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Tolloid cleavage activates latent GDF8 by priming the pro-complex for dissociation

Viet Q. Le, Roxana E. Iacob, Yuan Tian, William McConaughy, Justin Jackson, Yang Su, Bo Zhao, John R. Engen, Michelle Pirruccello-Straub, Timothy A. Springer

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Transaction Report:

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

1st Editorial Decision 06 October 2017

Thank you again for submitting your manuscript for consideration by the EMBO Journal. We have now received all three reports on your manuscript, which are included below for your information.

As you can see from the comments, all reviewers express interest in the presented analysis of GDF8 structure and the proposed mechanism of its activation, and the reviewers appreciate the high quality of presented data. Therefore I would like to invite you to submit your revised manuscript in which you address the comments of all reviewers, but particularly focusing on the inhibitor sensitivity of mature versus primed myostatin as requested by reviewer #1.

Referee #1:

The main finding of this paper is that cleavage of the latent myostatin complex by TLL2 does not cause complete dissociation of the propeptide/C-terminal dimer complex but rather destabilizes the complex and primes it for activity. The observations are interesting and potentially important. In addition to providing insights into the molecular/structural events occurring during activation of the latent complex, this observation has potential implications regarding the overall biology of myostatin. In my opinion, perhaps the most interesting possibility is that the fact that the complex does not completely fall apart may render it resistant to regulation by other binding proteins, like FST, FSTL-3, etc. The authors state this possibility on page 10 in the discussion. However, the authors do not show any data to this effect. The paper would be greatly strengthened by experiments documenting that under the conditions in which in the experiments in Figure 2 were carried out, mature myostatin is completely blocked by these inhibitors but that the primed myostatin is resistant.

On page 5, the authors state: "Moreover, the concentration . . . showed that rebinding of dissociated

prodomain fragments to the GF occurred. . . " I don't understand this statement. The authors need to show directly using purified fragments that rebinding can occur if they want to make this statement.

Referee #2:

This study aims to understand how protease cleavage of the prodomain of the TGF-beta family signaling protein myostatin (GDF-8) alters interactions with its corresponding GF domain to enable its release. The data reported includes analysis of three different forms of pro-GDF8, unprocessed, furin processed ('latent'), and furin- and Tld-processed ('primed') by SEC-MALS, MS detected HD exchange, and negative stain EM. The major findings are that a) unprocessed and latent GDF8 have similar overall open structures as proBMP9 and proActivinA, b) that unprocessed, as well as latent, proGDF-8 have considerably restricted H-D exchange in the prodomain straightjacket and edge b-strand of the arm domain, known from the structure of proTGF-b1 to be important in maintaining latency, and c) primed GDF8 does not exhibit the same restricted H-D exchange of unprocessed and latent GDF8 in the straighjacket and arm domains and that it is prone to disassociation.

The data and the presentation of this data are both of high quality - thus the authors conclusions are well-supported by the experimental data and these are effectively communicated through their manuscript.

The author's findings contribute to understanding of the biology of TGF-b family signaling proteins in at least two ways - first they show that the closed arm form of the pro-complexes, as in the three TGF-b isoforms, is not a requirement for a high degree of latency, but instead can be achieved in an open arm conformation, but with enhanced interactions between the prodomain straightjacket and b1-strand of the arm domain with the GF - second, they have shown how proteolysis, at a site distant from the furin-cleavable pro-mature boundary, can lead to increased dynamics and in turn GF activation - something long known to play a role in activation, but not yet studied at this level. Though not part of this study, these findings are nicely corroborate and complement the structure of the pro GDF-8 that was evidently submitted concurrently with this manuscript.

Overall, this is an important contribution that advances knowledge in the field - the only concerns pertain to some relatively minor points detailed below:

1. In the Discussion section, it is stated that "Previous studies have shown that prodomain cleavage by Tolloid proteases activates GDF8 and GDF11 signaling (Ge et al., 2005, Wolfman et al., 2003); however, whether cleavage immediately released the GF from embrace by either or both of the two cleaved prodomain fragments was not examined". While true, this point has in fact been addressed for GDF11 in a very recently published paper by Pepinsky (Biochemistry 2017, 56, 4405−4418) - accordingly, the authors should adjust this statement according to the findings reported by Pepinsky.

2. In the Discussion section, there is little mention of the extent to which the elements they have identified that have reduced exchange and are believed to contribute to latency are conserved or not in other TGF-b family GFs.

3. In the Discussion section, fifth paragraph, second sentence, it woud be best to rephrase this from "At a bowtie knot at the end of the arm domain" to "At the bowtie knot end of the arm domain"

4. In the legend for Figure 1, I believe it should be Asn71 (as in the Figure), not Asn48.

5. In the text, it is argued that the two adjacent cysteines in b-strand 2 of the arm domain form a vicinal disulfide, while in the legend for Fig. 3 it indicates that these are free cysteines; this discrepancy should be clarified.

Referee #3:

Timothy Springer and coworkers at Harvard Medical School and environs have amassed an

impressive and highly technical body of work toward dissecting the structural and functional basis underlying the robust latency of the promyostatin (GDF-8) complex which is cleaved in the trans-Golgi network during the secretory process prior to deposition into the extracellular matrix of skeletal muscle tissue. Given the tremendous clinical interest in developing therapeutic means of inhibiting the inhibitor of muscle growth, i.e. the mature dimeric growth factor (myo-statin), a broad readership might take interest in the results of the extensive studies.

That said, given the major contributions to the field that have been produced by the Springer Laboratory through crystallographic studies, the findings reported here from more indirect methods were most likely in lieu of or meant to support a crystal structure. To their credit, the group was able to gain significant insight from the diverse, highly technical and complementary characterization methods of size-exclusion chromatography combines with multi-angle light scattering, hydrogen-deuterium exchange mass spectrometry and negative-stain electron microscopy.

Similarly, expression and preparation of the three forms of proprotein studied relied on production from stably transfected mammalian or insect cell lines, which provided samples of adequate purity and integrity, however inferior to bacterially expressed, engineered variant protein that enabled formation of well-diffracting crystals by a laboratory at Cambridge University referenced within:

Cotton TR, Fischer G, Wang X, McCoy JC, Czepnik M, Thompson TB, Hyvonen M (2017) Structure of the human proXmyostatin precursor and determinants of growth factor latency. bioRxiv

Although the indirect Harvard analyses are complementary to and supportive of the direct crystallographic studies successfully conducted at Cambridge, and vice-versa, the impact of the indirect results, despite the impressive technical abilities and expertise that are evident, is less so than of the three-dimensional structure. Hence the technically dense alternative studies might not be suitable for the broad readership of The EMBO Journal, but rather perhaps for EMBO reports.

Regardless, the publication of the Cambridge, Harvard and Cincinnati (also referenced within) studies in concert online, whether in the same journal or not, would garner much attention and boost the field, hopefully spurring on the development of therapeutics to treat muscle wasting that stems from an array of all-too-common diseases and disorders.

1st Revision - authors' response 10 November 2017

Referee 1
"On page 5, the authors state: "Moreover, the concentration . . . showed that rebinding of dissociated prodomain fragments to the GF occurred. . . " I don't understand this statement. The authors need to show directly using purified fragments that rebinding can occur if they want to make this statement."

We have revised that section for clarity:

> *"Dissociation has first order kinetics and thus the same proportion of dissociation must have occurred at all concentrations. Therefore, the concentration-dependence of the molecular mass of the main peak and the increasing proportion of the secondary peak with decreasing primed GDF8 concentration strongly suggest that at higher concentrations, dissociation was partially balanced by reassociation. These results suggest dissociation constants in the range of experimentally used concentrations."*

Referee 2
Overall, this is an important contribution that advances knowledge in the field the only concerns pertain to some relatively minor points detailed below:

1. In the Discussion section, it is stated that "Previous studies have shown that prodomain cleavage by Tolloid proteases activates GDF8 and GDF11 signaling (Ge et al., 2005, Wolfman et al., 2003); however, whether cleavage immediately released the GF from embrace by either or both of the two cleaved prodomain fragments was not examined". While true, this point has in fact been addressed

for GDF11 in a very recently published paper by Pepinsky (Biochemistry 2017, 56, 4405−4418) accordingly, the authors should adjust this statement according to the findings reported by Pepinsky.

We now take into consideration the findings of Pepinsky et al. 2017 in the Discussion section.

> *"Moreover, recent work on GDF11 showed that in vitro cleavage by the endoproteinase AspN generated a prodomain fragment capable of maintaining association with the GF without inhibiting GDF11 activity (Pepinsky, Gong et al., 2017). This fragment aligns in part with the α1- through α2-helix region of the GDF8 prodomain (i.e., TLD-cleaved N-terminal fragment) and improves solubility of the GDF11 GF. Association of the N-Frag in primed GDF8 may similarly maintain solubility of the GF until it reaches and binds to downstream signaling receptors."*

2. In the Discussion section, there is little mention of the extent to which the elements they have identified that have reduced exchange and are believed to contribute to latency are conserved or not in other TGFb family GFs.

We now include a discussion on the implications of our findings for GDF11 maturation and activation.

> *"The HDX-MS studies of GDF8 also provide insight into latency and activation of GDF11. Of the 33 members of the TGF-β family, GDF11 is most similar in sequence to GDF8 (64% identity). In particular, sequences that correspond to the α1-helix, latency lasso (including the 6-residue latency helix insertion), α1-helix, fastener, and β1 strand in the prodomain and the β6'–7' strands in the GF of GDF8 are strongly conserved in GDF11 (Hinck, 2016). Although GF factor structures of GDF8 and 11 vary in conformation, follistatin288-bound structures of both are remarkably alike (RMSD = 0.657 Å) (Apgar et al., 2016, Cash, 2009, Padyana, Vaidialingam et al., 2016, Walker et al., 2017a) suggesting that interaction with the same binding partner imposes similar structural constraints on the GDF8 and 11 GFs. These observations combined with conservation of overall domain architecture and secondary structure in the family (Cotton et al., 2017, Hinck et al., 2016, Mi et al., 2015, Shi et al., 2011, Wang et al., 2016) suggest that latent GDF11 forms similar prodomain–GF interfaces. Activation of GDF11 occurs via cleavage at a conserved TLD-site in the prodomain (Ge et al., 2005) (Fig. 5). Moreover, in vitro cleavage of GDF11 has shown that an N-terminal prodomain fragment that corresponds in part to the expected TLD-cleaved product remains associated with the GF (Pepinsky et al., 2017). Thus, we propose that TLD cleavage similarly destabilizes conserved prodomain-GF interfaces in GDF11 and primes the pro-complex for dissociation."*

3. In the Discussion section, fifth paragraph, second sentence, it woud be best to rephrase this from "At a bowtie knot at the end of the arm domain" to "At the bowtie knot end of the arm domain"

The text has been edited for clarity accordingly.

4. In the legend for Figure 1, I believe it should be Asn71 (as in the Figure), not Asn48.

We have fixed this typo in the Figure 1 legend.

5. In the text, it is argued that the two adjacent cysteines in bstrand 2 of the arm domain form a vicinal disulfide, while in the legend for Fig. 3 it indicates that these are free cysteines; this discrepancy should be clarified.

The figure legend for Figure 3 has been edited to:

> *"Asterisks (*) mark cysteines in the GDF8 prodomain that are discussed in the text."*

Referee #3:
Timothy Springer and coworkers at Harvard Medical School and environs have amassed an impressive and highly technical body of work toward dissecting the structural and functional basis underlying the robust latency of the promyostatin (GDF-8) complex which is cleaved in the trans-Golgi network during the secretory process prior to deposition into the extracellular matrix of skeletal muscle tissue. Given the tremendous clinical interest in developing therapeutic means of inhibiting the inhibitor of muscle growth, i.e. the mature dimeric growth factor (myo-statin), a broad readership might take interest in the results of the extensive studies.

Thank you, we completely agree.

That said, given the major contributions to the field that have been produced by the Springer Laboratory through crystallographic studies, the findings reported here from more indirect methods were most likely in lieu of or meant to support a crystal structure. To their credit, the group was able to gain significant insight from the diverse, highly technical and complementary characterization methods of size-exclusion chromatography combines with multi-angle light scattering, hydrogen-deuterium exchange mass spectrometry and negative-stain electron microscopy.

While it is true that we were also engaged in crystallography, we were not as successful as hoped. We did in fact express and purify from S2 cells the pro-GDF8 precursor and obtained crystals that diffracted to 4.1Å. However, we were unable to solve a structure in a timely manner. Furthermore, if we did have it in time, it would just duplicate what the Hyvonen group has so elegantly achieved. We believe that the HDX, EM, MALS, and gel filtration data adds much more information to an understanding of GDF8 biology than a duplicate crystal structure would have. Also, the HDX work stands on its own, and we engaged in it for its own sake, not to complement a crystal structure.

Similarly, expression and preparation of the three forms of proprotein studied relied on production from stably transfected mammalian or insect cell lines, which provided samples of adequate purity and integrity, however inferior to bacterially expressed, engineered variant protein that enabled formation of well-diffracting crystals by a laboratory at Cambridge University referenced within: Cotton TR, Fischer G, Wang X, McCoy JC, Czepnik M, Thompson TB, Hyvonen M (2017) Structure of the human proXmyostatin precursor and determinants of growth factor latency. bioRxiv

We would like to clarify that none of the mammalian expressed pro-complex forms were tested for crystallization; as mentioned above, we obtained crystals of S2-cell expressed pro-GDF8.

Although the indirect Harvard analyses are complementary to and supportive of the direct crystallographic studies successfully conducted at Cambridge, and vice-versa,
the impact of the indirect results, despite the impressive technical abilities and expertise that are evident, is less so than of the three-dimensional structure. Hence the technically dense alternative studies might not be suitable for the broad readership of The EMBO Journal, but rather perhaps for EMBO reports.

First, as asked by the other reviewers, we have made the MS less dense. Second, it is important to note that our work makes important contributions to our understanding of GDF8 activation that are not evident in the crystal structure from Dr. Hyvonen's group. We have shown that after Tolloid cleavage the GDF8 prodomain fragments and growth factor remain partially associated in a primed GDF8 pro-complex. Importantly, HDX revealed prodomain–growth factor interfaces that become destabilized by Tolloid cleavage during activation. These insights were only made possible by studying the conformational dynamics of the precursor, latent, and primed forms of the pro-complex. We are also puzzled by the referee's suggestion that "our studies might not be suitable for the broad readership of The EMBO Journal", but previously stated "a broad readership might take interest in the results of the extensive studies." In short, we believe that the referee makes arguments both for and against the MS, and we prefer the ones in favor of publication. Overall, the reviewer reads like a crystallographer more than an HDX expert. I also love crystal structures. However, HDX has lots of strengths, which I appreciate more and more, and HDX really does provide insights

orthogonal to crystallography. We would argue against HDX being "indirect", because it does reveal many things that structures don't.

Regardless, the publication of the Cambridge, Harvard and Cincinnati (also referenced within) studies in concert online, whether in the same journal or not, would garner much attention and boost the field, hopefully spurring on the development of therapeutics to treat muscle wasting that stems from an array of all-too-common diseases and disorders.

We agree and feel that co-publication with Dr. Hyvonen's manuscript in EMBO J would synergize and amplify better than if the manuscripts were to be published in separate journals.

2nd Editorial Decision 11 December 2017

Thank you for submitting a revised version of your manuscript. We have now received reports from two of the original referees, who find that all their main concerns have now been addressed. There are just a few minor mainly editorial issues to be dealt with before formal acceptance here. Congratulations on a nice study!

1. Please include error estimates in the Figure 2C as requested by reviewer #2.

REFEREE COMMENTS

Referee #1:

no additional comments

Referee #2:

The revised manuscript submitted by Le and co-workers adequately addresses the concerns raised in the initial review. The only minor issue I see is that the newly reported IC50s for BMP antagonists now included as part of Fig. 2C should include error estimates.

2nd Revision - authors' response 14 December 2017

Please include error estimates in the Figure 2C as requested by reviewer #2.

Figure 2 has been revised to report fitting errors for the EC50 and IC50 values. After reanalyzing the data, we have decided to omit the IC50 values of each antagonist obtained for 10 nM mature and primed GDF8 as there were not enough intermediate values around the inflection point to reliably calculate the error.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Timothy A. Springer
Journal Submitted to:	EMBO Journal
Manuscript Number:	EMBOJ-2017-97931R

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
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4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA

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Is there an estimate of variation within each group of data?	Yes. The error bars in each plot represent the standard deviation of three measurements.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA