

CTGF/ CCN2 facilitates LRP4-mediated formation of the embryonic neuromuscular junction

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Dear Dr. Ohkawara,

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

As you will see, all referees acknowledge that the findings are potentially interesting. However, they also all point out that the data are not sufficiently strong to support the main conclusions, and that the technical quality of the study is rather low. I think all referee comments are reasonable and should be addressed.

I would thus like to invite you to revised your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO Reports

Referee #1:

The authors are investigating the role of CTGF, an extracellular matrix protein in acetylcholine receptor (AChR) clustering at the neuromuscular junction. They show that CTGF is binding to LRP4, the co-receptor of MuSK and stimulates MuSK phosphorylation and AChR clustering. This function of CTGF is probably the consequence of a higher level of LRP4 at the membrane and thus an increased binding of agrin to LRP4. These new findings are potentially interesting. However a number of data are not fully convincing.

Figure 1 : when performing IP flag, CTGF is only clearly visualized when MuSK and LRP4 are co-expressed (A, I5). The signal for CTGF and LRP4 ectoflag is very weak (A, I6). In B, the signal in the same condition as A, I6 is also very weak. CTGF could bind propeller 3 but since the signal with the full LRP4 ecto is much weaker than $\Delta 1-2$, it is difficult to draw a conclusion. In C, the western blot is overexposed and there is a lot of unspecific signals for CTGF (see comparison without CTGF) making it difficult to conclude.

F : Agrin binding to LRP4 is not affected by increasing concentration of CTGF but does increasing concentrations of CTGF increase LRP4 at the membrane and agrin binding to LRP4 ?

Figure 2 : Blots A and B should be quantified and immunocytochemistry should be provided
Title should be « in C2C12 cells and embryonic muscle ». How old are the embryos ?

Figure 3 : Blot A should be quantified. Since CTGF has been shown to induce LRP tyrosine phosphorylation (Yang et al., 2004, FASEB J), it would be interesting to see if LRP4 is phosphorylated. In B, the effect of agrin is surprisingly low and it is not clear if AChR clusters are at the membrane (confocal microscopy ?). Measuring the length of clusters (which at best appear like spots) in this condition is not possible. More convincing images should be provided. Same comments for F.

Figure 4 : C : do these images result from confocal microscopy ? Spinal cord versus diaphragm expression of CTGF : it does not exclude that motoneuron CTGF could be accumulated at the axon terminal.

Figure 5. B : the presence of synaptophysin along the axon is a hallmark of nerve immaturity. It could explain that less synaptic vesicles are observed at the terminal. Are the glial processes normally localized at the periphery of the synapse in the mutant ?

Figure S2 : it is hard to see in E but is axonal branching different in mutant compared to wt (number

and length of branches) ?

Other comments :

Are the CTGF mutant mice myasthenic ?

In discussion, I would not say that CTGF is critical for formation of the nerve terminal. The nerve terminal forms but is immature since synaptophysin is detected along the axon.

Referee #2:

The present manuscript by Kobayakawa and colleagues addresses the role of CTGF on NMJ. A series of apparently well executed experiments using HEK293 and C2C12 cells as well as CTGF $-/-$ mouse embryos demonstrates different novel aspects: i) interaction between CTGF and LRP4 and the domains necessary for interaction, ii) effects of CTGF on LRP4 membrane recruitment, MuSK phosphorylation, and AChR clustering, iii) enrichment of CTGF at NMJ and embryonic expression profile, iv) effects of lack of CTGF on NMJ formation, innervation, and NMJ fine structure. In general, the manuscript is written in an intelligible manner, although it would profit from proofreading by a native speaker. The data are well laid out, coherent, and do well fit to the text. They are of interest to the general readership and this referee has only a number of minor comments and suggestions.

- 1) In Figure 2A and B, the WB for CTGF are missing, the scheme in Figure 2D is shown at a premature place, since at least the legend to it also mentions MuSK phosphorylation as a role for CTGF. But that is first shown in Figure 3.
- 2) In Figure 3, please add the WB raw data for the downregulation of CTGF upon shRNA treatment.
- 3) In Table 1, the parameter "Mitochondria numbers" is not sufficiently specified: are these pre- or postsynaptic mitochondria?
- 4) I think, it would be useful to add some experimental data on the potential interaction between CTGF and agrin as well as an expression profile for CTGF during early postnatal development and in adults.

Referee #3:

In this manuscript, the authors demonstrated a role of connective tissue growth factor (CTGF) in the formation of the mammalian neuromuscular junction (NMJ). They found that CTGF facilitates agrin-MuSK signaling and AChR clustering by interaction with Lrp4, the co-receptor for agrin. Biochemical analysis showed that CTGF directly binds to the third b-propeller domain of Lrp4, the same domain which binds to MuSK. Furthermore, CTGF enhances the Lrp4/MuSK interaction, up-regulates the level of Lrp4, and promotes MuSK phosphorylation and AChR clustering. Finally, they found some defects in the NMJ structure or function, including smaller AChR clusters, abnormal distribution of synaptic vesicles, and impaired neuromuscular transmission in *ctgf* knockout mice. Although this work addresses an interesting question of how Lrp4 is regulated in the process of NMJ formation, the mild phenotypes of *ctgf* mutant mice in AChR clustering, the lack of essential controls, and un-satisfactory quality of some data lowered my enthusiasm on the significance of this work.

Major points:

1. Fig. 1B, to conclude that the third b-propeller domain of Lrp4 is required for CTGF binding, the authors should generate delb1, delb2, delb3, and delb4 of Lrp4 and test binding of these mutated forms of Lrp4 to CTGF, respectively.
2. Fig. 1C, CT domain of CTGF was required for binding with Lrp4. Is it sufficient for binding?
3. Fig. 1D, whether the mutated form of CTGF with the deletion of CT domain binds to Lrp4ect-Flag in the overlay assay?
4. Fig. 2A, the blot results should be quantified. The authors interpret that CTGF stabilizes Lrp4 and thus promotes the formation of the agrin-Lrp4-MuSK complex. To reach this conclusion, the authors should determine whether CTGF treatment promotes the association between Lrp4 and MuSK, and also test the effect of mutated form of CTGF with the deletion of CT domain.
5. Fig. 2B, what happened to MuSK phosphorylation in *ctnf* mutant mice?
6. Fig. 3A-C, the mutated form of CTGF with the deletion of CT domain is an essential control to conclude that the effect of CTGF on MuSK signaling and AChR clustering is through the interaction with Lrp4. Fig. 3A, the authors should test the Lrp4/MuSK interaction under indicated conditions with essential loading controls.
7. Fig. 3B, the quality of signals is very poor. Better focused images should be shown. Besides the length of AChR clusters (Fig. 3C), numbers should also be quantified. Again, the mutated form of CTGF is needed as an essential control.
8. Fig. 3D-F, it seems that shCtgf-1 works better in down-regulating the expression of *ctgf* and suppressing MuSK phosphorylation than shCtgf-2. But the representative image shows lesser effect on AChR clustering. Please clarify. Fig. 3E should be quantified.
9. Fig. 3B, F, how are the contours of myotubes delineated? How do you judge whether the clusters are in the same rather than overlapped other myotubes. Given the blurry signals, it's hard for me to tell the contour of myotubes. I suggest the authors to use some internal markers, e.g., transfected fluorescent protein or muscle membrane marker, to outline muscle contour.
10. Whether the decrease in MuSK signaling (Fig. 3E) and AChR clustering (Fig. 3F) restored by forced expression of Lrp4?
11. Fig. 5A-D, how were the numbers of AChR and synaptophysin quantified? The authors should show whole mount staining images with high quality.
12. Table 2, mEPP frequency is about 0.69/sec in this work, which is much higher than other studies (Chen et al., *Nature Neuroscience* 2011). The original recording traces should be included in figures.
13. The statement that "Lrp4 at the presynaptic nerve terminal induces clustering of synaptic vesicles" (page 9, last sentence) in discussion is NOT correct, because no presynaptic deficits were observed in HB9-Lrp4-/- mice, where Lrp4 is knocked out in motor neurons (Wu et al., *Neuron*, 2012). It has been suggested that Lrp4 expressed in muscle cells regulates presynaptic differentiation through a retrograde signaling mechanism (Yumoto N et al. *Nature* 2012). This point should be appropriately discussed.
14. Fig. S1B, what is the physiological basis for using ATF2-luciferase as a readout for agrin/MuSK signaling? More explanation is needed. Was the effect of MuSK over-expression significant? It seems that MuSK had little effect in this assay? It's very strange here because overexpression MuSK leads to self-phosphorylation, which has been shown in many early studies.
15. Fig. S2C, E, the quality of AChR staining is very poor. The quantification for AChR clusters based on these is not convincing (Fig. 5C and 5D). As mentioned in point 11, whole mount staining with high quality is needed for reliable quantification.

Minor points

1. For electrophysiology study, which muscles were recorded? The description is not consistent in Methods and Results sections.
2. The authors should describe the CMAP recording protocol in detail in Method section and show

the original CMAP recording traces in figures.

3. Fig. 1E, 1F, S1A, S1B, S2F, S2G, what are the letters above the error bars representing? These are not mentioned in either main text or figure legends.

Specific response to reviewer's comments

Referee #1

The authors are investigating the role of CTGF, an extracellular matrix protein in acetylcholine receptor (AChR) clustering at the neuromuscular junction. They show that CTGF is binding to LRP4, the co-receptor of MuSK and stimulates MuSK phosphorylation and AChR clustering. This function of CTGF is probably the consequence of a higher level of LRP4 at the membrane and thus an increased binding of agrin to LRP4. These new findings are potentially interesting. However a number of data are not fully convincing.

Comment 1-1: Figure 1: When performing IP flag, CTGF is only clearly visualized when MuSK and LRP4 are co-expressed (A, 15). The signal for CTGF and LRP4 ectoflag is very weak (A, 16). In B, the signal in the same condition as A, 16 is also very weak. CTGF could bind propeller 3 but since the signal with the full LRP4ect is much weaker than $\Delta 1-2$, it is difficult to draw a conclusion. In C, the western blot is overexposed and there are a lot of unspecific signals for CTGF (see comparison without CTGF) making it difficult to conclude.

Answer 1-1: Thank you for your suggestions. We analyzed bindings between CTGF and LRP4 by making new constructs (CTGF-CT, LRP4- $\Delta 1$, LRP4- $\Delta 2$, LRP4- $\Delta 3$, and LRP4- $\Delta 4$). Fig. 1A shows that full-length CTGF and CTGF-CT were immunoprecipitated with LRP4ecto-Flag (lanes 6 and 7). We also confirmed binding of LRP4ecto to CTGF-CT, but not to CTGF- Δ CT, by a cell-free plate-binding assay in Fig. 1D. Fig. 1B shows that LRP4- $\Delta 1$, LRP4- $\Delta 2$, and LRP4- $\Delta 4$, but not LRP4- $\Delta 3$, were immunoprecipitated with full-length CTGF (lanes 3 to 6). As suggested, we re-performed Western blotting of Fig. 1B (previously Fig. 1C). We revised relevant statements in Materials & Methods, Results, and legends for Fig. 1ABCD.

Added statements in Materials and Methods

phLRP4ecto-Flag, was used for constructing Flag-tagged human LRP4 lacking one of the four β -propeller domains via the site-directed mutagenesis kit (BioLabs, E0554S). Human LRP4- $\Delta 1$ lacked codons 456-717, LRP4- $\Delta 2$ lacked codons 761-022, LRP4- $\Delta 3$ lacked codons 1069-1330, and LRP4- $\Delta 4$ lacked codons 1373-1634. Human full-length CTGF cDNA was PCR-amplified, and cloned into a pcDNA3.1(+) mammalian expression vector to generate phCTGF. CTGF lacking both TSP1 and CT domains (Δ TSP1_ Δ CT construct retaining codons 1-190) and CTGF lacking the CT domain (Δ CT construct retaining codons 1-252) were similarly generated from phCTGF. cDNAs for the ectodomain of mouse MuSK carrying codons 1-454, partial rat Agrin carrying codons 1141-1937 [24], mouse full-length CTGF carrying codons 1-348, mouse CTGF- Δ CT retaining codons 1-252, mouse CTGF-CT carrying codons 252-348, and human C-terminal-deleted RSPO2 carrying codons 1-218 as a control were cloned into APtag-5 (GenHunter) at HindIII and SnaBI sites to generate MuSKect/pAPtag-5, Agrin/pAPtag-5, CTGF/pAPtag-5, CTGF- Δ CT/pAPtag-5, CTGF-CT/pAPtag-5, and Control/pAPtag-5, respectively.

Added statements in Results

In order to determine the associations between CTGF and the agrin-LRP4-MuSK complex, we first examined the binding of CTGF to LRP4 and MuSK. An immunoprecipitation assay revealed that CTGF bound to the Flag-tagged ectodomain of LRP4 (LRP4ecto-Flag) but not to that of MuSK (MuSKect-Flag) (Fig. 1A, lanes 3 and 6). We observed that the CT domain of CTGF was sufficient for binding to LRP4 (Fig. 1A, lane 7), and that the deletion of the CT domain abrogated binding to LRP4 (Fig. 1B). Conversely, deletion of each β -propeller domain of LRP4 (LRP4- $\Delta 1$, - $\Delta 2$, - $\Delta 3$, and - $\Delta 4$) showed that the 3rd β -propeller domain of LRP4 was required for binding to CTGF (Fig. 1C). We also confirmed direct binding of CTGF and LRP4 by a cell-free plate-binding assay. Purified full-length CTGF and the CT domain of CTGF that were tagged with myc and alkaline-phosphatase (CTGF-mycAP and CTGF-CT-mycAP, respectively) indeed bound to the plate-coated ectodomain of LRP4 (LRP4ecto-Flag), but not to that of MuSK

(MuSKect-Flag) (Fig. 1D). In contrast, purified CTGF lacking the CT domain (CTGF- Δ CT-mycAP) had weak binding compared to full-length CTGF.

Figure 1. Interaction between the CT domain CTGF and the third β -propeller domain of LRP4 enhances the binding of LRP4 to MuSK

(A-C) Representative immunoprecipitation (IP) of HEK293 cells transfected with indicated cDNAs. (A) CTGF interacts with LRP4 but not with MuSK. Full-length CTGF (CTGF-full-myc) was immunoprecipitated with the ectodomain of LRP4 (LRP4ect-Flag, lane 6) but not with the ectodomain of MuSK (MuSKect-Flag, lane 3). However, when CTGF-full-myc, LRP4ect, and MuSKect-Flag were expressed together, CTGF-full-myc was immunoprecipitated with MuSKect-Flag (lane 5). The CT domain of CTGF (CTGF-CT-myc) was sufficient to immunoprecipitate LRP4ect-Flag (lane 7). (B) Deletion of the CT domain of human CTGF (CTGF- Δ CT- Δ TSP1 and CTGF- Δ CT) impairs the interaction between CTGF and LRP4ect-Flag. (A, B) Predicted positions of full-length and shortened CTGF proteins are indicated by bars and/or asterisks. (C) Anti-CTGF antibody immunoprecipitated human LRP4 deleted with the 1st, 2nd, and 4th β -propeller domains (Δ 1, Δ 2, and Δ 4) but not with the 3rd β -propeller domain (Δ 3).

(D-E) Cell-free plate-binding assays. A purified tagged-protein was attached to a plastic plate and a purified recombinant protein fused to alkaline phosphatase (AP) was overlaid. Bound protein was quantified by measuring AP activity. (D) LRP4ect-Flag, but not MuSKect-Flag, bound to overlaid CTGF-full-mycAP and CTGF-CT-mycAP. In contrast, LRP4ect-Flag did not bind to CTGF- Δ CT-mycAP. (E, upper panel) LRP4ect-Flag bound to overlaid MuSKect-mycAP in a dose-dependent manner of CTGF-full-myc, but not of CTGF- Δ CT-myc. (E, lower panel) LRP4ect-Flag bound to overlaid agrin-mycAP independent of CTGF-full-myc. (D-E) Rspo2-mycAP and Rspo2-myc were used as Control-mycAP and control-myc, respectively. Mean and SD ($n = 9$ wells; 3 wells in 3 independent experiments) are indicated. p -value < 0.05 by two-way repeated measures ANOVA for the three panels in (D) and (E). $p < 0.05$ by post-hoc Tukey test is indicated by a single letter representing each group.

Comment 1-2: Figure 1F: Agrin binding to LRP4 is not affected by increasing concentration of CTGF but does increasing concentrations of CTGF increase LRP4 at the membrane and agrin binding to LRP4?

Answer 1-2: Thank you for a valuable suggestion. We quantified the amount of LRP4 in the plasma membrane fraction in C2C12 myotubes (Fig. 2A) and in the mouse diaphragms (Fig. 2C). Agrin alone (lane 2 in Fig. 2A) or CTGF alone (lane 3 in Fig. 2A) had no effect on the amount of the membrane-bound LRP4 in C2C12 myotubes, but a combination of agrin and CTGF increased it (lane 4 in Fig. 2A). We also showed that the absence of *Ctgf* reduced the amount of LRP4 on the plasma membrane in mouse embryonic muscles (Fig. 2C). However, CTGF did not increase the binding of agrin to LRP4 on the surface of HEK293 cells (Fig. EV1AB). CTGF is thus likely to stabilize LRP4 on the plasma membrane in the presence of NMJ-specific proteins like agrin and MuSK. We added relevant statements in Results.

Added statement in Results.

We also observed in agrin-treated C2C12 myotubes that CTGF increased the amount of Lrp4 on the plasma membrane, but not that in whole cells (Fig. 2A). In addition, CTGF increased phosphorylation of Lrp4 in agrin-treated C2C12 myotubes (Fig. 2B). Similarly, the absence of *Ctgf* reduced the amount of Lrp4 on the plasma membrane in mouse embryonic muscles (Fig. 2C). However, transgenic (lanes 2 to 4 in Fig. EV1A) or exogenous (lanes 3 to 5 in Fig. EV1B) CTGF failed to increase the binding of agrin to LRP4 on the surface of HEK293 cells. CTGF, thus, likely stabilizes LRP4 on the plasma membrane in the presence of NMJ-specific proteins like agrin and MuSK.

Comment 1-3: Figure 2: Blots A and B should be quantified and immunocytochemistry should be provided. Title should be « in C2C12 cells and embryonic muscle ». How old are the embryos?

Answer 1-3: As suggested, we quantified the amounts of LRP4 in Fig. 2AC (previously Fig. 2AB). We revised the title for Fig. 2, as suggested. We tried to immunostain LRP4 using two anti-LRP4 antibodies that are advertised to be applicable for immunostaining. As indicated below, the R&D antibody stained cytoplasm of some muscle fibers in wild-type adult mouse. In contrast, the Abcam antibody stained cell membrane of muscle fibers in wild-type adult mouse, but LRP4 was not enriched at the NMJ. We could not optimize the staining conditions for C2C12 myotubes and skeletal muscles at E18.5 (see attached). As far as we know, no previous report showed immunostaining of LRP4 in adult or embryonic mouse skeletal muscle. We apologize that we could not show the suggested immunostaining images. The embryos were at E18.5. We revised relevant statements in a legend for Fig. 2.

Revised statement in a legend for Fig. 2AC.

Figure 2. CTGF stabilizes Lrp4 on the plasma membrane of C2C12 myotube and the embryonic muscle

- (A) Representative immunoblotting of Lrp4, MuSK, Transferrin Receptor (TfR), CTGF, and β -actin in whole-cell lysates and the plasma membrane fraction of C2C12 myotubes treated with 1 pM (1 ng/ml) agrin and/or 250 pM (100 ng/ml) CTGF for 24 h. CTGF increases Lrp4 on the plasma membrane in the presence of agrin.
- (C) Representative immunoblotting of Lrp4, MuSK, CTGF, and TfR in whole tissue lysates and the plasma membrane fraction of lower limb muscles at E18.5 of wild-type (WT) and *Ctgf*^{-/-} mice.
- (A-D) Mean and SD ($n = 3$ independent experiments) are indicated. p -value < 0.05 by one-way ANOVA. $p < 0.05$ by post-hoc Tukey test is indicated by a single letter representing each group.

[Figures for referees not shown.]

Comment 1-4: Figure 3: Blot A should be quantified. Since CTGF has been shown to induce LRP4 tyrosine phosphorylation (Yang et al., 2004, FASEB J), it would be interesting to see if LRP4 is phosphorylated. In B, the effect of agrin is surprisingly low and it is not clear if AChR clusters are at the membrane (confocal microscopy?). Measuring the length of clusters (which at

best appear like spots) in this condition is not possible. More convincing images should be provided. Same comments for F.

Answer 1-4: Thank you for valuable suggestions. As suggested, we showed quantification of the blots of Fig. 3A. As suggested, we showed that agrin and CTGF cooperatively increased LRP4 phosphorylation (Fig. 2B). In Fig. 3B, we reduced the concentration of agrin to 5 ng/ml to minimally induce AChR clusters so that AChR clustering is not saturated and that the effect of CTGF can be observed. As suggested, in Fig. 3B, we stained plasma membrane of C2C12 myotubes to corroborate that AChR clusters were on the plasma membrane. As suggested, we quantified the lengths and the numbers of AChR clusters of Fig. 3B. We also showed more distinct images in Fig. 3BD.

Revised statement in a legend for Fig. 2B.

Figure 2. CTGF stabilizes Lrp4 on the plasma membrane of C2C12 myotube and the embryonic muscle

(B) C2C12 myotubes were treated with 1 pM agrin and/or 250 pM CTGF for 6 h. Lrp4 was immunoprecipitated (IP) with an anti-Lrp4 antibody, and phosphorylated Lrp4 was immunoblotted with an anti-phosphotyrosine (p-Tyr) antibody.

Figure 3. CTGF enhances agrin-mediated AChR clustering in C2C12 myotubes

(A) C2C12 myotubes were treated with indicated concentrations of agrin-mycAP, CTGF-mycAP, and/or CTGF- Δ CT-mycAP for 3 h. Total MuSK was immunoprecipitated (IP) with an anti-MuSK antibody, and phosphorylated MuSK was immunoblotted with an anti-phosphotyrosine (p-Tyr) antibody. CTGF-mycAP, but not CTGF- Δ CT-mycAP, enhanced agrin-mediated MuSK phosphorylation.

(B) C2C12 myotubes were treated with 5 pM agrin-mycAP, 250 pM CTGF-mycAP, and/or 250 pM CTGF- Δ CT-mycAP for 18 h. BSA was added to the control medium. AChRs and the plasma membrane were visualized with Alexa594-conjugated α -bungarotoxin (red) and CellMask (green), respectively. Bar = 20 μ m. CTGF enhances agrin-mediated AChR clustering.

(A-D) Mean and SD ($n = 3$ independent immunoblotting experiments) are indicated in A and C. Blinded morphometric analysis of myotube and AChR signals are shown by mean and SD ($n = 60-75$ myotubes in 3 independent experiments) in the right panels in B and D. The axis length of an AChR cluster with 4 μ m or more was recognized and measured by the MetaMorph software, and p -value < 0.05 by one-way ANOVA in A to D except for myotube length in D. $p < 0.05$ by post-hoc Tukey test is indicated by a single letter representing each group.

Revised statement in Materials and Methods.

The lengths of the AChR clusters and myotubes were defined as the longest axes of Alexa594 signals and GFP signals, respectively, in C2C12 myotubes stained with the CellMask green plasma membrane stain (Invitrogen, C37608) in Fig. 3B or in C2C12 myotubes infected with lentivirus expressing shRNA and GFP in Fig. 3F.

Revised statement in a legend for Fig. 3B.

(B) C2C12 myotubes were treated with 5 pM agrin-mycAP, 250 pM CTGF-mycAP, and/or 250 pM CTGF- Δ CT-mycAP for 18 h. BSA was added to the control medium. AChRs and the plasma membrane were visualized with Alexa594-conjugated α -bungarotoxin (red) and CellMask (green), respectively. Bar = 20 μ m. CTGF enhances agrin-mediated AChR clustering.

Comment 1-5: Figure 4C: do these images result from confocal microscopy? Spinal cord versus diaphragm expression of CTGF: it does not exclude that motoneuron CTGF could be accumulated at the axon terminal.

Answer 1-5: Yes, Fig. 4C are confocal microscopy images of thick frozen muscle sections of 20 μ m at E18.5. We revised Materials and Methods, and Results. Because an identical CTGF

isoform is expressed in the spinal motor neurons and the diaphragm (Fig. 4D), we could not identify the origin of CTGF at the NMJ. We revised relevant statements in Results.

Revised statement in Materials and Methods.

Frozen sections (20 μm thickness) of the thigh muscles of mouse embryos were fixed with acetone for 10 min at -20°C , washed three times with PBS, and then covered with PBS containing 1% goat serum for 60 min.

Revised statements in Results.

Although the diaphragm produced more *Ctgf* mRNA than the spinal cord, the origin of CTGF concentrated at the NMJs remains undetermined.

Comment 1-6: Figure 5. B: the presence of synaptophysin along the axon is a hallmark of nerve immaturity. It could explain that less synaptic vesicles are observed at the terminal. Are the glial processes normally localized at the periphery of the synapse in the mutant?

Answer 1-6: Thank you for valuable suggestions. As suggested, we performed diaphragm staining for S100 β , a marker for Schwann cells. Fig. EV4A-C showed that almost all NMJ synapses were adjacent to S100 β signals. In Fig. EV4D, representative electron microscopic image shows that Schwann cell is adjacent to the pre-synaptic terminal. Taken together, we concluded that immaturity of pre-synaptic terminal in *Ctgf*^{-/-} diaphragm was not due to abnormality in Schwann cells.

Revised statement in Results.

Almost all NMJ synapses were adjacent to S100 β signals, a marker for Schwann cells (Fig. EV4ABC). In electron microscopy, the pre-synaptic nerve terminal was also adjacent to terminal Schwann cell (Fig. EV4D).

Revised statement in Results.

In addition, synaptophysin-positive areas were abnormally observed along motor axons in the *Ctgf*^{-/-} diaphragm (Fig. 5B and Fig. EV5A), which may represent the immaturity of the nerve terminal. Blinded morphometric analysis revealed that the area of AChR clusters was smaller in the *Ctgf*^{-/-} diaphragm than that in the wild-type diaphragm, while the numbers and intensities of AChR-positive areas and of synaptophysin-positive areas were unchanged (Fig. 5CD). Furthermore, the ratio of synaptophysin-positive areas that were not colocalized with AChR signals was increased in *Ctgf*^{-/-} mice (Fig. 5E), which likely represents dispersion of synaptophysin along the motor axon (Fig. 5B). The ultrastructure of NMJs of the *Ctgf*^{-/-} diaphragm at E18.5 showed a lower density of synaptic vesicles, fewer active zones, and significantly fewer mitochondria in the pre-synaptic nerve terminals (Fig. 5F and Table 1). These structural analyses suggest that CTGF is important for the maturation of the presynaptic nerve terminal.

Revised legends for Fig.EV4

Figure EV4. *Ctgf*^{-/-} diaphragm shows no significant differences in number of Schwann cells at the NMJ

(A-C) Representative low **(A)** and high **(B)** magnification images of the whole-mount left diaphragms at E18.5 stained for S100 β (green) and AChR (α -bungarotoxin, red). Bar = 1 mm. **(C)** Blinded morphometry to examine the ratio of AChR signals adjacent to S100 β signals. Mean and SD ($n = 30$ NMJs in 5 left diaphragms) are indicated. Bar = 20 μm **(A)** and 2 μm **(B)**. p -value > 0.05 by Student's t -test. Lack of statistical difference is indicated by a single letter "a".

(D) Representative electron micrographs of the NMJs in the diaphragm of wild-type and *Ctgf*^{-/-} mice at embryonic day (E) 18.5. Nerve terminal (red) is juxtaposed with Schwann cells (yellow). Bar = 1 μm .

Comment 1-7: Figure S2: it is hard to see in E but is axonal branching different in mutant compared to wt (number and length of branches) ?

Answer 1-7: Thank you for the suggestion. We used new representative images in Fig. EV3D (previously Fig. S2E) to show peripherin and AChR signals more clearly. We also quantified the number and length of branches in Fig. EV3F. We concluded that *Ctgf* knockout had no essential effect on axonal branching in the diaphragm at E18.5. We added Fig. EV3CDF and their legends. **Added legends for Fig. EV3DF.**

Figure EV3. *Ctgf*^{-/-} diaphragm shows no significant differences in myogenic gene expressions, myofibril ultrastructure, width of the AChR band, or axonal branches of the phrenic nerves

(C-F) Representative low (C) and high (D) magnification images of the whole-mount left diaphragms at E18.5 stained for peripherin (anti-peripherin antibody, upper panels) and AChR (α -bungarotoxin, lower panels). Bar = 1 mm. (E) Blinded morphometry of the endplate bandwidth of AChR clusters in C. (F) Blinded morphometry of the 2nd and 3rd branches in C. Mean and SD ($n = 6$ left diaphragms) are indicated. p -value > 0.05 by one-way ANOVA for the three panels in E and F. Lack of statistical difference is indicated by a single letter “a”.

Comment 1-8: Are the CTGF mutant mice myasthenic ?

Answer 1-8: *Ctgf* deficiency resulted in lethality just after birth because of respiratory distress caused by thoracic skeletal defects, so that we could not measure muscle strength. Instead, we applied repetitive nerve stimulation on the sciatic nerve, and observed a CMAP decrement to 55.6% in *Ctgf*^{-/-} mice (Table 2), which is a commonly used hallmark of myasthenia in patients.

Revised statement in Results

Ctgf deficiency resulted in lethality immediately after birth because of respiratory failure caused by thoracic skeletal defects [20].

Revised statement in Results

Defective NMJ signal transmission was also indicated by abnormally decreased CMAP amplitudes of the TA muscle after repetitive stimulation of the sciatic nerve (Table 2 and Fig. EV5D), which is a diagnostic hallmark of myasthenia gravis and congenital myasthenic syndromes [22].

Comment 1-9: In discussion, I would not say that CTGF is critical for formation of the nerve terminal. The nerve terminal forms but is immature since synaptophysin is detected along the axon.

Answer 1-9: Thank you for your valuable suggestion. As suggested, we revised our statement in Discussion.

Revised statement in Results

These structural analyses suggest that CTGF is important for the maturation of the presynaptic nerve terminal.

Revised statement in Discussion

CTGF is also critical for the maturation and function of the presynaptic nerve terminal.

Referee #2:

The present manuscript by Kobayakawa and colleagues addresses the role of CTGF on NMJ. A series of apparently well executed experiments using HEK293 and C2C12 cells as well as CTGF^{-/-} mouse embryos demonstrates different novel aspects: i) interaction between CTGF and LRP4 and the domains necessary for interaction, ii) effects of CTGF on LRP4 membrane recruitment, MuSK phosphorylation, and AChR clustering, iii) enrichment of CTGF at NMJ and embryonic expression profile, iv) effects of lack of CTGF on NMJ formation, innervation, and NMJ fine structure. In general, the manuscript is written in an intelligible manner, although it would profit from proofreading by a native speaker. The data are well laid out, coherent, and do well fit to the

text. They are of interest to the general readership and this referee has only a number of minor comments and suggestions.

Answer 2-0: We appreciate valuable and encouraging comments. Our manuscript has been proofread by a native English speaker.

Comment 2-1: In Figure 2A and B, the WB for CTGF are missing, the scheme in Figure 2D is shown at a premature place, since at least the legend to it also mentions MuSK phosphorylation as a role for CTGF. But that is first shown in Figure 3.

Answer 2-1: As suggested, we showed CTGF expression in C2C12 myotubes in Fig. 2A and in *Ctgf*^{-/-} embryos in Fig. 2C (previously Fig. 2B). As suggested, we moved the summary scheme for CTGF functions (previously Fig. 2D) to Fig. 5G, which is the final figure panel.

Revised legend for Fig. 2AC

Figure 2. CTGF stabilizes Lrp4 on the plasma membrane of C2C12 myotube and the embryonic muscle

- (A) Representative immunoblotting of Lrp4, MuSK, Transferrin Receptor (TfR), CTGF, and β -actin in whole-cell lysates and the plasma membrane fraction of C2C12 myotubes treated with 1 pM (1 ng/ml) agrin and/or 250 pM (100 ng/ml) CTGF for 24 h. CTGF increases Lrp4 on the plasma membrane in the presence of agrin.
- (C) Representative immunoblotting of Lrp4, MuSK, CTGF, and TfR in whole tissue lysates and the plasma membrane fraction of lower limb muscles at E18.5 of wild-type (WT) and *Ctgf*^{-/-} mice.

Comment 2-2: In Figure 3, please add the WB raw data for the downregulation of CTGF upon shRNA treatment.

Answer 2-2: Thank you for your valuable suggestions. As suggested, we performed immunoblotting of CTGF in C2C12 myotubes with shCtgf-1 or shCtgf-2, and showed the results in Fig. EV2B.

Added legend for Fig. EV2B

Figure EV2. CTGF enhances agrin- and LRP4-mediated AChR clustering in C2C12 myotubes

- (A, B) C2C12 myotubes were infected with lentivirus expressing shControl, shCtgf-1, or shCtgf-2. Doxycycline was added for 2 days to induce shRNA expression. (A) Quantitative RT-PCR. (B) Immunoblotting. Mean and SD ($n = 3$ independent experiments) are indicated. p -value < 0.05 by two-way repeated measures ANOVA for both A and B. $p < 0.05$ by post-hoc Tukey test is indicated by a single letter representing each group.

Comment 2-3: In Table 1, the parameter "Mitochondria numbers" is not sufficiently specified: are these pre- or postsynaptic mitochondria?

Answer 2-3: We apologize for insufficient explanation. We quantified the number of mitochondria in the presynaptic terminal. We revised the label in Table 1 as follows.

Added statement in Table 1

Number of presynaptic mitochondria (/synapse)

Comment 2-4: I think, it would be useful to add some experimental data on the potential interaction between CTGF and agrin as well as an expression profile for CTGF during early postnatal development and in adults.

Answer 2-4: Thank you for valuable suggestions. As suggested, we performed a cell-free plate-binding assay with CTGF and agrin. Fig. 1E showed that purified partial agrin-mycAP binds to LRP4ect but not to full-length CTGF. As suggested, we performed quantitative RT-PCR for *Ctgf* and neural *Agrin* in the diaphragm and in the spinal cord from embryonic day 13.5 to

postnatal day 126 (Fig. 4D). We revised relevant statements in Results, and legends for Fig. 1E and Fig. 4D.

Revised statement in Results

Cell-free plate-binding assay also revealed that purified full-length CTGF-myc on a plate had no binding to MuSK-mycAP or partial agrin-mycAP (Fig. 1E).

Added statement in Results

Expression of *Ctgf* was detected in the diaphragm from E13.5 and peaked at E17.5, when the *Ctgf* expression was 4 times higher than that in the spinal cord (Fig. 4D). Expression of neural agrin was also detected in the spinal cord from E14.5 and peaked from E17.5 to postnatal day (P) 7.

Revised legends for Fig. 1E

(E, upper panel) LRP4ect-Flag bound to overlaid MuSKect-mycAP in a dose-dependent manner of CTGF-full-myc, but not of CTGF- Δ CT-myc. (E, lower panel) LRP4ect-Flag bound to overlaid agrin-mycAP independent of CTGF-full-myc.

Revised legend for Fig. 4D

Figure 4. *Ctgf* is enriched at the neuromuscular junction

(D) Quantitative RT-PCR of *Ctgf* and neural *Agrn* in the spinal cord and the diaphragm in mouse. Mean and SD ($n = 3$ mice) are indicated.

Referee #3:

In this manuscript, the authors demonstrated a role of connective tissue growth factor (CTGF) in the formation of the mammalian neuromuscular junction (NMJ). They found that CTGF facilitates agrin-MuSK signaling and AChR clustering by interaction with Lrp4, the co-receptor for agrin. Biochemical analysis showed that CTGF directly binds to the third β -propeller domain of Lrp4, the same domain which binds to MuSK. Furthermore, CTGF enhances the Lrp4/MuSK interaction, up-regulates the level of Lrp4, and promotes MuSK phosphorylation and AChR clustering. Finally, they found some defects in the NMJ structure or function, including smaller AChR clusters, abnormal distribution of synaptic vesicles, and impaired neuromuscular transmission in *ctgf* knockout mice. Although this work addresses an interesting question of how Lrp4 is regulated in the process of NMJ formation, the mild phenotypes of *ctgf* mutant mice in AChR clustering, the lack of essential controls, and un-satisfactory quality of some data lowered my enthusiasm on the significance of this work.

Major points:

Comment 3-1: Fig. 1B, to conclude that the third β -propeller domain of Lrp4 is required for CTGF binding, the authors should generate delb1, delb2, delb3, and delb4 of Lrp4 and test binding of these mutated forms of Lrp4 to CTGF, respectively.

Answer 3-1: Thank you for valuable suggestion. As suggested, we constructed delb1(Δ 1), delb2(Δ 2), delb3(Δ 3), and delb4(Δ 4) of Flag-tagged Lrp4ect, and performed the immunoprecipitation assays. We found that only LRP4- Δ 3 was not immunoprecipitated with the full-length CTGF [Fig. 1C (previously Fig. 1B)].

Added statements in Materials and Methods.

phLRP4ect-Flag, which we previously generated [24], carried the extracellular domain (codons 1-1722) of human *LRP4* cDNA between the HindIII and XbaI sites upstream of the 3xFlag epitope in a mammalian expression vector p3xFlag-CMV-14. phLRP4ect-Flag, was used for constructing Flag-tagged human LRP4 lacking one of the four β -propeller domains via the site-directed mutagenesis kit (BioLabs, E0554S). Human LRP4- Δ 1 lacked codons 456-717, LRP4- Δ 2 lacked codons 761-1022, LRP4- Δ 3 lacked codons 1069-1330, and LRP4- Δ 4 lacked codons 1373-1634.

Added statements in Results.

Conversely, deletion of each β -propeller domain of LRP4 (LRP4- Δ 1, - Δ 2, - Δ 3, and - Δ 4) showed that the 3rd β -propeller domain of LRP4 was required for binding to CTGF (Fig. 1C).

Revised legend for Fig. 1C.

Figure 1. Interaction of the CT domain CTGF to the third β -propeller domain of LRP4 enhances the binding of LRP4 to MuSK

(C) Anti-CTGF antibody immunoprecipitated human LRP4 deleted with the 1st, 2nd, and 4th β -propeller domains ($\Delta 1$, $\Delta 2$, and $\Delta 4$) but not with the 3rd β -propeller domain ($\Delta 3$).

Comment 3-2: Fig. 1C, CT domain of CTGF was required for binding with Lrp4. Is it sufficient for binding?

Answer 3-2: Thank you for valuable suggestion. As suggested, we constructed myc- and mycAP-tagged CT domain of CTGF (CTGF-CT-myc and CTGF-CT-mycAP), and performed immunoprecipitation and plate-binding assays, respectively. Fig. 1A showed that CTGF-CT is sufficient for binding to ectodomain of LRP4 (LRP4ect). The plate-binding assay also showed that CTGF-CT bound to LRP4ect (Fig. 1D). We added relevant statements in Materials and Methods, as well as in Results. We also revised legends for Fig. 1A-E.

Revised statements in Materials and Methods.

cDNAs for the ectodomain of mouse MuSK carrying codons 1–454, partial rat Agrin carrying codons 1141-1937 [24], mouse full-length CTGF carrying codons 1-348, mouse CTGF- Δ CT retaining codons 1-252, mouse CTGF-CT carrying codons 252-348, and human C-terminal-deleted RSPO2 carrying codons 1-218 as a control were cloned into pAPtag-5 (GenHunter) at HindIII and SnaBI sites to generate MuSKect/pAPtag-5, Agrin/pAPtag-5, CTGF/pAPtag-5, CTGF- Δ CT/pAPtag-5, CTGF-CT/pAPtag-5, and Control/pAPtag-5, respectively. The pAPtag-5 clones carried the I κ gk-originated signal peptide upstream of the insert and the myc-tag/alkaline phosphatase downstream of the insert. cDNAs for mouse full-length CTGF, mouse CTGF- Δ CT, mouse CTGF-CT, and human C-terminal-deleted RSPO2 were also cloned into pcDNA3.1/3x myc at EcoRI sites to generate myc-CTGF/pcDNA3.1, myc-CTGF- Δ CT/pcDNA3.1, myc-CTGF-CT/pcDNA3.1, and myc-Control/pcDNA3.1, respectively.

Revised statement in Results.

An immunoprecipitation assay revealed that CTGF bound to the Flag-tagged ectodomain of LRP4 (LRP4ect-Flag) but not to that of MuSK (MuSKect-Flag) (Fig. 1A, lanes 3 and 6). We observed that the CT domain of CTGF was sufficient for binding to LRP4 (Fig. 1A, lane 7), and that the deletion of the CT domain abrogated binding to LRP4 (Fig. 1B). Conversely, deletion of each β -propeller domain of LRP4 (LRP4- $\Delta 1$, - $\Delta 2$, - $\Delta 3$, and - $\Delta 4$) showed that the 3rd β -propeller domain of LRP4 was required for binding to CTGF (Fig. 1C). We also confirmed direct binding of CTGF and LRP4 by a cell-free plate-binding assay. Purified full-length CTGF and the CT domain of CTGF that were tagged with myc and alkaline-phosphatase (CTGF-mycAP and CTGF-CT-mycAP, respectively) indeed bound to the plate-coated ectodomain of LRP4 (LRP4ect-Flag), but not to that of MuSK (MuSKect-Flag) (Fig. 1D). In contrast, purified CTGF lacking the CT domain (CTGF- Δ CT-mycAP) had weak binding compared to full-length CTGF.

Revised legend for Fig.1.

Figure 1. Interaction between the CT domain CTGF and the third β -propeller domain of LRP4 enhances the binding of LRP4 to MuSK

(A-C) Representative immunoprecipitation (IP) of HEK293 cells transfected with indicated cDNAs. (A) CTGF interacts with LRP4 but not with MuSK. Full-length CTGF (CTGF-full-myc) was immunoprecipitated with the ectodomain of LRP4 (LRP4ect-Flag, lane 6) but not with the ectodomain of MuSK (MuSKect-Flag, lane 3). However, when CTGF-full-myc, LRP4ect, and MuSKect-Flag were expressed together, CTGF-full-myc was immunoprecipitated with MuSKect-Flag (lane 5). The CT domain of CTGF (CTGF-CT-myc) was sufficient to immunoprecipitate LRP4ect-Flag (lane 7). (B) Deletion of the CT domain of human CTGF (CTGF- Δ CT_ Δ TSP1 and CTGF- Δ CT) impairs the interaction between CTGF and LRP4ect-Flag. (A, B) Predicted positions of full-length and

shortened CTGF proteins are indicated by bars and/or asterisks. (C) Anti-CTGF antibody immunoprecipitated human LRP4 deleted with the 1st, 2nd, and 4th β -propeller domains ($\Delta 1$, $\Delta 2$, and $\Delta 4$) but not with the 3rd β -propeller domain ($\Delta 3$).

(D-E) Cell-free plate-binding assays. A purified tagged-protein was attached to a plastic plate and a purified recombinant protein fused to alkaline phosphatase (AP) was overlaid. Bound protein was quantified by measuring AP activity. (D) LRP4ect-Flag, but not MuSKect-Flag, bound to overlaid CTGF-full-mycAP and CTGF-CT-mycAP. In contrast, LRP4ect-Flag did not bind to CTGF- Δ CT-mycAP. (E, upper panel) LRP4ect-Flag bound to overlaid MuSKect-mycAP in a dose-dependent manner of CTGF-full-myc, but not of CTGF- Δ CT-myc. (E, lower panel) LRP4ect-Flag bound to overlaid agrin-mycAP independent of CTGF-full-myc. (D-E) Rspo2-mycAP and Rspo2-myc were used as Control-mycAP and control-myc, respectively. Mean and SD ($n = 9$ wells; 3 wells in 3 independent experiments) are indicated. p -value < 0.05 by two-way repeated measures ANOVA for the three panels in (D) and (E). $p < 0.05$ by post-hoc Tukey test is indicated by a single letter representing each group.

Comment 3-3: Fig. 1D, whether the mutated form of CTGF with the deletion of CT domain binds to Lrp4ect-Flag in the overlay assay?

Answer 3-3: As suggested, we performed cell-free plate-binding assay with CTGF- Δ CT-mycAP and Lrp4ect-Flag, and showed that CTGF- Δ CT reduced its binding affinity to Lrp4ect (Fig. 1D). Revisions were pasted in Answer 3-2 above.

Comment 3-4: Fig. 2A, the blot results should be quantified. The authors interpret that CTGF stabilizes Lrp4 and thus promotes the formation of the agrin-Lrp4-MuSK complex. To reach this conclusion, the authors should determine whether CTGF treatment promotes the association between Lrp4 and MuSK, and also test the effect of mutated form of CTGF with the deletion of CT domain.

Answer 3-4: Thank you for valuable suggestions. As suggested, we performed cell-free plate-binding assays with full-length CTGF (CTGF-full) and CT-deleted CTGF (CTGF- Δ CT) in Fig. 1E. We showed that adding CTGF-full but not CTGF- Δ CT enhanced binding of Lrp4ect to MuSKect (upper panel in Fig. 1E). As suggested, we also performed cell surface binding assays. We showed that expression of CTGF (Fig. EV1A), as well as addition of purified CTGF in the medium (Fig. EV1B), enhanced the binding of MuSKect-mycAP to LRP4 on the surface of HEK293 cells. We revised relevant statements in Materials and Methods, Results, and legends for Figs. EV1AB. Revised Materials and Methods and revised legends for Fig. 1DE were pasted in Answer 3-2.

Revised statement in Results.

Cell-free plate-binding assay also revealed that purified full-length CTGF-myc on a plate had no binding to MuSK-mycAP or partial agrin-mycAP (Fig. 1E). However, full-length CTGF, but not CTGF- Δ CT, enhanced the binding of LRP4 and MuSKect-mycAP on the plate, while CTGF had no effect on the binding of LRP4 and agrin-mycAP (Fig. 1E). To examine whether CTGF enhances the binding of LRP4 and MuSK on the cell membrane, we performed a cell surface binding assay. Expression of CTGF (Fig. EV1A), as well as addition of CTGF in the medium (Fig. EV1B), enhanced the binding of MuSKect-mycAP, but not of agrin-mycAP, to LRP4 on the surface of HEK293 cells.

Added statement in a legend for Fig. EV1AB

Figure EV1. CTGF functions with LRP4

(A, B) Cell surface binding assays. Variable concentrations of LRP4 cDNA and CTGF cDNA were introduced to HEK293 cells. Purified MuSKect-mycAP (30 nM) or purified agrin-mycAP (30 nM), both of which carried alkaline phosphatase in fusion, was added to the cultured medium. In B, 30 (+) or 60 (++) nM CTGF was also added as indicated.

Bound protein was quantified by measuring alkaline phosphatase activity. CTGF enhanced binding of LRP4 to MuSKect-mycAP but not to Agrn-mycAP. Mean and SD ($n = 9$ wells; 3 wells in 3 independent experiments) are indicated. p -value < 0.05 by two-way repeated measures ANOVA for both **A** and **B**. $p < 0.05$ by post-hoc Tukey test is indicated by a single letter representing each group.

Comment 3-5: Fig. 2B, what happened to MuSK phosphorylation in *ctgf* mutant mice?

Answer 3-5: We appreciate the scrutinizing suggestion. We found in E18.5 *Ctgf*^{-/-} diaphragm that Y755-phosphorylated MuSK was decreased compared to that in wild-type and *Ctgf*^{+/-} mice (Fig. 2D). We added statements in Materials and Methods, Results, and a legend for Fig. 2D.

Revised statement in Materials and Methods.

The membrane was incubated overnight at 4°C either with mouse monoclonal anti-myc (Abcam, 9E10), anti-MuSK (1:1000, Santa Cruz Biotechnology, sc-6009), anti-LRP4 (1:2000, Abcam, ab85679), anti-CTGF (1:200, Santa Cruz Biotechnology, sc-14939), anti-Flag M2 (1:4000, Sigma-Aldrich, F1804), anti-transferrin receptor (TfR, 1:1000, Abcam, ab84036), anti-phosphoY755 MuSK (1:1000, abcam, ab192583), anti-phosphotyrosine (1:1000, Millipore, 05-321), or anti-β-actin (1:200, Santa Cruz Biotechnology, sc-47778) antibody.

Revised statement in Results.

We then analyzed the effects of CTGF on the agrin-LRP4-MuSK complex. We first analyzed the effect of CTGF on MuSK activation *in vivo*. Tyrosine (Y) 755 is located in an activation loop of the MuSK kinase domain, and is self-phosphorylated by MuSK to induce AChR clustering. In the mouse gastrocnemius, Y755-phosphorylated MuSK is localized at the NMJ [23]. The absence of *Ctgf* reduced phosphorylation of MuSK Y755 in the mouse diaphragm at E18.5 (Fig. 2D).

Revised legend for Fig. 2D.

Figure 2. CTGF stabilizes Lrp4 on the plasma membrane of C2C12 myotube and the embryonic muscle

(D) Representative immunoblotting of Y755-phosphorylated MuSK, total MuSK, and β-actin in the diaphragm at E18.5 of wild-type (WT), *Ctgf*^{-/-}, and *Ctgf*^{+/-} mice.

Comment 3-6: Fig. 3A-C, the mutated form of CTGF with the deletion of CT domain is an essential control to conclude that the effect of CTGF on MuSK signaling and AChR clustering is through the interaction with Lrp4. Fig. 3A, the authors should test the Lrp4/MuSK interaction under indicated conditions with essential loading controls.

Answer 3-6: Thank you for valuable suggestions. As suggested, we analyzed MuSK phosphorylation and AChR clustering with CTGF-ΔCT as a control in Fig. 3AB (previously Fig. 3A-C). Fig. 3A showed that full-length CTGF but not CTGF-ΔCT enhanced agrin-induced MuSK phosphorylation. Fig. 3B similarly showed that full-length CTGF but not CTGF-ΔCT enhanced agrin-induced AChR clustering. With these results, we concluded that CTGF increased agrin-induced MuSK phosphorylation and AChR clustering via CT domain, which bound to Lrp4. We revised relevant statements in Materials and Methods, Results, and legends for Fig. 3AB. Revised Materials and Methods were pasted in Answer 3-2.

Revised statement in Results.

In C2C12 myotubes, addition of full-length CTGF, but not CTGF-ΔCT, to the culture medium enhanced agrin-induced MuSK phosphorylation (Fig. 3A), and its downstream AChR clustering (Fig. 3B).

Revised legend for Fig. 3AB.

Figure 3. CTGF enhances agrin-mediated AChR clustering in C2C12 myotubes

(A) C2C12 myotubes were treated with indicated concentrations of agrin-mycAP, CTGF-mycAP, and/or CTGF-ΔCT-mycAP for 3 h. Total MuSK was immunoprecipitated (IP) with an anti-MuSK antibody, and phosphorylated MuSK was

- immunoblotted with an anti-phosphotyrosine (p-Tyr) antibody. CTGF-mycAP, but not CTGF- Δ CT-mycAP, enhanced agrin-mediated MuSK phosphorylation.
- (B) C2C12 myotubes were treated with 5 pM agrin-mycAP, 250 pM CTGF-mycAP, and/or 250 pM CTGF- Δ CT-mycAP for 18 h. BSA was added to the control medium. AChRs and the plasma membrane were visualized with Alexa594-conjugated α -bungarotoxin (red) and CellMask (green), respectively. Bar = 20 μ m. CTGF enhances agrin-mediated AChR clustering.

Comment 3-7: Fig. 3B, the quality of signals is very poor. Better focused images should be shown. Besides the length of AChR clusters (Fig. 3C), numbers should also be quantified. Again, the mutated form of CTGF is needed as an essential control.

Answer 3-7: As suggested, in Fig. 3B, we stained plasma membrane of C2C12 myotubes to visualize the plasma membrane and to corroborate that AChR clusters were on the plasma membrane. With these images, we quantified the length and the number of AChR clusters [Fig.3B (previously Fig. 3C)]. We revised relevant statements in a legend for Fig. 3B, which were pasted in Answer 3-6.

Comment 3-8: Fig. 3D-F, it seems that shCtgf-1 works better in down-regulating the expression of ctgf and suppressing MuSK phosphorylation than shCtgf-2. But the representative image shows lesser effect on AChR clustering. Please clarify. Fig. 3E should be quantified.

Answer 3-8: Thank you for valuable suggestions. Based on our knowledge that infection efficiencies were different from batch to batch of lentivirus, we re-performed all experiments with an identical large batch of lentivirus [Fig. 3CD (previously Fig. 3D-F) and Fig. EV2AB]. We showed that both shCTGF-1 and -2 similarly reduced CTGF expression (Fig. EV2AB), MuSK phosphorylation (Fig. 3C), and AChR clustering (Fig. 3D). We revised these figure panels.

Comment 3-9: Fig. 3B, F, how are the contours of myotubes delineated? How do you judge whether the clusters are in the same rather than overlapped other myotubes. Given the blurry signals, it's hard for me to tell the contour of myotubes. I suggest the authors to use some internal markers, e.g., transfected fluorescent protein or muscle membrane marker, to outline muscle contour.

Answer 3-9: As suggested, we stained plasma membrane of C2C12 myotube with CellMuSKTM green plasma membrane stain for visualizing cell membrane, and quantified AChR signals for Fig. 3B. For visualizing GFP signals originating from lentivirus carrying shRNA in C2C12 myotubes, we used a higher amount of lentivirus and quantified AChR signals for Fig. 3D (previously Fig. 3F). Revised Materials and Methods, and revised legends for Fig. 3B were pasted in Answer 3-6. We showed new representative images in Fig. 3D.

Comment 3-10: Whether the decrease in MuSK signaling (Fig. 3E) and AChR clustering (Fig. 3F) restored by forced expression of Lrp4?

Answer 3-10: Thank you for valuable suggestions. As suggested, we treated shCTGF-1-transfected C2C12 myotube with exogenous ectodomain of LRP4 (Lrp4ect), which activates MuSK phosphorylation and AChR clustering (Wu et al., *Neuron* 2012, 75(1): 94). We showed that LRP4ect rescued shCTGF-reduced MuSK phosphorylation (Fig. EV2C) and partially rescued shCTGF-reduced AChR clustering (Fig. EV2D). Therefore, we concluded that lack of CTGF can be compensated for by the ectodomain of LRP4. We revised relevant statements in Results and legends for Fig. EV2CD.

Revised statement in Results

In addition, the ectodomain of LRP4 restored shCTGF-induced reduction of MuSK phosphorylation (Fig. EV2C) and AChR clustering (Fig. EV2D) in C2C12 myotubes. These

results suggest that CTGF enhances MuSK phosphorylation and promotes AChR clustering via LRP4 in C2C12 myotubes.

Revised legend for Fig. EV2CD.

Figure EV2. CTGF enhances agrin- and LRP4-mediated AChR clustering in C2C12 myotubes

(C, D) C2C12 myotubes were infected with GFP-expressing lentivirus carrying shControl or shCtgf-1. Doxycycline was added for 2 days to induce shRNA expression. C2C12 myotubes were treated with indicated concentrations of purified agrin-mycAP and/or purified LRP4ect-Flag for 3 h. **(C)** Total MuSK was immunoprecipitated (IP) with anti-MuSK antibody, and phosphorylated MuSK was immunoblotted with anti-phosphotyrosine (p-Tyr) antibody. Quantification of immunoblots is shown in the right panel. *Ctgf* knockdown decreased MuSK phosphorylation, which was rescued by adding LRP4ect-Flag. **(D)** AChRs were visualized with Alexa594-conjugated α -bungarotoxin (red signals) in the infected myotube (green GFP signals). AChR cluster with an axis length of 4 μ m or more was recognized and measured by the MetaMorph software. Analysis was performed in a blinded manner. *Ctgf* knockdown decreased the number and the length of AChR clusters, which was partially rescued by adding LRP4ect-Flag. Bar = 20 μ m. Mean and SD are indicated in the right panels ($n = 60-75$ myotubes in 3 independent experiments). p -value < 0.05 by one-way ANOVA for the three panels in **C** and **D**. $p < 0.05$ by post-hoc Tukey test is indicated by a single letter representing each group.

Comment 3-11: Fig. 5A-D, how were the numbers of AChR and synaptophysin quantified? The authors should show whole mount staining images with high quality.

Answer 3-11: As suggested, we explained our quantification method in detail in Materials and Methods. Briefly, Olympus FSX100 was used to take 10 to 20 overlapping images of the whole left diaphragm and to automatically reconstitute a whole mount diaphragm image. For quantifying signals for AChR and synaptophysin, we used original images before reconstitution. We showed representative low magnification images for AChR clustering and synaptophysin in Fig. EV5A.

Revised statements in Materials and Methods.

Fluorescence images were obtained using an Olympus FSX100 fluorescence microscope for the whole mount diaphragms ($n = 10-20$ images for each left diaphragm, 5 diaphragms), and Zeiss LSM710 or Nikon A1Rsi confocal microscope for high magnification images of the NMJ ($n = 40-50$ images for each left diaphragm, 6 diaphragms). The numbers, intensities, and areas of AChR signals and synaptophysin signals were blindly quantified using images taken by Olympus FSX100. Ratios of synaptophysin signals not colocalized with AChR signals were quantified using images taken by Zeiss LSM710 or Nikon A1Rsi confocal microscope. All signal values were quantified by two blinded researchers using MetaMorph software (Molecular Devices).

Revised legend for Fig. EV5A

Figure EV5. *Ctgf*^{-/-} diaphragm shows mild to moderate electrophysiological abnormalities and different expression patterns for CCN family genes

(A) Representative low magnification images of the whole-mount left diaphragms at E18.5 stained for synaptophysin (green) and AChR (α -bungarotoxin, red). Confocal images of the NMJ are indicated in Fig. 5B. Bar = 1 mm.

Comment 3-12: Table 2, mEPP frequency is about 0.69/sec in this work, which is much higher than other studies (Chen et al., Nature Neuroscience 2011). The original recording traces should be included in figures.

Answer 3-12: Thank you for your scrutinizing comment. Chen and colleagues recorded mouse MEPP at 22°C (*Nat Neurosci* 2011, 14: 570). We usually record human MEPP at 30°C (e.g.

Selcen et al. *JAMA Neurol* 2015, 72: 889). As MEPPs were infrequent at the embryonic NMJs at these temperatures, we took MEPP recordings at $37 \pm 2^\circ\text{C}$. We suppose that this was why MEPP frequency was high compared to the other reports. As suggested, we showed representative raw MEPP recordings in Fig. EV5B, and representative averaged MEPPs in Fig. EV5C. We explained the electrophysiology methods in detail in Materials and Methods. We also added a legend for Fig. EV5BC.

Revised statement in Materials and Methods.

Electrophysiology

Miniature and evoked endplate potentials (MEPPs) were recorded essentially as described previously [40]. *Ctgf*^{-/-} mice and their wild-type littermates were delivered by cesarean section of anesthetized pregnant mice. The diaphragm was dissected in Tyrode solution containing 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1.3 mM Na₂HPO₄, 12.5 mM NaHCO₃, and 11 mM D-glucose at pH 7.2, and pinned to a Sylgard-coated dish. The solution was continuously gassed with 95% O₂ and 5% CO₂ at 37°C. The endplate region was inserted with a glass micropipette (20-40 MΩ) filled with 2 M KCl. The signal was amplified by an AxonClamp 900A amplifier (Molecular Devices), digitized at 10 kHz by Digidata 1550B (Molecular Devices), and was analyzed with AxoGraph X 1.5.0 (AxoGraph Scientific). To record compound muscle action potentials (CMAPs), the delivered E18.5 embryos were kept at 37°C with manual respiratory assist. The stimulation needle electrode (Inter Medical, IMK2-1001) was inserted near the sciatic nerve in the right thigh. The recording needle electrode was inserted into the calf muscle, and was connected to a Neuropack S1 system (Nihon Koden, MEB-9404). The stimulation of the sciatic nerve was triggered with a series of 10 stimuli at 2 Hz, and CMAP amplitudes at the first and fifth stimuli were measured.

Added legends for Fig. EV5BC.

Figure EV5. *Ctgf*^{-/-} diaphragm shows mild to moderate electrophysiological abnormalities and different expression patterns for CCN family genes

- (B)** Representative raw MEPP traces for 7 sec of wild-type and *Ctgf*^{-/-} mice at E18.5.
- (C)** Representative averaged MEPP recordings of wild-type and *Ctgf*^{-/-} mice at E18.5.

Comment 3-13: The statement that "Lrp4 at the presynaptic nerve terminal induces clustering of synaptic vesicles" (page 9, last sentence) in discussion is NOT correct, because no presynaptic deficits were observed in HB9-Lrp4^{-/-} mice, where Lrp4 is knocked out in motor neurons (Wu et al., *Neuron*, 2012). It has been suggested that Lrp4 expressed in muscle cells regulates presynaptic differentiation through a retrograde signaling mechanism (Yumoto N et al. *Nature* 2012). This point should be appropriately discussed.

Answer 3-13: We apologize for our misunderstanding of the previous reports. As suggested, we revised statements in Discussion.

Revised statement in Discussion

Previous studies demonstrated that a lack of muscle LRP4 leads to defective AChR clustering in skeletal muscle, as well as sparse synaptic vesicles and fewer active zones in the presynaptic nerve terminal [7,8,11,12]. Although the lack of CTGF induces more prominent defects at the nerve terminal than at the motor endplate, both presynaptic and postsynaptic defects are milder compared to those in the lack of muscle LRP4. As stated above, the lack of APPs [29] and SGα [30] induces presynapse-dominant defects at the NMJ, as we observed in *Ctgf*^{-/-} mice. APPs, SGα, and CTGF enhance the AChR-clustering activity mediated by LRP4 at the motor endplate. Similarly, APPs, SGα, and CTGF enhance the organization of the nerve terminal mediated by LRP4, and the effects of these modulators are more prominent at the nerve terminal than those at the motor endplate. LRP4 at the postsynaptic skeletal muscle induces the clustering of synaptic vesicles and active zones [11,12]. However, binding partner(s) for this LRP4 remain elusive. CTGF is one of candidate molecules that may enhance the functions of LRP4 in pre- and post-synaptic regions.

Comment 3-14: Fig. S1B, what is the physiological basis for using ATF2-luciferase as a readout for agrin/MuSK signaling? More explanation is needed. Was the effect of MuSK over-expression significant? It seems that MuSK had little effect in this assay? It's very strange here because overexpression MuSK leads to self-phosphorylation, which has been shown in many early studies.

Answer 3-14: Thank you for your suggestion. We screened three luciferase constructs in the JNK signaling pathway (Burden, *Cell* 2011, 144: 826), and found that only ATF2-luciferase responded to the agrin-LRP4-MuSK signaling pathway (Ohkawara *et al. Hum Mol Genet* 2014, 23:1856). We also showed that the effect of agrin can be estimated by the ATF2-luciferase activity in HEK293 cells that were transfected with optimized low doses of MuSK and LRP4 cDNAs (Nakashima *et al. Sci Rep* 2016, 6: 28512). Figure 2D of the Nakashima's *Sci Rep* paper is pasted below. In Fig. EV1C, we used the minimal amounts of MuSK and Lrp4 cDNAs to enable the detection of the effect of CTGF. We explained the method in detail including the optimization of the amounts of MuSK and Lrp4 in Materials and Methods.

Figure 2D of Nakashima *et al. Sci Rep* 2016, 6: 28512 to for reviewer's reference [Figures for referees not shown.]

A legend for Fig. 2D of Nakashima et al. Sci Rep 2016, 6: 28512

ATF2-luciferase reporter assay to quantify agrin (10 ng/ml)- and Rspo2 (100 ng/ml)-mediated activation of MuSK signaling in transfected HEK293 cells. Relative luciferase activities (RLA) are normalized to that with empty vectors. Mean and SD are indicated ($n = 3$). ** $p < 0.01$ by t -test. n.s., no significant difference.

Revised statement in Materials and Methods

ATF-2 luciferase assay in HEK293 cells

The mouse *Musk* cDNA in pExpress-1 was purchased from Open Biosystems. Partial rat *Agrin* (codons 1141-1937) ([24]) was cloned into APTag-5 (GenHunter) at the HindIII and SnaBI sites. ATF2-Luc to quantify the JNK signaling activity [24,25] and phRL-TK Renilla luciferase vector (Promega) were used to monitor agrin-Lrp4-MuSK signaling. HEK293 cells (1×10^4 cells) in a 96-well plate were transfected with 5 ng ATF2-Luc and 0.5 ng phRL-TK, along with 1, 2 or 5 ng pExpress-1-*Musk* encoding full-length MuSK, 5 ng phLRP4 encoding full-length LRP4, and 5 ng Agrin/pAPtag-5 encoding rat partial *Agrin*. The amounts of these constructs were optimized to lower levels to enable the detection of the effect of CTGF. The cells were cultured for 24 h in the presence of CTGF (250 pM, ProSpec, cyt-541).

Comment 3-15: Fig. S2C, E, the quality of AChR staining is very poor. The quantification for AChR clusters based on these is not convincing (Fig. 5C and 5D). As mentioned in point 11, whole mount staining with high quality is needed for reliable quantification.

Answer 3-15: As suggested, we re-performed diaphragm stainings with higher concentrations of α -bungarotoxin, and showed representative images in Fig. EV3CD and Fig. EV5A. We also

explained the quantification method in detail in Materials and Methods. We revised relevant statements in Materials and Methods and legends for Figs. EV3CD and EV5A.

Revised statement in Materials and Methods.

Diaphragm staining

The diaphragms of *Ctgf*^{-/-} embryos at E18.5 were fixed in 2% paraformaldehyde in PBS for 4 h at 4°C, and rinsed with PBS. After dissection of the connective tissue, the muscles were permeabilized with 0.5% Triton X-100 in PBS for 10 min and then incubated with α -bungarotoxin conjugated with biotin-XX (1:600, Invitrogen, B1606), anti-peripherin antibody (1:800, Millipore, AB1530), anti-S100 beta antibody (1:100, abcam, ab52642), and anti-synaptophysin antibody (1:100, Invitrogen, 180130) overnight. After washing, the sections were incubated with streptavidin conjugated with Alexa 564 (1:500, Invitrogen) or anti-mouse IgG conjugated with Alexa 488 (1:500, Invitrogen). Fluorescence images were obtained using an Olympus FSX100 fluorescence microscope for the whole mount diaphragms ($n = 10-20$ images for each left diaphragm, 5 diaphragms), and Zeiss LSM710 or Nikon A1Rsi confocal microscope for high magnification images of the NMJ ($n = 40-50$ images for each left diaphragm, 6 diaphragms). The numbers, intensities, and areas of AChR signals and synaptophysin signals were blindly quantified using images taken by Olympus FSX100. Ratios of synaptophysin signals not colocalized with AChR signals were quantified using images taken by Zeiss LSM710 or Nikon A1Rsi confocal microscope. All signal values were quantified by two blinded researchers using MetaMorph software (Molecular Devices).

Revised legends for Fig. EV3CD and 5A

Figure EV3. *Ctgf*^{-/-} diaphragm shows no significant differences in myogenic gene expressions, myofibril ultrastructure, width of the AChR band, or axonal branches of the phrenic nerves

(C-F) Representative low (C) and high (D) magnification images of the whole-mount left diaphragms at E18.5 stained for peripherin (anti-peripherin antibody, upper panels) and AChR (α -bungarotoxin, lower panels). Bar = 1 mm. (E) Blinded morphometry of the endplate bandwidth of AChR clusters in C. (F) Blinded morphometry of the 2nd and 3rd branches in C. Mean and SD ($n = 6$ left diaphragms) are indicated. p -value > 0.05 by one-way ANOVA for the three panels in E and F. Lack of statistical difference is indicated by a single letter “a”.

Figure EV5. *Ctgf*^{-/-} diaphragm shows mild to moderate electrophysiological abnormalities and different expression patterns for CCN family genes

(A) Representative low magnification images of the whole-mount left diaphragms at E18.5 stained for synaptophysin (green) and AChR (α -bungarotoxin, red). Confocal images of the NMJ are indicated in Fig. 5B. Bar = 1 mm.

Minor points

Comment 3-16: For electrophysiology study, which muscles were recorded? The description is not consistent in Methods and Results sections.

Answer 3-16: For MEPP, the diaphragm muscle in E18.5 embryo was recorded. For CMAPs, sciatic nerve-calf muscle in E18.5 embryo was recorded. As suggested, we rewrote the electrophysiology section to explain the methods in detail. A revised electrophysiology section in Materials and Methods was pasted in Answer 3-12 above.

Comment 3-17: The authors should describe the CMAP recording protocol in detail in Method section and show the original CMAP recording traces in figures.

Answer 3-17: Thank you for the suggestion. We explained the CMAP protocol in detail in Materials and Methods. A revised electrophysiology section in Materials and Methods was pasted in Answer 3-12. As suggested, we added representative CMAP traces in Fig. EV5D.

Added legend for Fig. EV5D.

Figure EV5. *Ctgf*^{-/-} diaphragm shows mild to moderate electrophysiological abnormalities and different expression patterns for CCN family genes

(D) Representative CMAP traces of wild-type and *Ctgf*^{-/-} mic at E18.5 in response to repetitive nerve stimulation at 2 Hz. Quantifications of MEPP and CMAP recordings are indicated in Table 2.

Comment 3-18: Fig. 1E, 1F, S1A, S1B, S2F, S2G, what are the letters above the error bars representing? These are not mentioned in either main text or figure legends.

Answer 3-18: We apologize for our lack of explanation. Each experimental condition was grouped according to the post-hoc Tukey test ($p < 0.05$) after one-way or two-way ANOVA. For example, values obtained from experimental conditions in the ‘a’ group were not statistically different. We explained this annotation in the section "Statistical analysis" in Materials and Methods, as well as in each instance of this annotation in relevant figure legends.

Revised statement in Materials and Methods

Statistical analysis

Statistical analyses including unpaired Student’s *t*-test, one-way or two-way ANOVA, and post-hoc Tukey test were performed using SPSS ver. 23 (IBM Corp). *P* values less than 0.05 were considered to be statistically significant. After one-way or two-way ANOVA, statistically similar items were grouped together according to the post-hoc Tukey test ($p < 0.05$), and each group was labeled by an identical single lowercase letter.

Dear Mrs. Ohkawara,

Thank you for the submission of your revised manuscript. We have now received the enclosed comments from the referees that were asked to assess it. Referee 3 has also assessed how well the concerns by referee 1 were addressed. S/he still raises a few points that will need to be addressed before we can proceed with the official acceptance of your manuscript.

A few other changes are also required:

Your manuscript has 5 main figure and should therefore be a scientific report with combined Results and Discussion sections. Please combine these sections, or add one more main figure to change to a full article. Given the rather short text, I think that a scientific report would be more appropriate.

Fig 3 has callouts to panels E+F, but they are missing from the figure itself.
Fig 5G callout is missing. Please correct/add.

Please upload the source data as one file per figure.

Fig EV4D is missing origin boxes and magnification bars for the inserts.

The 2 supplementary tables should be uploaded either as EV tables or should be regular tables in the manuscript file. Please also correct the callouts to these tables.

The funding information in EJP is JP15H05015 versus JP15H05014 in the manuscript. Please correct.

The synopsis image has incorrect dimensions. Please send us an image that is 550 pixels wide and up to 400 pixels high.

I would like to suggest a few changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

At the neuromuscular junction (NMJ), lipoprotein-related receptor 4 (LRP4) mediates agrin-induced MuSK phosphorylation that leads to clustering of acetylcholine receptors (AChRs) in the postsynaptic region of the skeletal muscle. Additionally, the ectodomain of LRP4 is necessary for differentiation of the presynaptic nerve terminal. However, the molecules regulating LRP4 have not been elucidated yet. Here we show that the CT domain of connective tissue growth factor (CTGF/CCN2) directly binds to the third beta-propeller domain of LRP4. CTGF/CCN2 enhances the binding of LRP4 to MuSK and facilitates the localization of LRP4 on the plasma membrane. CTGF/CCN2 enhances agrin-induced MuSK phosphorylation and AChR clustering in cultured myotubes. Ctgf-deficient mouse embryos (Ctgf^{-/-}) have small AChR clusters and abnormal dispersion of synaptic vesicles at the nerve terminal into the axon [what is "at the nerve terminal into the axon"? This is unclear, please rewrite]. Ultrastructurally, the presynaptic nerve terminals have reduced numbers of active zones and mitochondria. Functionally, Ctgf^{-/-} embryos exhibit impaired NMJ signal transmission. These results indicate that CTGF/CCN2 interacts with LRP4 to facilitate clustering of AChRs at the motor endplate and maturation of the nerve terminal.

Please also let me know if you agree with this summary and these bullet points:

The connective tissue growth factor CTGF/CCN2 is required for the formation of neuromuscular junctions (NMJ). CTGF functions together with the lipoprotein-related receptor 4 (LRP4) in pre- and post-synaptic regions of NMJ.

(i) CTGF/CCN2 increases agrin-mediated MuSK phosphorylation and induces clustering of acetylcholine receptors (AChR) in cultured myotubes via LRP4 binding.

(ii) *Ctgf/Ccn2*-deficient mouse embryos have severer defects at the presynapse than the postsynapse: (a) abnormal dispersion of synaptic vesicles into the axon, and (b) reduced numbers of active zones and mitochondria at the nerve terminal.

(iii) *Ctgf/Ccn2*-deficient mouse embryos have defective neuromuscular signal transmission.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript file.

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

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #2:

All my concerns were properly addressed.

Referee #3:

The revised manuscript has addressed all my previous concerns.

In my view, the authors have addressed most of three Reviewers' concerns, including Reviewer #1. I only have a few points that needs authors' clarification and/or discussion. These may also be concerned by Referee 1.

1. Fig. 1A, why there are bands with same size of CTGF-full-myc in the samples without CTGF-full-myc (lanes 2, 4, 7) in blot of lysates using Myc antibody? The authors should clarify or replace with another example.

2. Fig. 1B, In IP with Flag, CTGF signals are smear rather than clear bands. Is it due to any posttranslational modification, such as glycosylation? The authors should discuss this point.

3. Molecular weight (kDa) should be added to the blots of Fig. 1A and B to clearly indicate the size of individual proteins.

Specific comments for Editor

Thank you for your suggestions. As suggested, we corrected and highlighted in our manuscript.

Query 1: Your manuscript has 5 main figure and should therefore be a scientific report with combined Results and Discussion sections. Please combine these sections, or add one more main figure to change to a full article. Given the rather short text, I think that a scientific report would be more appropriate.

Answer 1: Thank you for your suggestion. As suggested, we combined Results and Discussion into a single section.

Query 2: Fig 3 has callouts to panels E+F, but they are missing from the figure itself.

Answer 1: We apologize for our mistakes. We changed Fig.3E and Fig. 3F in our manuscript to Fig. 3C and Fig. 3D, respectively.

Query 3: Fig 5G callout is missing. Please correct/add.

Answer 3: Thank you for pointing this out. We cited Fig. 5G at the end of the "Results and Discussion" section.

Query 4: Please upload the source data as one file per figure.

Answer 4: We made a total of 5 files showing the source data for Fig. 1ABC, Fig. 2ABCD, Fig. 3AE, Fig. EV1D and Fig. EV2BE.

Query 5: Fig EV4D is missing origin boxes and magnification bars for the inserts.

Answer 5: We added origin boxes in Fig. EV4 and a scale bar for the inserts.

Query 6: The 2 supplementary tables should be uploaded either as EV tables or should be regular tables in the manuscript file. Please also correct the callouts to these tables.

Answer 6: We renamed the supplementary tables to Table EV1 and Table EV2 in the Supplemental Information.

Query 7: The funding information in EJP is JP15H05015 versus JP15H05014 in the manuscript. Please correct.

Answer 7: We apologize for our mistake. JP15H05014 is correct. We corrected the error in EJP.

Query 8: The synopsis image has incorrect dimensions. Please send us an image that is 550 pixels wide and up to 400 pixels high.

Answer 8: We changed a synopsis image to 550 pixels wide and 400 pixels high.

Query 9: I would like to suggest a few changes to the abstract that needs to be written in present tense. Please let me know whether you agree with the following:

Answer 9: Thank you for your editions. We agreed with your corrections and revised our abstract as suggested.

Query 10: Please also let me know if you agree with this summary and these bullet points:

Answer 10: We agreed with your corrections and revised the summary and the bullet points as suggested.

Specific response to reviewer's comments

Referee #1

The revised manuscript has addressed all my previous concerns.

In my view, the authors have addressed most of three Reviewers' concerns, including Reviewer #1. I only have a few points that needs authors' clarification and/or discussion. These may also be concerned by Referee 1.

Thank you for your encouragement and valuable suggestions.

Comment 1-1: Fig. 1A, why there are bands with same size of CTGF-full-myc in the samples without CTGF-full-myc (lanes 2, 4, 7) in blot of lysates using Myc antibody? The authors should clarify or replace with another example.

Answer 1-1: As suggested, we optimized the immunoblotting condition to eliminate artifacts overlapping with CTGF-full-myc, and showed representative gel image in Fig. 1A.

Comment 1-2: Fig. 1B, In IP with Flag, CTGF signals are smear rather than clear bands. Is it due to any posttranslational modification, such as glycosylation? The authors should discuss this point.

Answer 1-2: Thank you for your valuable comment. Yes, CTGF protein has different posttranslational modifications including glycosylation. Multiple bands are indeed visible in a previous report by others (see attached below). We used high concentrations of acrylamide and glycerol to detect truncated short CTGF, which further made the CTGF bands broader. As suggested, we revised a relevant statement in "Results and Discussion".

Revised statement in Results and Discussion for Fig. 1AB.

CTGF protein has posttranslational modifications including glycosylation, and often showed multiple bands around 30- to 40-kDa in immunoblotting assays (Fig. 1A-C)[14,24].

An example of CTGF protein in an immunoblotting assay (Yang D. et al., Identification of glycosylated 38-kDa connective tissue growth factor (IGFBP-related protein 2) and proteolytic fragments in human biological fluids, and up-regulation of IGFBP-rP2 expression by TGF- β in Hs578T human breast cancer cells, 1998, Journal of Clinical Endocrinology and Metabolism. 83(7):2593-2596) [Figures for referees not shown.]

Comment 1-3. Molecular weight (kDa) should be added to the blots of Fig. 1A and B to clearly indicate the size of individual proteins.

Answer 1-2: As suggested, we indicated molecular weight markers in Fig. 1A and 1B for CTGF proteins.

Mrs. Bisei Ohkawara
Nagoya University Graduate School of Medicine
Division of Neurogenetics, Center for Neurological Diseases and Cancer
65 Tsurumai
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Nagoya, Aichi 466-8550
Japan

Dear Mrs. Ohkawara,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Journal Submitted to: EMBO reports

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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 χ^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?	Preliminary data originated in the laboratory and results from parallel experiments were used to estimate sample size.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We include a statement in Materials and Methods
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	When the animals became lethal, animals were excluded from CMAP analysis
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
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randomly used before the genotyping
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	All data were analyzed by two blind reviewers and the statement is included in Materials and Methods section
5. For every figure, are statistical tests justified as appropriate?	Yes. Every statistical analysis performed on the data is explained in the corresponding figure legend as well as in the materials and methods.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used SPSS software for reasonable data analysis.
Is there an estimate of variation within each group of data?	Yes

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<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor>

<http://grants.nih.gov/grants/olaw/olaw.htm>
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>
<http://ClinicalTrials.gov>
<http://www.consort-statement.org>
<http://www.consort-statement.org/checklists/view/32-consort/66-title>

<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum>

<http://datadryad.org>

<http://figshare.com>

<http://www.ncbi.nlm.nih.gov/gap>

<http://www.ebi.ac.uk/ega>

<http://biomodels.net/>

<http://biomodels.net/miriam/>
<http://jij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

Is the variance similar between the groups that are being statistically compared?	Yes
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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).	All antibodies and reagents are listed in Materials and Methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Name of cell lines are all written in Materials and Methods

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mouse strain and their ages are all written in Materials and Methods
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Statement for experiments involving mouse is written in Materials and Methods
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Mouse experiments were approved by the Animal Care and Use Committee of the Nagoya University, and were performed in accordance with the relevant guidelines.

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
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

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).	Raw data will be available for graphs and statistics upon request, and this is clearly stated in our manuscript for readers.
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 Biocompare (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
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