Multiple regions of junctin drive interaction with calsequestrin-1 and localization at triads in skeletal muscle

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DOI: 10.1242/jcs.259185

Editor: John Heath

Review timeline

Original submission:	26 July 2021
Editorial decision:	2 September 2021
First revision received:	29 October 2021
Accepted:	8 December 2021

Original submission

First decision letter

MS ID#: JOCES/2021/259185

MS TITLE: Multiple regions of junctin drive interaction with calsequestrin and localization at triads in skeletal muscle

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ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

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

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to

all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The muscle SR contains two major compartments; the junctional SR contributing to Ca2+ release and the longitudinal SR dedicated to Ca2+ uptake. Reflecting the different roles, the junctional and longitudinal regions have distinct protein component profiles. Despite of no barrier between the compartments, it is wondering why transmembrane and luminal SR proteins are correctly segregated.
This question is now one of main focuses in muscle biology, and the authors are probably working in this line. In the manuscript, they now report important findings in direct interactions between the junctional SR proteins, that regulate CSQ polymerization, modulate SR Ca2+ release and contribute to the triad targeting of junction. The present data in the manuscript are of high quality and largely exciting, and thus will attract broad attention from skeletal and cardiac muscle researchers.

Comments for the author

Minor comments:
1. The authors identified two KEKE domains in junction. Although the authors gave two reference reports, there seems to be no tight consensus sequence for the core domain. Briefly explain the criteria for the KEKE domains in the first Results section.
2. CSQ polymer formation in non-muscle cultured cells is an attractive finding (Fig. 2). Previous in vitro studies demonstrated that CSQ can be Ca2+-dependently polymerized. Do CSQ polymers disassemble in depleted ER elements? ER depletion in the cultured cells is probably induced by ionomycin treatments under Ca2+-free bathing conditions. It will be interesting to compare fluorescence images before and after ER depletion.

Reviewer 2

Advance summary and potential significance to field

The authors in this work aim to find the JNT protein sequence which interacts with CSQ by using deletions of the JNT protein in its luminal fragment. Their results might contribute to the understanding of the formation of a protein complex that assembles in the process of calcium release from the RS during the contraction of the skeletal muscle. Though the KO mouse for JNT in previous studies did not show an effect on SR calcium release experiments in skeletal muscle.

The models used include myotubes, myofibrils, isolated microsomes from skeletal and cardiac muscle, and transfected proteins (DNA) of interest (RyR, CSQ, and JNT) into HEK293T cells. They succeed in the recognition of three JNT sequences that interact with CSQ 1 and 2, whose physiological importance is not mentioned.

Comments for the author

Main concerns
Pag. 6 last paragraph states: “However, in experiments with solubilized skeletal muscles microsomes, calsequestrin-1, being more abundant might compete with calsequestrin-2 for binding...”
In the materials and methods and in the results section the muscles are not specified from where the microsomes were isolated. It is possible to isolate specific membrane fractions (Triads, SR terminal cisternae, junctional SR, and longitudinal SR) from fast and slow SM. Using specific membrane fractions it would be possible to obtain clearer results, my concern is why not obtain these fractions if already isolating microsomes from SM.
It is my assumption that the microsomes from SM were isolated from a mixed group of muscles, therefore there are fast and slow SM fibers, one containing mainly CSQ isoform 1 and the other

containing the CSQ isoform 2. If microsomes are isolated from a mixed group of muscles the fast SM is more abundant than slow SM in rodent mix fiber muscles. Therefore, that could lead to a misunderstanding in CSQ isoform participation in JNT interaction.
The GST-JNT fraction 49-140, which binds to both CSQ isoforms not necessarily is the result of competition in this experimental setup, as stated in the paragraph since the source of the CSQ is mixed. Perhaps if the authors search for the competitiveness of these JNC fragments (deletions) it could be done by other means in SM but still might not have a cell physiological meaning.

Page 9, second paragraph, “Assembly of mature triads is a multistep process beginning in the embryonic life and proceeding until the first weeks after birth.”

During myotube differentiation, fiber development, and the first period after birth, slow SM predominates, with the isoforms 2 for RyR, SERCA, and CSQ. The isoform 1 of fast SM is expressed in fast SM sometime after birth, at which time the fast SM is the predominant form in the adult rodent SM.
Since authors are interested in the interaction of JNT with both CSQ 1 and 2 in SM and given the major differences between fast and slow skeletal muscles, I recommend discussing more in terms of the physiological meaning of the difference found in this work. It is not necessary to include cardiac muscle to study the CSQ 2-JNT interaction, since there is a natural source of CSQ 2 within SM. However, as this is the case in this study, it would be relevant to discuss potential meaning in terms of the cardiac and SM differences to regulate calcium release during excitation-contraction coupling.

Page 9, paragraph 3, “To investigate the role of the intraluminal region of junction in the localization at the junctional compartment of the SR,”

The idea of expressing the suggested proteins in HEK293 cells, to avoid the interaction of other proteins, may not be ideal to understand the formation of the complex that regulates the calcium release of SR in the process of excitation-contraction coupling.
Caffeine-induced calcium release in the presence of either JNT or CSQ independently or in combination seems to have a very limited effect on the activity of RYR (Fig 2Q), thus raising doubts about the relevance of this interaction and its location in the triad, for the (physiological) function of RyR.
Deletions in JNT results in delocalization of the protein from the junctional SR observed by the sarcomere disruption shown in Figure 4, suggesting protein-membrane redistribution, and therefore JNT is no longer present in the JSR.
However, it may be considered to study in a direct manner the JNT fragments distribution and interactions in isolated SR terminal cisternae, junctional SR longitudinal SR from developed SM fibrils that express either specific CSQ 1 for fast SM or CSQ 2 in slow SM.
In the same Figure 4, it seems that the size bar=3um, is not the same in all myotubes shown in the boxes, it can be observed by the number and size of sarcomeres included in the bar.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

Minor comments:

1. The authors identified two KEKE domains in junctin. Although the authors gave two reference reports, there seems to be no tight consensus sequence for the core domain. Briefly explain the criteria for the KEKE domains in the first Results section.

The referee comment is correct since there in no tight consensus sequence for the core domain. As stated by Realini and coworkers in their 1994 paper, a KEKE motif is defined as an amino acid sequence greater than 12 residues in length, devoid of W, Y, F or P residues and containing more

than 60% K and E/D amino acids and lacking five positive or negatively charged residues in a row. In addition to the first KEKE motif previously identified based on sequence homology with the KEKE motif described in triadin, we report the identification of a second sequence which matches the KEKE definition, in the region between amino acids 118-132. A brief description of the criteria for KEKE domain identification has been added to the Result section to better clarify this point (**page 5, lines 120-127**). In addition, to improve the description of junctin sequence analysis, we also prepared a new **Figure 1S**, to better highlight the identified domains.

2. CSQ polymer formation in non-muscle cultured cells is an attractive finding (Fig. 2). Previous in vitro studies demonstrated that CSQ can be Ca2+-dependently polymerized. Do CSQ polymers disassemble in depleted ER elements? ER depletion in the cultured cells is probably induced by ionomycin treatments under Ca2+-free bathing conditions. It will be interesting to compare fluorescence images before and after ER depletion.

The point raised by the reviewer is correct. Following reviewer' suggestion, we performed experiments to deplete intracellular Ca2+ stores in HeLa cells expressing CASQ1-GFP or co-expressing CASQ1-GFP and junctin by means of ionomycin treatment. Time lapse images of CASQ1-GFP distribution were acquired by confocal microscope and are reported in **figure S4**. Analysis of acquired images showed that, as expected, Ca2+ depletion resulted in disassembly of CASQ1-GFP polymers with a redistribution of the GFP signal in the entire ER network. Data are shown in **new figure 2Q and 2R** and comments have been added in the Results section **(page 8, lines 214-224)** and in the Discussion section **(page 14, lines 387-390)**. The Materials and Method section has been accordingly modified (**page 22-23, lines 633-642**).

Reviewer 2

Advance Summary and Potential Significance to Field: The authors in this work aim to find the JNT protein sequence which interacts with CSQ by using deletions of the JNT protein in its luminal fragment. Their results might contribute to the understanding of the formation of a protein complex that assembles in the process of calcium release from the RS during the contraction of the skeletal muscle. Though the KO mouse for JNT in previous studies did not show an effect on SR calcium release experiments in skeletal muscle.

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In the materials and methods and in the results section the muscles are not specified from where the microsomes were isolated. It is possible to isolate specific membrane fractions (Triads, SR terminal cisternae, junctional SR, and longitudinal SR) from fast and slow SM. Using specific membrane fractions it would be possible to obtain clearer results, my concern is why not obtain these fractions if already isolating microsomes from SM.

The reviewer is correct when stating that it is possible to isolate specific membrane fractions from SM. However, the majority of protocols applied for similar experiments over the last decades, has made use of total microsomal fractions and there is no evidence that the presence of longitudinal SR and plasma membrane proteins might affect the results of pull-down or immunoprecipitation experiments.

It is my assumption that the microsomes from SM were isolated from a mixed group of muscles, therefore there are fast and slow SM fibers, one containing mainly CSQ isoform 1 and the other containing the CSQ isoform 2. If microsomes are isolated from a mixed group

of muscles the fast SM is more abundant than slow SM in rodent mix fiber muscles. Therefore, that could lead to a misunderstanding in CSQ isoform participation in JNT interaction. The GST-JNT fraction 49-140, which binds to both CSQ isoforms not necessarily is the result of competition in this experimental setup, as stated in the paragraph since the source of the CSQ is mixed. Perhaps if the authors search for the competitiveness of these JNC fragments (deletions) it could be done by other means in SM but still might not have a cell physiological meaning.

The reviewer's assumption about isolation of microsomes from a mixed group of mouse leg muscles is correct (**page 6, line 136**). We agree with the reviewer that expression of CASQ1 and CASQ2 varies among skeletal muscles. Indeed, the levels of CASQ2 in skeletal muscle are low even in slow muscle like soleus, while CASQ1 is always the most abundant isoform in all muscles, as shown now in **Figure S2,** which we included to make this point clearer (**page 5-6, lines 132-135**).

Actually, in re-reading the manuscript in light of the reviewer's comments we realized that the way these results were presented could be confusing for the readers. We did not want to analyze a potential competition between CASQ1 and CASQ2, as this is not the interest of this study. Therefore, we have simplified data presentation by showing CASQ1 binding using total microsomal fractions from hindlimbs muscles, which contain only minute amounts of CASQ2, and using microsomal fractions from cardiac muscle which only contains CASQ2, as shown in Figure S2. As presented now in the **new Figure 1A**, we are confident the evidence that both CASQ1 and CASQ2 can similarly bind most of the GST-junctin fusion proteins is more straightforward (**page 6, lines 150-163**).

Page 9, second paragraph, "Assembly of mature triads is a multistep process beginning in the embryonic life and proceeding until the first weeks after birth." During myotube differentiation, fiber development, and the first period after birth, slow SM predominates, with the isoforms 2 for RyR, SERCA, and CSQ. The isoform 1 of fast SM is expressed in fast SM sometime after birth, at which time the fast SM is the predominant form in the adult rodent SM. Since authors are interested in the interaction of JNT with both CSQ 1 and 2 in SM and given the major differences between fast and slow skeletal muscles, I recommend discussing more in terms of the physiological meaning of the difference found in this work. It is not necessary to include cardiac muscle to study the CSQ 2-JNT interaction, since there is a natural source of CSQ 2 within SM. However, as this is the case in this study, it would be relevant to discuss potential meaning in terms of the cardiac and SM differences to regulate calcium release during excitation-contraction coupling.

We thank the reviewer for his/her comment. We agree that there is a natural source of CASQ2 in skeletal muscle; however, in skeletal muscles CASQ2 is present in minute amounts and, even in slow muscles, it is always co-expressed with the predominant calseqeustrin-1 isoform. That is the reason why, for comparison, we included in our studies, as a source of calsequestrin-2, microsomes from cardiac muscle, where only CASQ2 is expressed. Based on the results obtained, we can conclude that CASQ1 and CASQ2 can both interact with junctin at the same binding sites (**page 6, lines 150-163)**

At the same time, we were not interested and data reported in our manuscript do not allow us to assess whether the regulation of excitation contraction coupling in cardiac and skeletal muscle is significantly influenced by interaction of junctin with CASQ1 or CASQ2.

Page 9, paragraph 3, "To investigate the role of the intraluminal region of junction in the localization at the junctional compartment of the SR," The idea of expressing the suggested proteins in HEK293 cells, to avoid the interaction of other proteins, may not be ideal to understand the formation of the complex that regulates the calcium release of SR in the process of excitation- contraction coupling. Caffeine-induced calcium release in the presence of either JNT or CSQ independently or in combination seems to have a very limited effect on the activity of RYR (Fig 2Q), thus raising doubts about the relevance of this interaction and its location in the triad, for the (physiological) function of RyR.

We agree with the reviewer observation that the effect of junctin and/or calsequestrin on caffeine- induced calcium release mediated by RyR1 in HeLa cells is limited. Indeed, as reported

in the discussion, the physiological function of this interaction is still debated and has not been completely defined. We thus moved Figure 2Q to supplemental material (**Figure S3**).

Deletions in JNT results in delocalization of the protein from the junctional SR observed by the sarcomere disruption shown in Figure 4, suggesting protein- membrane redistribution, and therefore JNT is no longer present in the JSR. However, it may be considered to study in a direct manner the JNT fragments distribution and interactions in isolated SR terminal cisternae, junctional SR, longitudinal SR from developed SM fibrils that express either specific CSQ 1 for fast SM or CSQ 2 in slow SM.

Data shown in figure 4 were obtained with in vitro differentiating skeletal muscle myotubes, in the attempt to follow localization of deletion mutants and full-lenght junctin proteins during differentiation. Unfortunately, since there is no skeletal muscle expressing exclusively CASQ1 or CASQ2 such studies are not possible to perform.

In the same Figure 4, it seems that the size bar=3um, is not the same in all myotubes shown in the boxes, it can be observed by the number and size of sarcomeres included in the bar.

We apologize for the inaccuracy. Size bars have been resized in each panel set.

Second decision letter

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AUTHORS: Daniela Rossi, Stefania Lorenzini, Enrico Pierantozzi, Filip Van Petegem, David Osamwonuyi Amadsun, and Vincenzo Sorrentino
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In response to all of the comments, the authors made wholehearted responses. The revised manuscript includes adequate modifications and new results, and thus is now in very good shape and ready for publication.

Comments for the author

none