallel, the issue of necessary or sufficient remains unresolved, hence the interpretation should be qualified, unless of course the control is eventually included and shows that the swapped segment is sufficient.

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9) p.9 "...due to the flexibility of the ligand, which is likely to be lacking in myostatin". Because no other crystal structure of myostatin has been determined, no evidence supports this conjecture, which actually runs contrary to the documented behavior of the most closely related ligand activin. Perhaps advisable to simply speculate "may".

Reviewer suggestion has been taken.

10) p.10 Here and subsequently in the discussion, perhaps the energetic cost of induced fit could be mentioned to account for the order of magnitude lower affinity of follistatin for myostatin relative to activin. Might this also contribute to the strongly diminished affinity for BMPs? Induced fit mechanisms have been shown to be as prevalent as rigid body, hence ought not be ignored here.

A discussion on the possibility of an induced fit mechanism has been introduced in the discussion section.

11) p.12 "...more suited to interact with myostatin than activin A due to this interface". Disconcerting if not qualified with "Nevertheless the affinity for myostatin is lower, hence despite the more suitable interaction, other aspects of binding appear to play an important role" or something to that effect.

This clause has been added.

12) p.12, bottom of page- on and off rates in support of role of electrostatics in binding affinity. Wouldn't these same rates be consistent with an induced fit mechanism or contribution thereof?

A discussion on the possibility of an induced fit mechanism has been introduced in the discussion section.

13) p.13 If myostatin enhances Fst288/Fst315 binding, doesn't myostatin alone also bind? If so, then wouldn't myostatin:Fstl3 be expected to bind? Perhaps the basic surfaces of the myostatin dimer are insufficient. Have myostatin:heparin interactions been described elsewhere and could this conundrum be discussed?

We agree that myostatin alone might have some affinity for heparin and have indicated this possibility in the text. During one particular purification of myostatin (Lee and McPherron, 2001, PNAS 98(16):9306-11) a heparin affinity column was used to bind the propeptide-mature complex and this eluted at low (200mM) salt. It is also possible this is simply an interaction with a cation exchange column. As far as we know this is the only published evidence that myostatin has an affinity for heparin. As noted in the experiment methods, we also purify myostatin with a cation exchange column and elute with a relatively low ionic strength solution. We would expect a stronger interaction with these columns if indeed myostatin were able to bind heparin. We did observe slightly increased levels of cell-surface binding of radio-labeled myostatin versus radio-labeled activin A to the cell surface, which might indicate an increased affinity for heparin sulfate proteoglycans (although receptor binding is also a possible explanation). We have shown that myostatin bound to Fstl3 does not bind to heparin. Therefore, if myostatin did have a low affinity for heparin, it is likely neutralized by an interaction with Fstl3.

14) p.14 "...whether or not Alk5 actually interacts more strongly with myostatin itself than it does with TGF- ." Indeed, Alk5 may interact more strongly with myostatin than TGF- , because abolishing T RII: T RI (Alk5) interaction abolishes assembly and signaling, indicating that TGF- binding to Alk5 alone is negligible and requires cooperative recruitment, whereas in the myostatin signaling complex, the type II and type I receptors likely do not interact directly, hence the ligand-type receptor interaction may be greater. Along those lines, even weak interactions could be compensated for by a membrane-mediated mechanism, as postulated for BMP assembly (Greenwald et al., 2003; Sebald, Mueller Review, 2003), which may also be noteworthy here.

We agree that small changes in receptor affinity might lead to signaling through Alk5 without the need for receptor-receptor interactions. We have added a statement in the discussion of the manuscript to that effect.

15) p.15 "Type II receptor sites are conserved within classes of TGF- ligands,...". Although the point is understood, the statement is inaccurate because the TGF- site is literally and figuratively an outlier.

We have altered this statement to be more specific and convey what the authors actually intended.

16) p.15 "Fittingly, the ND of Fst288 uses this site as a specificity determinant...". Perhaps more conservative to describe as "appears to use", in keeping with the last sentence of the paragraph?

This has been changed as per reviewer suggestion.

17) p.15 This paragraph may be an appropriate venue for mentioning broader concepts, such as the role of the prehelix segment as a switch (Kotzsch et al., 2009) and of the role of short loops or extensions, which evolutionarily are readily acquired, in conferring altered binding properties. In the TGF- ternary complex (Groppe et al., 2008), these segments were introduced into the receptors, including Alk5 (and Alk4). On the ligand side, myostatin appears to have lost a segment (or activin gained an insertion, depending on which appeared first). With respect to the antagonists, Noggin acquired an N-terminal extension (Groppe et al., 2002) and Follistatin an N-terminal domain.

We agree that it is interesting to speculate on concepts regarding the prehelix loop as a specificity feature for receptors, but think that broader speculation would be better presented in a review article

and by persons with more knowledge on such evolutionary concepts. In addition, Groppe et al. (2008 Mol Cell) have already emphasized some of these points in their discussion.

18) p.17 "It is also interesting to speculate that the combination of TGF- family ligands with other antagonists may also create new cell surface binding properties, conferring similar forms of regulation." Although an attractive notion, unfortunately other family members are not good candidates. TGF- ligands do not bind heparin, nor activin as shown here (and likely neither Nodal), and BMPs bind through a highly basic N-terminal extension that is highly flexible (protease sensitive, not modeled in crystal structures) that could not form a continuous interface, if the implication.

We would like to clarify the speculation that combinations of antagonist with ligands may also create new cell surface properties, conferring similar forms of regulation. First, we were not limiting this to binding heparin, and we have clarified this in the manuscript. Secondly, although a ligand does not have a particular property, this does not exclude it from augmenting one in a binding partner. Furthermore, one could imagine the complex could generate a surface with a novel property that is not present in the individual proteins. We have bolstered this speculative argument in the manuscript. We argue that this type of speculation is helpful in exploring unconsidered areas of investigation.

19) p.17 If indeed the implication, wouldn't non-contiguous interactions, for example those provided by the Noggin:BMP complex (both bind heparin with high affinity from opposite surfaces) enhance binding through avidity effects, not as a result of a composite electropositive surface? Maybe the above statement was in reference to such a situation. Regardless, a composite surface such as that in the myostatin:Fst complexes could conceivably yield a composite heparin binding site that, unlike the Noggin:BMP complex, required a contiguous surface for enhanced affinity. Once more, maybe this is indeed the whole point, but if so, wasn't laid out completely enough to be grasped. Moosa Mohammadi at NYU has published stellar structures showing the role of a composite site in the FGF dimer in assembly of the ligand receptor complex that might be worth mentioning in this context.

Certainly several reasons could explain a higher affinity of the myostatin:follistatin complexes over unbound or activin A-bound follistatin. We have tried to work these ideas into the manuscript. We had to replace a significant section of the discussion since we have included data to support our original statement, "It would be interesting to determine if our findings translated into both myostatin:Fst288 and Fst315 complexes having a greatly increased affinity for the cell surface and an increased ability to degrade ligand, as would be suggested by Hashimoto et al."

20) p.20-24 Unfortunately EndNote downloads references from PubMed without the original Greek symbols, thus throughout the references the Greek symbol for beta is corrupted and would require manual editing to restore to the published form.

We have manually edited the corruption of the Greek symbol for beta.

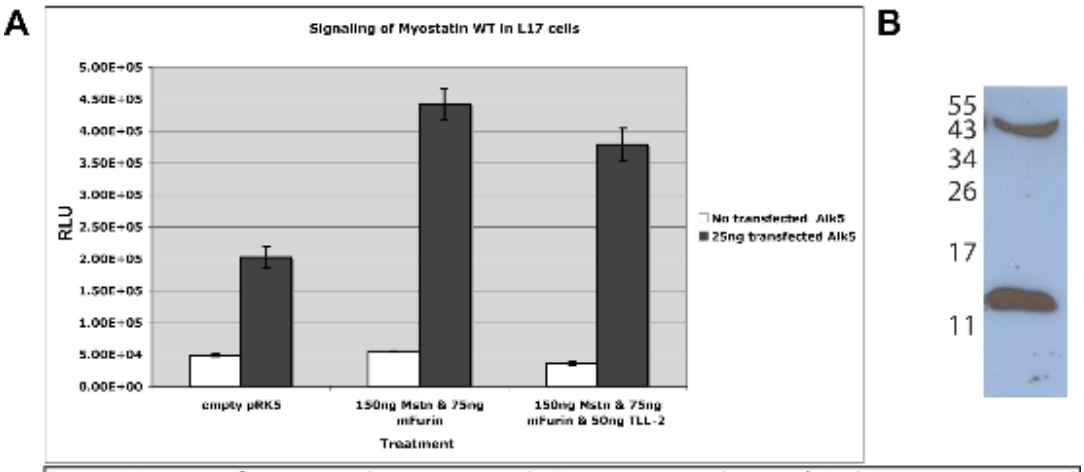
21) Figure 1 A review panel described in the Introduction, that while helpful to a broad audience, may be more suitable for a Supplementary Figure in the interest of space reduction.

The authors would like to keep this figure in the main text, as we hope that the manuscript will be utilized by those interested not only in the TGF- field in general, but also specifically in the myostatin field. This may be an audience less familiar with the information in such a review panel.

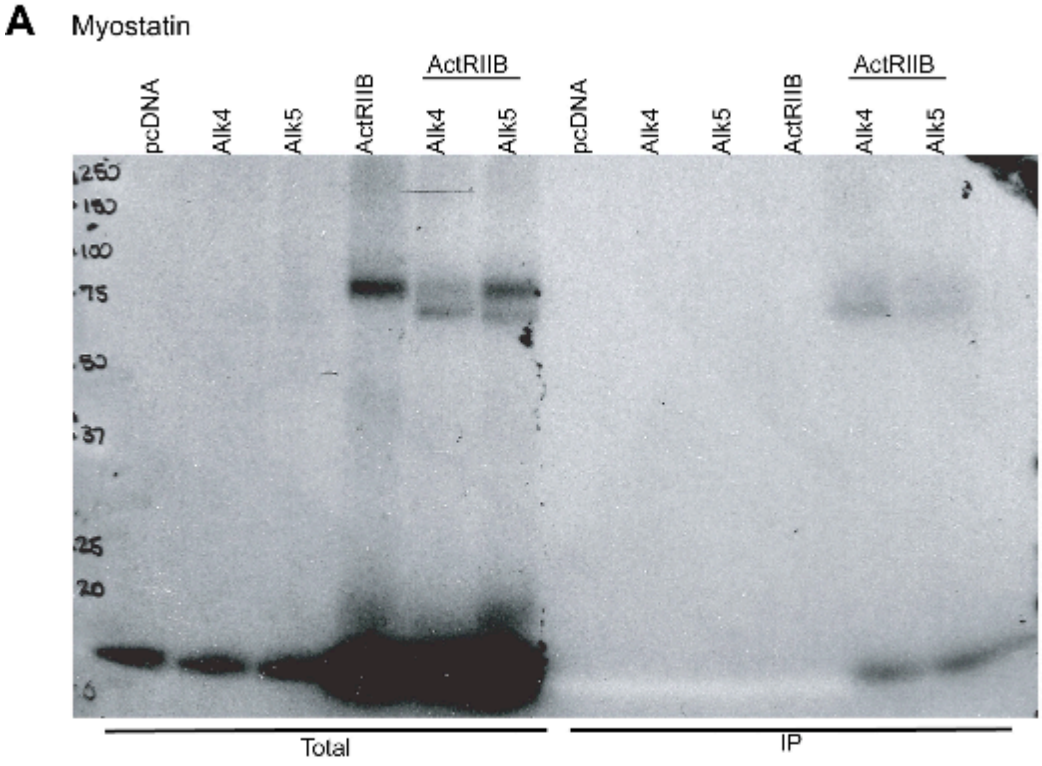
22) Figure 2E Perspective is somewhat misleading, in that the majority of the surface of Alk5 proximal to the prehelix loop is actually raised well above. Also, perhaps the ligands were aligned toward the ends of the fingers, but near the palm the strands do not appear to align at all. If so, the figure again is somewhat misleading, because the disposition of the prehelix loop relative to the superimposed Alk5 extracellular domain may be highly speculative, one of several possible arrangements. Maybe additional views are needed to fairly depict and convince the reader of this very important aspect of the paper.

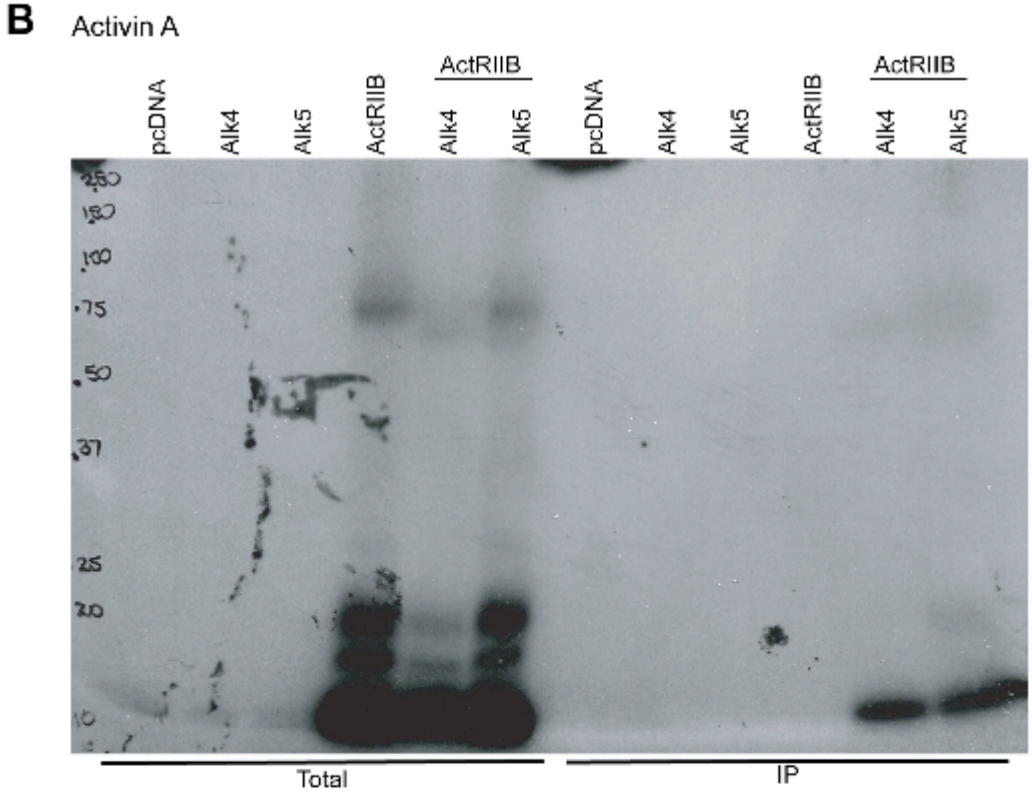
The authors agree with the reviewer and have included two new images (Fig. 3A). One image is a

view from the top of the ligand:receptor interaction, the other is a view looking directly at Alk5 through the prehelix and wrist regions, with the rest of the monomers removed. We feel that these views more fully and accurately depict this interaction.



Reviewer Figure 1: Luciferase assay and test expression with pRK5 myostatin WT. A) Furin or furin plus TLL-2 were coexpressed with myostatin WT in an attempt to relieve latent complex and activate signaling. However, very little signaling was observed. B) Test expression of pRK5 myostatin WT in 293T cells to confirm that protein can be made from this construct. Shown is a western blot of 20ul conditioned media (reduced) probed with anti-myostatin antibody.





Reviewer Figure 2: CHO cells were transfected with ActRIIB-Flag, Alk4-myc, or Alk5-myc alone or in combination. Affinity labelling was performed as described in Chapman et al. (2002; Mol Cell Endocrinol 196:79-93) with A) 125I-myostatin or B) 125I-activin A. Total cell lysates or anti-myc immunoprecipitates (IP) were resolved by SDS-PAGE and affinity labelled complexes visualized by autoradiography. Both ligands bound to ActRIIB alone and to Alk4 and Alk5 when co-expressed with ActRIIB. The IP results confirmed the binding of both ligands to both type I receptors.

2nd Editorial Decision 10 June 2009

Your revised manuscript has now been re-assessed by one of the original referees whose comments you will find enclosed. This scientist does have a specific remark that I would give you the opportunity to consider. Please provide us with the ultimate version of your paper as soon as you can to enable official acceptance.

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

Reviewer report on EMBOJ-2009-70618R entitled “The Structure of Myostatin:Follistatin 288: Insights into Receptor Utilization and Heparin Binding" by J.N. Cash, C.A. Rejon, A.C. McPherron, D.J. Bernard and T.B. Thompson

The manuscript describes the crystal structure analysis of the myostatin:follistatin complex. GDF-8 or myostatin, a member of the TGF- superfamily, has been identified as a key element in the regulation of muscle growth. Animals with deactivating mutations in GDF-8 or mice having null mutations in the GDF-8 gene exhibit a muscle hypertrophy phenotype showing that GDF-8/myostatin acts as a negative regulator of muscle growth. Knockout of myostatin in mice doesn't seem to have other deleterious effects. The very specific regulation of muscle growth by myostatin has fueled the idea of myostatin being an ideal target for therapeutic intervention in muscle disorder diseases, such as muscular dystrophies, e.g. Duchenne, or motor neuron diseases, e.g. amyotrophic lateral sclerosis.

The revised manuscript presented here yields important insights into the interaction of myostatin bound to its important antagonist follistatin. The specificity of the interaction of GDF-8 with its type I receptors was studied by functional analysis and mutagenesis. During the revision almost all criticism/issues were resolved by the authors, my only minor comment is to include the pre-helix single amino acid substitution results for the type I receptor specificity study as a table into the supplemental material, although the authors say that the amino acid exchanges do not affect the interaction. Having the table with the particular substitutions would make it easier for subsequent studies to reference these important results if it is not refering to a sentence which says "data not shown".

