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Peer Review File

Muscle-specific Ryanodine Receptor Properties Underlie Limb-Girdle Muscular Dystrophy 2B/R2 Progression.

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors demonstrate that the psoas muscle in mice, unlike the leg muscle (tibialis anterior), exhibits elevated RyR1 Ca leak. Dysferlin-null mice, a model for Limb-Girdle Muscular Dystrophy 2B, exhibit severe pathology in the psoas due to disrupted control over RyR1, leading to excessive Ca leak. This condition affects Ca handling and ROS production, contributing to disease onset and progression. Furthermore, aging exacerbates these effects through further alterations in Ca dynamics and ROS levels. The investigation provides significant insights into the pathological mechanisms of muscular dystrophy, however, there are critical concerns that need to be addressed to fully validate the findings.

Major concern:

1. The authors employed Rhod-5N to assess the Ca2+ levels within the sealed T-system, acknowledging that T-system Ca dynamics are influenced by changes in cytosolic Ca, such as leakage through RyR1. However, it is crucial to recognize that other factors and mechanisms may also impact these dynamics. For instance, alterations in the coupling between the SR and T-system could modify T-tubule Ca dynamics independently of changes in RyR1 Ca leakage. Moreover, given Dysferlin's critical roles in membrane and T-tubule integration, its deficiency could potentially affect the structure of t-tubules and their calcium coupling efficiency, suggesting that measurements of t-tubule Ca may not solely reflect SR Ca leakage. These concerns suggest that a more integrated methodological approach, incorporating direct measurements of both cytosolic and SR luminal Ca alongside t-tubule dynamics, might provide a more comprehensive understanding of calcium homeostasis alterations in muscular dystrophy. Addressing these methodological considerations is essential for accurately interpreting the results and could significantly strengthen the study’s conclusions.

2. The paper's title suggests a significant focus on the thermogenic properties underlying Limb-Girdle Muscular Dystrophy 2B progression. However, upon detailed review, it becomes apparent that the study primarily concentrates on the mechanistic insights into RyR1 Ca leak dynamics, dysferlin's role in muscle pathology, and alterations in calcium handling across muscle types, with less emphasis on directly linking these phenomena to thermogenesis. While the study suggests the psoas muscle as a thermogenic center and explores its unique protein expression related to thermogenesis, these findings are somewhat isolated within the broader context of the paper's focus on muscular dystrophy pathology. The analysis of thermogenic capacity, primarily through proteomic profiling indicating an enrichment of thermogenesis-related proteins in the psoas, offers valuable insights but is not thoroughly further investigated and integrated into the narrative of muscle-specific pathology development.

Minor concerns:
Figure 1J (video)
The wave frequency of TA starts at 3mM with some fast and robust waves initially. This does not happen in Psoas. Do all TAs behave like this? What is the potential explanation for this phenomenon?
Figure 2f
The connected dots should represent paired data. Here, it should not be paired since it is two different groups.
Figure 5
Lacking biochemical data for oxidation."

Reviewer #2

(Remarks to the Author)
The manuscript of Meizoso-Huesca and colleagues investigates changes in calcium (Ca^{2+}) cycling in a mouse model of Limb Girdle Muscular Dystrophy 2b (LGM2b). Moreover, the authors investigate changes in muscle Ca^{2+} cycling in two different muscles being the psoas and the tibialis anterior. The paper utilises a series of elegant and sophisticated techniques demonstrating muscle-dependent changes in Ca^{2+}cycling and storage due to altered phosphorylation of the ryanodine 1 receptor (RyR1) in the dysferlin-null BLaJ mice.
The following key findings were observed:
1. Altered Ca^{2+} handling was evident in the psoas muscle and not the tibialis anterior.
2. Dysferlin deletion was associated with increased Ser2844 phosphorylation of RyR1 and greater Ca^{2+} leak
3. The increased Ca^{2+} leak modified tubular handling of Ca^{2+} leading to increased cytosolic and mitochondrial Ca^{2+} levels
The authors have linked these key modifications to functional differences including disease progression and/or altered thermogenic and metabolic function.
Overall, the authors conclude that the psoas muscle has greater RyR1 leakage, leading to functional differences with altered metabolic and thermogenic functions. However, this is not directly tested. The proteomic and SWAT workflow analyses support altered expression patterns of thermogenic and metabolic factors. This would be greatly strengthened with the inclusion of physiological studies measuring heat production as a direct assessment of thermogenesis as well as differences in energy balance, glucose metabolism and fatty acid oxidation.
On the other hand, the authors suggest that altered Ca^{2+} handling increases the production of ROS leading to LGM2b disease progress. Can this be reversed with treatment of anti-oxidants?
The measurements of Ca^{2+} handling are indeed elegant and extensive. However, the significance of the work would be increased by showing that these changes have direct effect on physiological systems.

Reviewer #3

(Remarks to the Author)
Dysferlinopathies in man typically affect young adults and different muscle groups, but the reasons for the late onset and distinctive muscle specificity are unknown. This submission by Meizoso-Huesca, et al., presents evidence that begins to address these questions. The idea they test is that the leak of Ca ions through the ryanodine receptors, RyR1, is greater in muscles that are more severely affected than in less affected muscles, and that over time this leak leads to ROS-induced damage that further exacerbates the dysregulation of Ca homeostasis. The authors use an elegant method of imaging Ca leak through the RyR1 and its clearance across the t-tubule membrane to compare psoas muscles in dysferlin-null BlaJ mice to WT controls and to TA and EDL muscles as well. Psoas muscles are known to show more severe pathology than TA or EDL muscles in mice, and, the authors suggest, this property is linked to its predisposition to being more thermogenic. The results of an extensive series of experiments lend support to the central hypothesis of the paper, though some questions about their interpretation remain. The physiological studies are supplemented by mass spectrometric comparison of the proteomes of psoas and EDL muscles of WT mice, the differences in which are consistent with the a greater role of the psoas muscle in thermogenesis in mice. These are comprehensive and in many ways compelling studies that, with some additions and adjustments, will contribute significantly to our understanding of the role of dysferlin in skeletal muscle.

The paper would be strengthened by consideration of the following.

Relevance to LGMDR2: Human dysferlinopathies target the limb and limb girdle muscles first, which are not likely to be as thermogenic as the muscles of the chest, abdomen and back. How might the authors reconcile the pattern of pathogenesis in man with their observations?

Mouse strains: How many generations separate the C57Bl/6 mice and the BlaJ mice studied here? The differences in levels of psoas MYH7 (Sup. Fig. 5) are larger than one might expect for BlaJ mice that were rederived from the parent C57Bl/6 line recently.

Fatty infiltration: The Grounds laboratory has reported that dysferlin-null psoas muscle accumulates large amounts of lipid, especially with age. The authors do not mention making a similar observation. Does this not occur in their mice? If it does, how might it alter the interpretation of their results?

Proteomics: Fig. 4 shows that the proteome of WT TA and psoas muscles of 8 week old mice are different. Why was a similar analysis not performed with young BlaJ mice?
Fiber type: Although the authors examine the myosin heavy chains in Supp. Fig. 5, there is no discussion of the differences they report (e.g., in Myh7 between BlaJ and WT psoas), which may indicate a higher content of type 1A fibers in the BlaJ muscle. The possibility that the other proteomic differences which they report (in accord with earlier papers) may play a role in the outcome of their experiments would be consistent with their DDA analysis of WT psoas (Fig. 4), which shows the pathway with the highest level of significance to be “metabolic”, rather than “thermogenesis”.

DHPR-RyR1 coupling: The data in Fig. 2a,b, suggest that the absence of dysferlin alters the coupling of DHPR to RyR1. The data in Fig. 2b,c further suggest that most of the Ca in the t-system is due to leak through the DHPR and not the RyR1. If this is correct, how might the authors reconcile it with their hypothesis?

Additional concerns:

Is the difference in Fig. 1i between the psoas muscles of WT and BlaJ mice significant? If not, shouldn't it be?

Why is the Ca concentration in the t-system in BlaJ mice treated with cAMP lower than the concentration in its absence (compare Fig. 2b and 2e)? Shouldn't it be higher if cAMP promotes
phosphorylation of S2844 to enhance RyR1 Ca leak?

Are the curves shown in Fig. 5s significantly different? If not, shouldn't they be?

In Fig. 6b, BlaJ psoas +RyR1 leak is depicted as being lower than -RyR1 leak. Is this correct?

In Sup. Fig. 4, there is no significant difference in mitochondrial Ca content of psoas muscle in 8 and 36 week old BlaJ mice. If ROS generation associated with pathology is linked to accumulation of Ca in the mitochondria, shouldn't the older muscle have significantly higher levels?

Methods:

What is the range of concentrations of BAPTA used? Re BAPTA, the authors may wish to refer to PMID 36406982, which suggests that the triad junction is a privileged space where BAPTA is active when applied at very low concentrations.

How was the free [Ca] calculated? The reference is missing from the text.

Why was such a high concentration (50 mM) of EGTA chosen?

Editorial:

Re the MS results, it would be helpful to have a supplementary figure showing the amounts of the different peptides and their identities (sequences) that contributed to the conclusion that phosphorylation and oxidation were enhanced in psoas muscles. Extracting this information from the tables is a challenge!

A table of abbreviations would be helpful.

The Results section is sometimes difficult to read, as it includes aspects of the Methods and Discussion sections.
Psoas and dysferlin do not need to be capitalized in the text.

The formatting of the text when it refers to the figures is not consistent (such as Fig. X). Dots and spaces should be uniform.

Reviewer #4

(Remarks to the Author)
Summary
Limb Girdle Muscular Dystrophy 2B (LGMD2B) is caused by deficiency the protein dysferlin and presents with late-onset selective wasting of specific muscle groups. The functional role for dysferlin is most clearly established in membrane repair mechanisms, thereby implicating a possible disease pathogenesis in LGMD2B. Dysferlin is predominantly located in transverse tubule membranes, where in addition to promoting membrane repair, dysferlin interacts with other junctional proteins (RyR1, DHPR) at the triad formed by the T-tubule and terminal cisternae of the sarcoplasmic reticulum (SR) to regulate calcium homeostasis. In this study, the authors have explored the hypothesis that dysferlin-dependent mechanisms of Ca handling in the junctional space contribute to the selective vulnerability of psoas muscle as compared to much milder pathology of the tibialis anterior (TA) in a dysferlin-deficient mouse model (BlaJ) of LGMD2B.
The experimental approach is based on the exceptional access to measurement and manipulation of compartmentalized Ca in the T-tublules and of the sarcoplasmic reticulum with optical dyes in the skinned muscle fiber preparation. The primary observations are:

• Ca "leak" via the calcium release channel (RyR1) of the SR is prominent for WT psoas, but essentially undetectable for the TA muscle (Fig. 1). Phosphorylation of Ser2844 is known to increase RyR1 leakiness and was 3-fold higher for psoas compared to TA.

• Dysferlin deficiency exacerbated the RyR1 Ca leak in psoas muscle from young mice (8 week), but not for the TA.

• The RyR1 leak disrupts Ca distribution at the triad for psoas muscle in BlaJ mice such that: (1) SR Ca content is reduced, (2) mitochondrial Ca content is increased, (3) total Ca content is preserved (SR + mito + cytosol), Fig. 3.

• The increased Ca load to mito is predicted to increase ROS, and oxidation-dependent changes in Ca handling were detected by sensitivity to N-acetylcysteine (Fig. 5). Oxidation decreased the efflux rate for the plasmalemmal Ca ATPase pump (PMCA) and decreased the rate of Ca influx via store-operated Ca entry (SOCE).

• With aging (36 weeks vs 8 weeks) the pathologic changes of the psoas in dysferlin-deficient mice are exacerbated, with increased oxidation of RyR1, a more severe depletion of SR Ca, and additional anomalies of junctional Ca handling that may be a consequence of chronic persistent low-level SOCE.
The authors conclude that the more thermogenic profile of psoas muscle, better optimized for Ca-dependent ATPase activity and generation of heat compared to TA, produces an increased susceptibility to Ca overload, oxidative damage and dystrophy in dysferlin-deficient muscle. This mechanism may contribution to the muscle-specific dystrophic changes in LGMD2B.

Critique:
This study shows clear evidence of muscle-specific differences in Ca handling at the triad of skinned fibers. These changes are consistent with increased Ca leakiness of RyR1 for psoas relative to TA that are further exacerbated in the dysferlin-deficient mouse. This difference may contribute to the increased susceptibility for dystrophic changes in the psoas vs TA in the BlaJ mouse model of LGMD2B. The key question is the specificity for the interpretation of the Ca transients.

Specific Concerns.

(1) The authors are leaders in the field for the development and use of the skinned muscle fiber preparation to interrogate Ca handling of the triad. The preparation, however, is far from an intact muscle, depends on consensus agreement for interpreting the Ca transients (e.g. that tetracaine-sensitive fluorescence transients of the T-system are a robust measure of RyR1 leak), and rely on computational modeling (distribution of Ca amongst membrane compartments). This concern Is not meant to reject the use of the skinned fiber preparation to investigate muscle disease. The reader's confidence in the author's interpretation would be strengthened, however, if more detail or examples could be provided in the Discussion that these new mechanistic insights are consistent with observations from other studies using intact fibers, whole muscle, motor performance, or histopathological studies.

(2) The functional changes in PMCA and SOCE for BlaJ psoas (Fig. 5) raise concerns about the interpretation of [Ca]t-sys as a specific indicator of RyR1 leak. The steady-state [Ca]t-sys is expected to be set by a balance of extrusion (PMCA) and depletion (SOCE), which in turn depends on [Ca]js that is strongly influenced by the RyR1 leak. The tet-sensitive change in [Ca]t-sys is used to extract the RyR1 leak-dependent component from the total change. SOCE is also tet-sensitive (e.g. Fig 6), however, so how can the authors be confident that the tet-sensitive change in [Ca]t-sys in Figs. 1 and 2 are primarily a reflection of RyR1 leak versus a contribution from SOCE? Could the experiments of Fig. 1 be performed in the presence of BTP2 to exclude a contribution of SOCE to the tet-sensitive component of the [Ca]t-sys transient?

(3) In the analysis of Ca handling process for the T-system (Fig. 5), what about a contribution from the Na/Ca exchanger (NCX)?

(4) For the studies in aged mice (Fig. 6), the unexpected increase of psoas [Ca]t-sys by Tet is difficult to reconcile. While the proposal of an aberrant chronically active SOCE (also Tet-S) is consistent with the BTP2 data in Fig. 6C, other aspects are less satisfying. For example, if chronic activity of SOCE occurs form worsening RyR1 leak with aging, then why isn't the Tet-dependent decrease of [Ca]t-sys in the presence of BTP2 larger than for the comparable change in the psoas of young mice? Fig. 6 G does show progressively increased oxidation of psoas RyR1 with aging, but where are the data to quantitatively show increased Ca leak of psoas RyR1 with aging?

(5) Can confidence limits graphically indicated for the compartmental distribution of Ca shown for the model calculations of Figs 3f and 6h? The confidence levels of the underlying [Ca]t-sys measurements are clearly indicated, but it is difficult to understand how this level of uncertainty will propagate to the model calculations. This information is important because the age-dependent difference for psoas Ca_SR is modest (0.95 vs 0.79, 36 wk vs 8 wk) and unexpectedly there is a detectable difference for TA (1.27 vs 1.11, 36 wk vs 8 wk). The figure legend provides confidence levels for these differences (as p-values), but the SEM or some equivalent should be shown on the bar graphs.

Minor Points.

Page 7, 4th line from the bottom. Fig. 1e should be Fig. 1j.

Page 17, first line. Fig. 6e should be Fig. 6f.

Methods, page 23. The paragraph beginning with "For high temporal resolution real-time…" is duplicated.

Reviewer #5

(Remarks to the Author)
In the manuscript, muscle thermogenic properties underlie limb-girdle muscular dystrophy 2B (LGMD2B) progression, the author presents data that in murine psoas muscle, RyR1 channels operate in a leaky state and elaborate on that the loss of dysferlin further exacerbates RyR1 instability and leak, contributing to the localized progression of the disease in this muscle. The manuscript is focused on Ca2+ and the authors are world-renowned for their Ca2+ recording.

The manuscript provides a careful characterization of the Ca2+ signals in the early phase of the disease and compares two

muscle types with wildtype control. However, there are a range of unanswered questions that need to be clarified. For instance, limb-girdle muscular dystrophy (LGMD) belongs to a group of inherited muscle diseases where the muscle fibers slowly break down and are replaced by connective tissue and fat. The breakdown leads to muscle weakness and muscle atrophy. If RyR1 is an important driver of LGMD2B, the degree of leak should then be transferred onto other parameters. For instance, will a reduction of RyR1 Ca2+ leak protect muscle from increased fat/fibrosis accumulation and atrophic signaling?

Elevations in tetanic Ca2+ are required for force production and as the authors reference enhanced RyR1 leak has been shown to contribute to muscle weakness. The level of leak observed in LGMD2B psoas, is that making the muscle weaker or more fatigable already at this earlier timepoint?

The authors show that there is a difference in the mitochondrial Ca2+ content and that psoas from BlaJ mice have the highest mitoCa2+ uptake, but the manuscript lacks information regarding whether this difference is large enough to have any functional effects. There are Ca2+-dependent kinases in the mitochondria, are these affected by the alterations in mitoCa2+ content? Moreover, does differences in mitoCa2+ result in any differences in mitochondria oxidative phosphorylation capacity?

On the same note, the authors present interesting enrichment analyses of their proteomics data set and highlight that NADH regeneration appears altered, but it is not clear if that is a direct consequence of the mitochondrial Ca2+ or that the overall changes in Ca2+ (mito, cytosol, influx) has downstream effects on protein translation. Assessment of NAD/NADH levels and other metabolites e.g. AMP, ADP, ATP needs to be measured in the mouse models to understand this in more detail.

By utilizing targeted mass-spectrometry that authors show that psoas muscle (WT and BlaJ) exhibit higher RyR1 phosphorylation levels as compared to TA muscle (WT and BlaJ). This phosphorylation is assumed to be PKA derived and indeed the authors show that adding cAMP to the muscle enhances the RyR1 leak. The authors assume this to be PKA derived phosphorylation, and thus suggest that the beta-adrenergic signaling should be more pronounced in psoas fibers as compared with TA fibers. Hence suggest that muscle as baseline has intrinsic variation in the baseline beta-adrenergic signaling and further exacerbated in BlaJ genetic background, this needs to be further assessed.

In previous work, the phosphorylation of RyR1 has been linked to reduced FKBP12 binding and channel instability with the higher the phosphorylation the lower the FKB12 occupancy. What level of RyR1-FKBP12 binding is present in psoas and TA wildtype versus BlaJ?

Furthermore, the authors state that “comparable trends were observed across Blaj muscle Fig 1I”, although this does not match the graph. BlaJ psoas phosphorylation levels appear to have a very large spread, ranging from 1-10. Thus, it is not accurate to say that they are comparable. Instead, one wonders what this spread is the result of. Is there a vast difference in the b-adrenergic signaling and thus a difference in the phosphorylation levels or is there a difference in the phosphatase activities in these muscles. A careful assessment of the spread in phosphorylation levels is needed.

In addition to altered intramuscular Ca2+ levels linked to DHPR-RyR1, SOCE is also examined. The authors propose that there is reduced Ca2+ fluxes of the t-tubular system in BlaJ muscle as a result of increased oxidative stress in the muscle. However, it is unclear where the oxidative stress originates from which should be clarified. Also, RyR1 is known to be a redox sensitive channel and there is also data suggestion that b-adrenergic signaling may enhance ROS production. If SOCE in t-tubular system is altered by ROS it is likely that RyR is as well, please assess the RyR1’s redox state in the WT and BlaJ groups.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have changed the title of manuscript from “Muscle Thermogenic Properties Underlie Limb-Girdle Muscular Dystrophy 2B Progression” to “Muscle-specific Ryanodine Receptor Properties Underlie Limb-Girdle Muscular Dystrophy 2B/R2 Progression”. Such a topic has been reported by other investigators, which reduce the novelty of the current study.

Integration of Thermogenesis Findings

The paper's title initially suggested a primary focus on thermogenesis in the context of Limb-Girdle Muscular Dystrophy 2B (LGMD2B), which demonstrated great novelty. However, the actual content primarily addresses RyR1 Ca2+ leak dynamics and dysferlin's role in muscle pathology, with minimal data supporting the mechanism of thermogenesis. A more detailed investigation into how these thermogenic properties directly influence the progression of LGMD2B and their mechanistic interplay with Ca2+ handling anomalies would significantly strengthen the narrative. The current presentation isolates these findings rather than weaving them into a cohesive story that enhances our understanding of the disease's pathology.

Broader Implications for Muscle Pathology and Function

The broader implications of RyR1 Ca2+ leak for muscle pathology and disease progression of LGMD2B are not adequately

addressed. If RyR1 leak is a significant driver of LGMD2B, then mitigating this leak should demonstrably protect against muscle degeneration and disease progression. Demonstrating how interventions that reduce RyR1 leak translate to improved muscle integrity and reduced fibrosis would be critical. For instance, Khodabukus (2024) recently showed that dysferlin-deficient mice treated with the RyR inhibitor dantrolene or the L-type calcium channel blocker diltiazem restore LGMD2B functionality. They also demonstrated that chemically induced chronic RyR leak in healthy myobundles phenocopies LGMD2B contractile and metabolic deficits.

The authors need to address these substantial issues through a more integrated methodological approach, provide stronger quantitative evidence for their claims, and directly link their findings to functional outcomes in muscle pathology and thermogenesis regulation.

Reviewer #3

(Remarks to the Author)
The authors have responded adequately to many of my earlier comments. The following remain to be considered, however.

Relevance to LGMDR2: Human dysferlinopathies target the limb and limb girdle muscles first, which are not likely to be as thermogenic as the muscles of the chest, abdomen and back. How might the authors reconcile the pattern of pathogenesis in man with their observations?

Thanks for raising this interesting point. Extrapolating muscle-specific conditions from murine models to humans is quite challenging, and limitations as well as key differences between the organisms should be considered. Of great importance are the biomechanical properties of the limb muscles, which differ significantly between rodents and humans. For instance, the extent of muscle excursion during a gait cycle is higher in the majority of limb muscles of humans compared to their murine counterparts. Of particular interest, during a gait cycle, the human gastrocnemius (typically first muscle to show problems in dysferlinopathy patients) has an excursion ~2 times larger than mouse gastrocnemius. We believe this difference is key in providing a constitutively higher basal stress to human limb muscles compared to mouse muscles, which is likely a pathogenic contributor to the onset and development of the condition in humans. We have now raised this point in the Discussion.

Doesn't this mean that in man, the relationship between thermogenesis and dysferlinopathy is weak, at best? If other factors (the authors mention mechanical stress) play a more important role in pathogenesis, why not simply induce more stress rather than focus on thermogenesis?

Mouse strains: How many generations separate the C57Bl/6 mice and the BlaJ mice studied here? The differences in levels of psoas MYH7 (Sup. Fig. 5) are larger than one might expect for BlaJ mice that were rederived from the parent C57Bl/6 line recently.

Mice were purchased from a commercial colony. We cannot easily answer your question. Regardless, we have focussed our work on single fibres, and one fibre type.

This remains a serious question. If there has been genetic drift between the two strains of mice compared in this paper, as the data on MYH7 suggest, then the proteomic data become suspect.

Fatty infiltration: The Grounds laboratory has reported that dysferlin-null psoas muscle accumulates large amounts of lipid, especially with age. The authors do not mention making a similar observation. Does this not occur in their mice? If it does, how might it alter the interpretation of their results?

We show histology in Fig 1 and note fatty infiltration in older psoas muscle. Yet, this does not alter the interpretation of our results. We have focused on the changes in the physiology within the muscle fibres, which will be a primary event in dysferlinopathy. That is, Ca2+ handling changes in the psoas fibres occur in the young mouse, prior to fat infiltration.

This is certainly true for the physiological studies of Ca handling, but it is unlikely to be true for the proteomic analysis, which should show differences based at least in part on a shift from muscle to fat.

Proteomics: Fig. 4 shows that the proteome of WT TA and psoas muscles of 8 week old mice are different. Why was a similar analysis not performed with young BlaJ mice?

Yes, it was. However, our focus was on the differences in the wild type, between psoas and TA, which represented the primary deviation between the muscles that provide an explanation for the differences that are exacerbated in the Blaj. We decided these latter results add little to the manuscript.

I suggest including these results as Supplemental Information.

Fiber type: Although the authors examine the myosin heavy chains in Supp. Fig. 5, there is no discussion of the differences they report (e.g., in Myh7 between BlaJ and WT psoas), which may indicate a higher content of type 1 fibers in the BlaJ muscle. The possibility that the other proteomic differences which they report (in accord with earlier papers) may play a role in the outcome of their experiments would be consistent with their DDA analysis of WT psoas (Fig. 4), which shows the pathway with the highest level of significance to be “metabolic”, rather than “thermogenesis”.

We don’t see any statistically significant difference in the fibre type proportions among the groups that would affect or explain our results as any alternative to what we have presented in the manuscript. The fibre types expressed were 25-30% type IIx; 60-65% type IIb; and ~5% type I fibres across the four groups (Fig S5). Regarding Ca2+ handling, the results are drawn exclusively from type II fibres (we separate out the low probability of selecting any type I fibres with the functional response to high Ca2+ in the presence of myosin-II ATPase inhibitor BTS in the t-system experiments; and the response to Sr2+ in the bapta lysis experiments on the force transducer). We have now made these statements in the Methods.

The proteomic studies point to more significant differences in the “metabolic” than in the “thermogenic” pathways. These can occur even when fiber type analyses do not show significant differences. The authors should address this result, which does not fully support their hypothesis.

Additional concerns:

Is the difference in Fig. 1i between the psoas muscles of WT and BlaJ mice significant? If not, shouldn’t it be?

The p value for this comparison comes to 0.0527. Based on the values of BlaJ and the current spread of the population, our impression is that this trend would likely be significant if more experiments were conducted.

They should be done.

Why is the Ca concentration in the t-system in BlaJ mice treated with cAMP lower than the concentration in its absence (compare Fig. 2b and 2e)? Shouldn’t it be higher if cAMP promotes
phosphorylation of S2844 to enhance RyR1 Ca leak?

These are different, individual fibre experimental traces that come from different muscles; 2b corresponds to a psoas fiber experiment, where the effect of nifedipine was tested to explore a potential role of DHPR on the RyR1 leak. One the other hand, 2e corresponds to TA fiber experiments, where RyR1 phosphorylation was induced by cAMP exposure (Singh et al. 2023). It is possible that the cAMP treatment followed is not sufficient for the TA fiber to reach the levels observed in psoas, nor alter its physiology to the same extent. In addition, there may be natural variation between fibres that account for the difference in steady state [Ca2+]t-sys. Also, as with any experimental measurement, there is error between calibrations of each fibre. Regardless, we are confident in the summary of data that we present and the conclusions we have drawn. These traces accurately represent the physiology being described.

This point should be discussed in the text or in Supplemental Information.

In Fig. 6b, BlaJ psoas +RyR1 leak is depicted as being lower than -RyR1 leak. Is this correct?

Yes.

Unless I am missing something, this result is inconsistent with the argument that Ca leak into the t-system is greater in aged Blaj psoas muscle.

In Sup. Fig. 4, there is no significant difference in mitochondrial Ca content of psoas muscle in 8 and 36 week old BlaJ mice. If ROS generation associated with pathology is linked to accumulation of Ca in the mitochondria, shouldn’t the older muscle have significantly higher levels?

We suspect that the Ca2+ content of the mitochondria (the steady state) is approaching saturation of the relationship between mitochondrial Ca2+ and cytoplasmic Ca2+ in these old psoas fibres from BlaJ mice. As mitochondrial Ca2+ content is higher, the resolution power decreases, impeding us from resolving mild differences at those levels (Lamboley et al. 2021).

The Lamboley et al paper seems to show that the differences in mitochondrial Ca levels under conditions of high, moderate and low leak are almost aways significant. Thus, this result is unexpected and inconsistent with the hypothesis of the paper. At the least, it needs to be discussed.

Methods:

What is the range of concentrations of BAPTA used? Re BAPTA, the authors may wish to refer to PMID 36406982, which suggests that the triad junction is a privileged space where BAPTA is active when applied at very low concentrations.

Bapta was used in the µM to low mM range.

This should be stated in Methods.

Why was such a high concentration (50 mM) of EGTA chosen?

This was done to heavily buffer the bulk cytoplasmic [Ca2+]. This assists in detecting a difference in [Ca2+]cyto and [Ca2+]JS, which is critical for measuring RyR Ca2+ leak (Cully et al. 2018).

This should be stated in Methods.

Reviewer #4

(Remarks to the Author)
In the revised manuscript and the extensive point-by-point responses to the Reviewers, the authors have addressed all of the prior concerns. Important changes include: (1) a shift of emphasis to the well-documented pathologic Ca leak by RyR1 in dysferlin-deficient muscle, rather than emphasizing an inference to thermogenic properties, (2) new additional data in Fig. 4 (NE levels, metabolite ratios, mito membrane potential)to support a role for psoas muscle in thermogenesis.

Reviewer #5

(Remarks to the Author)
The authors have responded to the reviewers comments in an appropriate way. However, the response had not been added into the manuscript which is needed so not only reviewers will benefit from the responses but, more importantly, the potential readers.

Reviewer #6

(Remarks to the Author)
This manuscript provides an interesting finding which has been suspected but not directly shown . Especially the role of back psoas muscle in muscle thermogenesis . Most interesting finding is how an increased Ca2+ leak due to lack of coordination between DHPR and RYR can be detrimental to muscle physiology and metabolism due to increased Ca2+ leak in a dysferlin KO model. Key experiments document that this is the case .Data support the conclusions.

I dont believe that we need additional experiments to prove that Dysferlin is critical foe t-tubular integrity and RYR Ca2 release function . Ryr leak has been associated with thermogenesis ever since malignant Hyperthermia was discovered . Actually I am surprised that the phenotype is mild . Overall this is a manuscript that provides and establishes a new paradigm to study and understand muscle thermogenesis albeit in a dysferlin deficient model.

Version 2:

Reviewer comments:

Reviewer #7

(Remarks to the Author)
The responses to the reviews are well-handled. However, since the paper contains no actual thermogenesis data, it seems inconsistent to frame some of the findings within a thermogenic context.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this manuscript, the authors demonstrate that the psoas muscle in mice, unlike the leg muscle (tibialis anterior), exhibits elevated RyR1 Ca leak. Dysferlin-null mice, a model for Limb-Girdle Muscular Dystrophy 2B, exhibit severe pathology in the psoas due to disrupted control over RyR1, leading to excessive Ca leak. This condition affects Ca handling and ROS production, contributing to disease onset and progression. Furthermore, aging exacerbates these effects through further alterations in Ca dynamics and ROS levels. The investigation provides significant insights into the pathological mechanisms of muscular dystrophy, however, there are critical concerns that need to be addressed to fully validate the findings.

We thank the reviewer for their time and positive comments on our manuscript.

Major concern:

1. The authors employed Rhod-5N to assess the Ca2+ levels within the sealed T-system, acknowledging that T-system Ca dynamics are influenced by changes in cytosolic Ca, such as leakage through RyR1. However, it is crucial to recognize that other factors and mechanisms may also impact these dynamics. For instance, alterations in the coupling between the SR and T-system could modify T-tubule Ca dynamics independently of changes in RyR1 Ca leakage. Moreover, given Dysferlin's critical roles in membrane and T-tubule integration, its deficiency could potentially affect the structure of t-tubules and their calcium coupling efficiency, suggesting that measurements of t-tubule Ca may not solely reflect SR Ca leakage. These concerns suggest that a more integrated methodological approach, incorporating direct measurements of both cytosolic and SR luminal Ca alongside t-tubule dynamics, might provide a more comprehensive understanding of calcium homeostasis alterations in muscular dystrophy. Addressing these methodological considerations is essential for accurately interpreting the results and could significantly strengthen the study's conclusions.

Thank you for raising these concerns. We and others have previously shown how the skinned fibre reliably retains function as expected in the intact fibre while providing the advantage of providing unprecedented access to the intracellular environment (eg Lamb & Stephenson, 2018, JAP; Lamboley et al 2021, Sci Adv). Here, we have used multiple methods from which we have drawn our conclusions. Moreover, our results align with those from intact fiber preparations published by others.

Firstly, our method for detecting RyR Ca^{2+} leak uses the properties of the t-system of the fibre under investigation to measure the difference in junctional space Ca^{2+} concentration ($[Ca2+]_{JS}$) in the presence and absence of functional RyRs. Thus, variations between fibers, whether within or between groups, do not influence the detection of the leak's magnitude or the comparison of leaks across groups. The critical point is that each fibre serves as its own internal reference in this assay.

Supporting the findings obtained with our RyR Ca^{2+} leak assay that leak is higher in psoas compared to TA are: (i) RyR1 phosphorylation (identified via proteomics); (ii) norepinephrine; (iii) PKA phosphorylation substrates; (iv) RyR caffeine sensitivity; (v) metabolomic analysis; and (vi) bapta-lysis assays revealed that muscle groups with leaky RyRs showed reduced SR Ca^{2+} content and increased mitochondrial Ca^{2+} content (reduced SR Ca^{2+} content and increased mitochondrial Ca^{2+} content are a result of leaky RyRs, Lamboley et al 2021, Sci Adv). These assays (i-vi) are all independent of the t-system.

Furthermore, we can compare our results to those already obtained with intact fibre preparations. The results of Bloch's groups, who focus their study on intact FDB fibre preparations of dysferlin-positive and -negative mice, only find a difference in regard to Ca^{2+} handling between the genotypes after the dysferlin-null fibres have undergone osmotic stress (dysferlin-null muscle from the foot are sensitive to osmotic shock but wild type are not; Lukanyenko et al 2017, 2022). This is again consistent with the result we have shown in limb muscles (TA & EDL) that there are no differences in Ca^{2+} handling compared to wild type muscle without any applied stress. Additionally, Bloch's group find a point of weakness in the dysferlin null muscle is the DHPR – this is consistent with our result in skinned fibre that the DHPR-RyR interaction is affected in dysferlin null muscle (Fig 2). The consistency between the mechanically skinned fibre results and the results from the enzymatically dissociated fibres are acknowledged in Discussion.

Overall, this confirms that any differences in t-system Ca^{2+} handling properties between groups did not affect our ability to detect the respective RyR Ca^{2+} leak in each group nor affect the validity of the comparisons between groups.

2. The paper's title suggests a significant focus on the thermogenic properties underlying Limb-Girdle Muscular Dystrophy 2B progression. However, upon detailed review, it becomes apparent that the study primarily concentrates on the mechanistic insights into RyR1 Ca leak dynamics, dysferlin's role in muscle pathology, and alterations in calcium handling across muscle types, with less emphasis on directly linking these phenomena to thermogenesis. While the study suggests the psoas muscle as a thermogenic center and explores its unique protein expression related to thermogenesis, these findings are somewhat isolated within the broader context of the paper's focus on muscular dystrophy pathology. The analysis of thermogenic capacity, primarily through proteomic profiling indicating an enrichment of thermogenesis-related proteins in the psoas, offers valuable insights but is not thoroughly further investigated and integrated into the narrative of muscle-specific pathology development.

We take this point and have amended the title of the manuscript to better reflect the findings and focus of the study. Additionally, we have now performed metabolomic analysis to compare psoas vs TA as well as mitochondrial membrane potential measurements (now in Fig 4) and integrated this into the narrative of the manuscript.

Minor concerns:
Figure 1J (video)
The wave frequency of TA starts at 3mM with some fast and robust waves initially. This does not happen in Psoas. Do all TAs behave like this? What is the potential explanation for this phenomenon?

The initial response to caffeine (first transient) varies in robustness and reuptake behaviour on a fiber-to-fiber basis, regardless of the group. In fact, we have found this behaviour to be fiber segment-dependent. We think this could be the result of mild differences in calsequestrin polymerization status across the fiber, perturbing the local [Ca2+]SR in specific regions of the fiber during exposure to low mM caffeine, which can affect the release-uptake cycle analysed. Due to this, we assessed this feature (SR Ca^{2+} release) in a binary manner (presence or absence of transient). Once the fiber goes through its first release event and the initial calsequestrin depolymerization has occurred (Manno et al 2017) the following transients exhibit a more consistent and regular behaviour.

Figure 2f

The connected dots should represent paired data. Here, it should not be paired since it is two different groups.

In this assay, two skinned fibres are placed in the same chamber in parallel. The fibres share the same cytoplasmic solution and are imaged under identical conditions. We therefore consider these fibres experimentally paired. We find this is an important approach to reduce inter-assay noise in the recording of the small fluxes we are studying. We have now made our approach clear in the text.

Figure 5
Lacking biochemical data for oxidation."
We use NAC to change the function of the fibre, which is undoubtedly due to reversing oxidation. Such a functional characterization is more valuable than a biochemical characterization, where the latter may not reflect nor accurately predict the degree of functional change.
Oxidation can cause both positive and negative effects on muscle function. Therefore, alteration of the function using NAC, we demonstrate that oxidation plays a key functional role. Further molecular details of this mechanism will be interesting to explore and may uncover potential therapeutic options, but is currently beyond the scope of our investigation.

Reviewer #2 (Remarks to the Author):

The manuscript of Meizoso-Huesca and colleagues investigates changes in calcium (Ca2+) cycling in a mouse model of Limb Girdle Muscular Dystrophy 2b (LGM2b). Moreover, the authors investigate changes in muscle Ca2+ cycling in two different muscles being the psoas and the tibialis anterior. The paper utilises a series of elegant and sophisticated techniques demonstrating muscle-dependent changes in Ca2+cycling and storage due to altered phosphorylation of the ryanodine 1 receptor (RyR1) in the dysferlin-null BLaJ mice.
The following key findings were observed:
1. Altered Ca2+ handling was evident in the psoas muscle and not the tibialis anterior.
2. Dysferlin deletion was associated with increased Ser2844 phosphorylation of RyR1 and greater Ca2+ leak
3. The increased Ca2+ leak modified tubular handling of Ca2+ leading to increased cytosolic and mitochondrial Ca2+ levels
The authors have linked these key modifications to functional differences including disease progression and/or altered thermogenic and metabolic function.

We thank the reviewer for their time and positive comments on our manuscript.

Overall, the authors conclude that the psoas muscle has greater RyR1 leakage, leading to functional differences with altered metabolic and thermogenic functions. However, this is not directly tested. The proteomic and SWAT workflow analyses support altered expression patterns of thermogenic and metabolic factors. This would be greatly strengthened with the inclusion of physiological studies measuring heat production as a direct assessment of thermogenesis as well as differences in energy balance, glucose metabolism and fatty acid oxidation.

We have realigned the title of the manuscript to better represent the focus on Ca^{2+}-handling. Additionally, we have performed further experiments showing that psoas displays a higher norepinephrine concentration relative to TA, which offers a mechanism for the increased RyR1 phosphorylation and Ca^{2+} leak observed. This is further supported by higher presence of phosphorylated substrates of PKA in psoas (also included in the manuscript). Finally, we examined energy balance holistically using two complementary approaches, finding differences in 1) both the mitochondrial and cytosolic NADH/NAD+ ratio (measured the ratios of marker metabolites lactate/pyruvate and glutamate/α-ketoglutarate, as outlined by Krebs (Williamson et al., 1967)); and 2) the mitochondrial membrane potential between resting tissues (assayed by TMRE fluorescence). Together, these metabolic data provide support for greater potential for mitochondrial oxidation in the psoas muscle.

We agree that measuring flux rates of glucose and fatty oxidation would be valuable in this context, but this would require live tissue and ideally *in vivo* tracing, which is beyond the scope of this manuscript.

On the other hand, the authors suggest that altered Ca2+ handling increases the production of ROS leading to LGM2b disease progress. Can this be reversed with treatment of anti-oxidants?
The measurements of Ca2+ handling are indeed elegant and extensive. However, the significance of the work would be increased by showing that these changes have direct effect on physiological systems.

Our results clearly show that we can acutely reverse the effects of oxidation on Ca^{2+} handling in BlaJ psoas muscle fibres. At the mouse level, Garcia Campos et al 2020 showed that the chronic use of

antioxidant improved the general strength of the dysferlin null mouse. Together, these results are consistent with the accumulative negative effect of oxidation on Ca^{2+} handling and muscle function in Blaj muscle.

Reviewer #3 (Remarks to the Author):

Dysferlinopathies in man typically affect young adults and different muscle groups, but the reasons for the late onset and distinctive muscle specificity are unknown. This submission by Meizoso-Huesca, et al., presents evidence that begins to address these questions. The idea they test is that the leak of Ca ions through the ryanodine receptors, RyR1, is greater in muscles that are more severely affected than in less affected muscles, and that over time this leak leads to ROS-induced damage that further exacerbates the dysregulation of Ca homeostasis. The authors use an elegant method of imaging Ca leak through the RyR1 and its clearance across the t-tubule membrane to compare psoas muscles in dysferlin-null BlaJ mice to WT controls and to TA and EDL muscles as well. Psoas muscles are known to show more severe pathology than TA or EDL muscles in mice, and, the authors suggest, this property is linked to its predisposition to being more thermogenic. The results of an extensive series of experiments lend support to the central hypothesis of the paper, though some questions about their interpretation remain. The physiological studies are supplemented by mass spectrometric comparison of the proteomes of psoas and EDL muscles of WT mice, the differences in which are consistent with the a greater role of the psoas muscle in thermogenesis in mice. These are comprehensive and in many ways compelling studies that, with some additions and adjustments, will contribute significantly to our understanding of the role of dysferlin in skeletal muscle.

We thank the reviewer for their time and positive comments on our manuscript.

The paper would be strengthened by consideration of the following.

Relevance to LGMDR2: Human dysferlinopathies target the limb and limb girdle muscles first, which are not likely to be as thermogenic as the muscles of the chest, abdomen and back. How might the authors reconcile the pattern of pathogenesis in man with their observations?

Thanks for raising this interesting point. Extrapolating muscle-specific conditions from murine models to humans is quite challenging, and limitations as well as key differences between the organisms should be considered. Of great importance are the biomechanical properties of the limb muscles, which differ significantly between rodents and humans. For instance, the extent of muscle excursion during a gait cycle is higher in the majority of limb muscles of humans compared to their murine counterparts. Of particular interest, during a gait cycle, the human gastrocnemius (typically first muscle to show problems in dysferlinopathy patients) has an excursion ~2 times larger than mouse gastrocnemius. We believe this difference is key in providing a constitutively higher basal stress to human limb muscles compared to mouse muscles, which is likely a pathogenic contributor to the onset and development of the condition in humans. We have now raised this point in the Discussion.

Mouse strains: How many generations separate the C57Bl/6 mice and the BlaJ mice studied here? The differences in levels of psoas MYH7 (Sup. Fig. 5) are larger than one might expect for BlaJ mice that were rederived from the parent C57Bl/6 line recently.

Mice were purchased from a commercial colony. We cannot easily answer your question. Regardless, we have focussed our work on single fibres, and one fibre type.

Fatty infiltration: The Grounds laboratory has reported that dysferlin-null psoas muscle accumulates large amounts of lipid, especially with age. The authors do not mention making a similar observation. Does this not occur in their mice? If it does, how might it alter the interpretation of their results?

We show histology in Fig 1 and note fatty infiltration in older psoas muscle. Yet, this does not alter the interpretation of our results. We have focused on the changes in the physiology within the muscle fibres, which will be a primary event in dysferlinopathy. That is, Ca^{2+} handling changes in the psoas fibres occur in the young mouse, prior to fat infiltration.

Proteomics: Fig. 4 shows that the proteome of WT TA and psoas muscles of 8 week old mice are different. Why was a similar analysis not performed with young BlaJ mice?

Yes, it was. However, our focus was on the differences in the wild type, between psoas and TA, which represented the primary deviation between the muscles that provide an explanation for the differences that are exacerbated in the Blaj. We decided these latter results add little to the manuscript.

Fiber type: Although the authors examine the myosin heavy chains in Supp. Fig. 5, there is no discussion of the differences they report (e.g., in Myh7 between BlaJ and WT psoas), which may indicate a higher content of type 1A fibers in the BlaJ muscle. The possibility that the other proteomic differences which they report (in accord with earlier papers) may play a role in the outcome of their experiments would be consistent with their DDA analysis of WT psoas (Fig. 4), which shows the pathway with the highest level of significance to be “metabolic”, rather than “thermogenesis”.

We don’t see any statistically significant difference in the fibre type proportions among the groups that would affect or explain our results as any alternative to what we have presented in the manuscript. The fibre types expressed were 25-30% type IIx; 60-65% type IIb; and ~5% type I fibres across the four groups (Fig S5). Regarding Ca^{2+} handling, the results are drawn exclusively from type II fibres (we separate out the low probability of selecting any type I fibres with the functional response to high Ca^{2+} in the presence of myosin-II ATPase inhibitor BTS in the t-system experiments; and the response to Sr^{2+} in the bapta lysis experiments on the force transducer). We have now made these statements in the Methods.

DHPR-RyR1 coupling: The data in Fig. 2a,b, suggest that the absence of dysferlin alters the coupling of DHPR to RyR1. The data in Fig. 2b,c further suggest that most of the Ca in the t-system is due to leak through the DHPR and not the RyR1. If this is correct, how might the authors reconcile it with their hypothesis?

The use of nifedipine is used to increase the mechanical stabilization of the DHPR-RyR interaction. This manoeuvre clearly separates the cause of the increased leak in the BlaJ compared to the WT. In contrast, using 3 mM Mg^{2+} to directly block the RyR1 causes the same block of the leak in both

genotypes (Fig. S3). There is no significant leak of t-system Ca^{2+} through the DHPR. This is shown by the blockade of leak by tetracaine, which directly affects the RyR and is not additive with nifedipine-induced leak reduction. We now make this clearer in the text.

Additional concerns:

Is the difference in Fig. 1i between the psoas muscles of WT and BlaJ mice significant? If not, shouldn't it be?

The p value for this comparison comes to 0.0527. Based on the values of BlaJ and the current spread of the population, our impression is that this trend would likely be significant if more experiments were conducted.

Why is the Ca concentration in the t-system in BlaJ mice treated with cAMP lower than the concentration in its absence (compare Fig. 2b and 2e)? Shouldn't it be higher if cAMP promotes phosphorylation of S2844 to enhance RyR1 Ca leak?

These are different, individual fibre experimental traces that come from different muscles; 2b corresponds to a psoas fiber experiment, where the effect of nifedipine was tested to explore a potential role of DHPR on the RyR1 leak. One the other hand, 2e corresponds to TA fiber experiments, where RyR1 phosphorylation was induced by cAMP exposure (Singh et al. 2023). It is possible that the cAMP treatment followed is not sufficient for the TA fiber to reach the levels observed in psoas, nor alter its physiology to the same extent. In addition, there may be natural variation between fibres that account for the difference in steady state $[Ca^{2+}]_{t\text{-}sys}$. Also, as with any experimental measurement, there is error between calibrations of each fibre. Regardless, we are confident in the summary of data that we present and the conclusions we have drawn. These traces accurately represent the physiology being described.

Are the curves shown in Fig. 5s significantly different? If not, shouldn't they be?

Fig 5s shows a single experiment where three fibres (WT, Blaj & Blaj + NAC) are depleted of SR Ca^{2+} while imaging rhod-5N in the sealed t-system to observe delays in Ca^{2+} release-SOCE activation (this is a novel assay that we introduce in this manuscript). We have not analysed the declining phase of the rhod-5N signal post caffeine exposure, if this is what you are referring to, as it is not the focus of the experiment. The rate of SOCE is analysed in Fig 5h.

In Fig. 6b, BlaJ psoas +RyR1 leak is depicted as being lower than -RyR1 leak. Is this correct?

Yes.

In Sup. Fig. 4, there is no significant difference in mitochondrial Ca content of psoas muscle in 8 and 36 week old BlaJ mice. If ROS generation associated with pathology is linked to accumulation of Ca in the mitochondria, shouldn't the older muscle have significantly higher levels?

We suspect that the Ca^{2+} content of the mitochondria (the steady state) is approaching saturation of the relationship between mitochondrial Ca^{2+} and cytoplasmic Ca^{2+} in these old psoas fibres from BlaJ mice. As mitochondrial Ca^{2+} content is higher, the resolution power decreases, impeding us from resolving mild differences at those levels (Lamboley et al. 2021).

Methods:

What is the range of concentrations of BAPTA used? Re BAPTA, the authors may wish to refer to PMID 36406982, which suggests that the triad junction is a privileged space where BAPTA is active when applied at very low concentrations.

Bapta was used in the µM to low mM range. However, our lysis experiments that require bapta are to buffer global cytoplasmic Ca^{2+} (see Fryer & Stephenson, 1996) and are not equivalent to the experiments described in PMID 36406982.

How was the free [Ca] calculated? The reference is missing from the text.

Free $[Ca^{2+}]$ in our solutions were calculated as described in Stephenson & William (1981) and $[Ca^{2+}]_{t\text{-}sys}$ was determined as described in Cully et al 2016. These references are both now in the Methods.

Why was such a high concentration (50 mM) of EGTA chosen?

This was done to heavily buffer the bulk cytoplasmic $[Ca^{2+}]$. This assists in detecting a difference in $[Ca^{2+}]_{cyto}$ and $[Ca^{2+}]_{JS}$, which is critical for measuring RyR Ca^{2+} leak (Cully et al. 2018).

Editorial:

Re the MS results, it would be helpful to have a supplementary figure showing the amounts of the different peptides and their identities (sequences) that contributed to the conclusion that phosphorylation and oxidation were enhanced in psoas muscles. Extracting this information from the tables is a challenge!

Noted, we have included this now.

A table of abbreviations would be helpful.

If it is editorial policy at The Journal to publish such tables, we are happy to include one.

The Results section is sometimes difficult to read, as it includes aspects of the Methods and Discussion sections.

We have amended this now. Limiting methods-related information to methods section.

Psoas and dysferlin do not need to be capitalized in the text.

This has been corrected now.

The formatting of the text when it refers to the figures is not consistent (such as Fig. X). Dots and spaces should be uniform.
This has been corrected now.

Reviewer #4 (Remarks to the Author):

Summary
Limb Girdle Muscular Dystrophy 2B (LGMD2B) is caused by deficiency the protein dysferlin and presents with late-onset selective wasting of specific muscle groups. The functional role for dysferlin is most clearly established in membrane repair mechanisms, thereby implicating a possible disease pathogenesis in LGMD2B. Dysferlin is predominantly located in transverse tubule membranes, where in addition to promoting membrane repair, dysferlin interacts with other junctional proteins (RyR1, DHPR) at the triad formed by the T-tubule and terminal cisternae of the sarcoplasmic reticulum (SR) to regulate calcium homeostasis. In this study, the authors have explored the hypothesis that dysferlin-dependent mechanisms of Ca handling in the junctional space contribute to the selective vulnerability of psoas muscle as compared to much milder pathology of the tibialis anterior (TA) in a dysferlin-deficient mouse model (BlaJ) of LGMD2B.
The experimental approach is based on the exceptional access to measurement and manipulation of compartmentalized Ca in the T-tublules and of the sarcoplasmic reticulum with optical dyes in the skinned muscle fiber preparation. The primary observations are:

• Ca "leak" via the calcium release channel (RyR1) of the SR is prominent for WT psoas, but essentially undetectable for the TA muscle (Fig. 1). Phosphorylation of Ser2844 is known to increase RyR1 leakiness and was 3-fold higher for psoas compared to TA.

• Dysferlin deficiency exacerbated the RyR1 Ca leak in psoas muscle from young mice (8 week), but not for the TA.

• The RyR1 leak disrupts Ca distribution at the triad for psoas muscle in BlaJ mice such that: (1) SR Ca content is reduced, (2) mitochondrial Ca content is increased, (3) total Ca content is preserved (SR + mito + cytosol), Fig. 3.

• The increased Ca load to mito is predicted to increase ROS, and oxidation-dependent changes in Ca handling were detected by sensitivity to N-acetylcysteine (Fig. 5). Oxidation decreased the efflux rate for the plasmalemmal Ca ATPase pump (PMCA) and decreased the rate of Ca influx via store-operated Ca entry (SOCE).

• With aging (36 weeks vs 8 weeks) the pathologic changes of the psoas in dysferlin-deficient mice are exacerbated, with increased oxidation of RyR1, a more severe depletion of SR Ca, and additional anomalies of junctional Ca handling that may be a consequence of chronic persistent low-level SOCE.
The authors conclude that the more thermogenic profile of psoas muscle, better optimized for Ca-dependent ATPase activity and generation of heat compared to TA, produces an increased susceptibility to Ca overload, oxidative damage and dystrophy in dysferlin-deficient muscle. This mechanism may contribution to the muscle-specific dystrophic changes in LGMD2B.

We thank the reviewer for their time and positive comments on our manuscript.

Critique:
This study shows clear evidence of muscle-specific differences in Ca handling at the triad of skinned fibers. These changes are consistent with increased Ca leakiness of RyR1 for psoas relative to TA that are further exacerbated in the dysferlin-deficient mouse. This difference may contribute to the increased susceptibility for dystrophic changes in the psoas vs TA in the BlaJ mouse model of LGMD2B. The key question is the specificity for the interpretation of the Ca transients.

Specific Concerns.

(1) The authors are leaders in the field for the development and use of the skinned muscle fiber preparation to interrogate Ca handling of the triad. The preparation, however, is far from an intact muscle, depends on consensus agreement for interpreting the Ca transients (e.g. that tetracaine-sensitive fluorescence transients of the T-system are a robust measure of RyR1 leak), and rely on computational modeling (distribution of Ca amongst membrane compartments). This concern Is not meant to reject the use of the skinned fiber preparation to investigate muscle disease. The reader's confidence in the author's interpretation would be strengthened, however, if more detail or examples could be provided in the Discussion that these new mechanistic insights are consistent with observations from other studies using intact fibers, whole muscle, motor performance, or histopathological studies.

A prime example of our skinned fibre results being consistent with what intact fibres have been used to show is the failure point being the DHPR in dysferlin null fibres. Both intact fibres studies by Bloch's group and skinned fibre studies here (Fig 2) identify the DHPR as changing its behaviour in the absence of dysferlin. This point is raised in the Discussion. Additionally, we point out above (first point answered to reviewer 1) out that ever other assay performed for this study was consistent with the fact that skinned fibres showed leaky RyRs in psoas. The consistency between the mechanically skinned fibre results and the results from the enzymatically dissociated fibres are acknowledged in Discussion.

(2) The functional changes in PMCA and SOCE for BlaJ psoas (Fig. 5) raise concerns about the interpretation of [Ca]t-sys as a specific indicator of RyR1 leak. The steady-state [Ca]t-sys is expected to be set by a balance of extrusion (PMCA) and depletion (SOCE), which in turn depends on [Ca]js that is strongly influenced by the RyR1 leak. The tet-sensitive change in [Ca]t-sys is used to extract the RyR1 leak-dependent component from the total change. SOCE is also tet-sensitive (e.g. Fig 6), however, so how can the authors be confident that the tet-sensitive change in [Ca]t-sys in Figs. 1 and 2 are primarily a reflection of RyR1 leak versus a contribution from SOCE? Could the experiments of Fig. 1 be performed in the presence of BTP2 to exclude a contribution of SOCE to the tet-sensitive component of the [Ca]t-sys transient?

We have modelled the system previously and also undertaken an extensive examination of the properties of BTP2 in skeletal muscle (Barclay & Launikonis, 2022; Meizoso-Huesca & Launikonis, 2021). Of course, the steady state $[Ca^{2+}]_{t\text{-}sys}$ is achieved when pump and leak mechanisms are in equilibrium. In the healthy resting fibre, the t-system leaks Ca^{2+}. This is blocked by BTP2 in the presence of tetracaine, indicating that the leak is through Orai1 (SOCE channels) but the leak is not due to Ca^{2+} depletion inside the SR (SOCE). The fact that this influx is store-independent is not a surprise, as we expect leak through any ion channel. From this state observed in the WT, we have progressively leakier RyRs, for example in RYR1 variants and we see the difference in $[Ca^{2+}]_{t\text{-}sys}$ ($\Delta[Ca^{2+}]_{t\text{-}sys}$) increase when comparing steady state $[Ca^{2+}]_{t\text{-}sys}$ in the absence and presence of tetracaine. This can occur in WT, heterozygous and homozygous RYR1 knock in mice without any significant activation of SOCE. We know this because we see an increase in $\Delta[Ca^{2+}]_{t\text{-}sys}$ with the gene-dose effect of the RYR1 mutations (Lamboley et al 2021, Sci Adv). To begin to observe SOCE, RyR Ca^{2+} leak needs to increase passed a threshold to so that enough Ca^{2+} must dissociate from STIM1 that SOCE is activated. To clearly see this occur, we need to bath the fibre in a relatively high $[Ca^{2+}]_{cyto}$ (1.3 µM). While there may be very minor contributions of SOCE reducing the $\Delta[Ca^{2+}]_{t\text{-}sys}$ that we observe in Figs 1 & 2, it does not affect our detection of leak – we also apply a range of $[Ca^{2+}]_{cyto}$ (28-1300 nM) to the fibre to progressively increase the leak (the leak is increased by the $[Ca^{2+}]_{SR}$ being increased by the $[Ca^{2+}]_{cyto}$). The fact that at the highest $[Ca^{2+}]_{cyto}$ that the $[Ca^{2+}]_{t\text{-}sys}$ in the absence of tetracaine remains higher than the $[Ca^{2+}]_{t\text{-}sys}$ in the presence of tetracaine (eg Fig 1) indicates that the t-system Ca^{2+} pumps are saturated and the steady state $[Ca^{2+}]_{t\text{-}sys}$ in the absence of tetracaine is not

significantly influenced by SOCE. We need to observe the "cross-over" effect of the $[Ca^{2+}]_{cyto}$ vs $[Ca^{2+}]_{t\text{-}sys}$ curves in the presence and absence of tetracaine to conclude that a significant SOCE had been activated. This only occurs in the leakiest of RyRs (eg old Blaj psoas fibres in Fig 6). This interpretation of increased leak in aged Blaj psoas fibres is consistent with the fact that the SR Ca^{2+} content in these fibres significantly decreases with age (Fig S4a). Now we explain these points further in Methods (section named *Analysis of [Ca2+]t-sys measurements for estimating RyR Ca2+ leak*)

(3) In the analysis of Ca handling process for the T-system (Fig. 5), what about a contribution from the Na/Ca exchanger (NCX)?

We have previously tested for a significant contribution from NCX by introducing NCX blockers. We found little change in $[Ca^{2+}]_{t\text{-}sys}$ (Cully et al 2018, PNAS), which is consistent with PMCA being sensitive to the nM range of $[Ca^{2+}]_{cyto}$ that we apply and PMCA being the dominant extrusion protein in the t-system at resting $[Ca^{2+}]_{cyto}$. This is noted in the Results text associated with Fig 5.

(4) For the studies in aged mice (Fig. 6), the unexpected increase of psoas [Ca]t-sys by Tet is difficult to reconcile. While the proposal of an aberrant chronically active SOCE (also Tet-S) is consistent with the BTP2 data in Fig. 6C, other aspects are less satisfying. For example, if chronic activity of SOCE occurs form worsening RyR1 leak with aging, then why isn't the Tet-dependent decrease of [Ca]t-sys in the presence of BTP2 larger than for the comparable change in the psoas of young mice? Fig. 6 G does show progressively increased oxidation of psoas RyR1 with aging, but where are the data to quantitatively show increased Ca leak of psoas RyR1 with aging?

We believe the explanation to point (2) helps to explain this query. The point that the aged psoas Blaj mice show a "cross-over" effect of the $[Ca^{2+}]_{cyto}$ vs $[Ca^{2+}]_{t\text{-}sys}$ curves in the presence and absence of tetracaine but the young psoas Blaj fibres do not is the evidence that the older mouse fibres are more leaky than the young ones. In line with this, we show a decreased SR Ca^{2+} content in old vs young BlaJ psoas (Fig 6h; Sup. Fig. 5a) consistent with a leakier group.

(5) Can confidence limits graphically indicated for the compartmental distribution of Ca shown for the model calculations of Figs 3f and 6h? The confidence levels of the underlying [Ca]t-sys measurements are clearly indicated, but it is difficult to understand how this level of uncertainty will propagate to the model calculations. This information is important because the age-dependent difference for psoas Ca_SR is modest (0.95 vs 0.79, 36 wk vs 8 wk) and unexpectedly there is a detectable difference for TA (1.27 vs 1.11, 36 wk vs 8 wk). The figure legend provides confidence levels for these differences (as p-values), but the SEM or some equivalent should be shown on the bar graphs.

The absolute measurements of SR Ca^{2+} content and mitochondrial Ca^{2+} content is presented with +/- SD (Fig 3b & d; Sup. Fig. 5). Only the cytoplasmic Ca^{2+} content has been modelled. Because the SR and mitochondrial Ca^{2+} contents have errors presented we do not present them again for the projection of the percentage of total Ca^{2+} content that they represent. The cytoplasmic Ca^{2+} is an estimated value from an average, so we have left this without CI or errors.

The apparent modest increase in mitochondrial Ca^{2+} content with age in psoas Blaj fibres is expected to be due to the saturation of the steady state mitochondrial Ca^{2+} content achievable in skeletal muscle (Seng et al 2022, Am J Physiol- Cell). We should point out that the SR Ca^{2+} content in the

psoas Blaj significantly decreases with age, indicating indeed that the older muscle is leakier than the younger (Sup. Fig 4a).

Minor Points.

Page 7, 4th line from the bottom. Fig. 1e should be Fig. 1j.

This has been corrected now.

Page 17, first line. Fig. 6e should be Fig. 6f.

This has been corrected now.

Methods, page 23. The paragraph beginning with “For high temporal resolution real-time…” is duplicated.

This has been corrected now.

Reviewer #5 (Remarks to the Author):

In the manuscript, muscle thermogenic properties underlie limb-girdle muscular dystrophy 2B (LGMD2B) progression, the author presents data that in murine psoas muscle, RyR1 channels operate in a leaky state and elaborate on that the loss of dysferlin further exacerbates RyR1 instability and leak, contributing to the localized progression of the disease in this muscle. The manuscript is focused on Ca2+ and the authors are world-renowned for their Ca2+ recording.

We wish to thank the reviewer for their time and positive comments on our manuscript.

The manuscript provides a careful characterization of the Ca2+ signals in the early phase of the disease and compares two muscle types with wildtype control. However, there are a range of unanswered questions that need to be clarified. For instance, limb-girdle muscular dystrophy (LGMD) belongs to a group of inherited muscle diseases where the muscle fibers slowly break down and are replaced by connective tissue and fat. The breakdown leads to muscle weakness and muscle atrophy. If RyR1 is an important driver of LGMD2B, the degree of leak should then be transferred onto other parameters. For instance, will a reduction of RyR1 Ca2+ leak protect muscle from increased fat/fibrosis accumulation and atrophic signaling?

Increased fat-fibrosis will reduce force because that cross-sectional area of the contractile apparatus in the muscle is reduced. The infiltration of these tissues must be secondary to processes inside the muscle fibre that are caused by the absence of dysferlin (with RyR phosphorylation, as we have shown). As leak becomes the most severe when the population of RyRs are oxidized, we would expect that the avoidance of oxidation would prevent leak and the loss of muscle tissue and concomitant fat infiltration. Others have shown that treating the mouse with antioxidant does indeed prevent the loss of force, consistent with the prevention of increased leak leading to pathology.

Elevations in tetanic Ca2+ are required for force production and as the authors reference enhanced RyR1 leak has been shown to contribute to muscle weakness. The level of leak observed in LGMD2B psoas, is that making the muscle weaker or more fatigable already at this earlier timepoint?

The pathology that we have confirm here (Fig 1) is consistent with the aged psoas muscle becoming weaker. We wished to measure the force responses from the psoas muscle, which requires a tendon-to-tendon dissection. It is very difficult to achieve this, so we are unable to pursue this line. Regarding the leak, this can be compensated for by the redistribution of Ca^{2+} content so that the reduction in SR Ca content is offset by the increase in cytoplasmic Ca content. In this situation, less release of Ca from the SR allows a similar amount of force to be produced compared to the 'healthy' situation (Lamboley et al 2021, Sci Adv). However, persistent and significantly raised Ca in the cytoplasm and mitochondria (as we show in psoas Blaj aged mice) is the likely trigger point for degradation of the muscle.

The authors show that there is a difference in the mitochondrial Ca2+ content and that psoas from BlaJ mice have the highest mitoCa2+ uptake, but the manuscript lacks information regarding whether this difference is large enough to have any functional effects. There are Ca2+-dependent kinases in the mitochondria, are these affected by the alterations in mitoCa2+ content? Moreover, does differences in mitoCa2+ result in any differences in mitochondria oxidative phosphorylation capacity?

Others have shown that Ca^{2+} is an important regulator of processes inside the mitochondria (eg. Glancy et al 2013, Biochem). The range of steady state mitochondrial Ca^{2+} content measured in this study encompasses the range currently known to be possible (the steady state mitochondrial

Ca^{2+} content moves from values in healthy mice at 0.03 to plateau around 0.3 mmol/L fibre volume in the pathophysiological states; Fig 3 & Fig S4; Seng et al 2022, AJP-Cell). We now have performed metabolomic analysis showing a decreased NADH/NAD+ ratio in psoas vs TA in both cytosol and mitochondrial cisternae, revealed by a lower lactate:pyruvate and glutamate:α-ketoglutarate ratio, respectively (Williamson et al 1967). Differences are also supported by newly incorporated data regarding mitochondrial membrane potential, showing elevated TMRE uptake in psoas vs TA. Both metabolomics and TMRE data are now part of Fig. 4. We can conclude that the function of the mitochondria such as oxidative phosphorylation are altered by this.

On the same note, the authors present interesting enrichment analyses of their proteomics data set and highlight that NADH regeneration appears altered, but it is not clear if that is a direct consequence of the mitochondrial Ca2+ or that the overall changes in Ca2+ (mito, cytosol, influx) has downstream effects on protein translation. Assessment of NAD/NADH levels and other metabolites e.g. AMP, ADP, ATP needs to be measured in the mouse models to understand this in more detail.

This has now being address (comment above) and it is part of Fig. 4 now.

By utilizing targeted mass-spectrometry that authors show that psoas muscle (WT and BlaJ) exhibit higher RyR1 phosphorylation levels as compared to TA muscle (WT and BlaJ). This phosphorylation is assumed to be PKA derived and indeed the authors show that adding cAMP to the muscle enhances the RyR1 leak. The authors assume this to be PKA derived phosphorylation, and thus suggest that the beta-adrenergic signaling should be more pronounced in psoas fibers as compared with TA fibers. Hence suggest that muscle as baseline has intrinsic variation in the baseline beta-adrenergic signaling and further exacerbated in BlaJ genetic background, this needs to be further assessed.

Thank you for raising this point. We have now measured norepinephrine at the psoas and TA muscles, which show differences consistent with the hypothesis already put forward. This data is in Fig 4.

In previous work, the phosphorylation of RyR1 has been linked to reduced FKBP12 binding and channel instability with the higher the phosphorylation the lower the FKB12 occupancy. What level of RyR1-FKBP12 binding is present in psoas and TA wildtype versus BlaJ?

Thank you for your comments, we have now measured norepinephrine concentration in TA and psoas, observing higher norepinephrine concentration in psoas relative to TA. This is accompanied by higher signal of phosphorylated PKA substrates in psoas compared to TA. These data support the notion of a more pronounced adrenergic signalling in psoas compared to TA, providing a mechanism for the increased RyR1 phosphorylation and leak in this muscle. We have included these data in the manuscript (Fig. 4a-c).

We agree that others have shown that phosphorylation of RyR1 is linked to FKBP12 dissociation and leak. As this is well established, we think the inherent conceptual gain from further measures of FKBP12 is low since we have shown the leak is different between the groups and that the phosphorylation of the RyR is aligned with this functional feature of the muscles.

Furthermore, the authors state that “comparable trends were observed across Blaj muscle Fig 1I”, although this does not match the graph. BlaJ psoas phosphorylation levels appear to have a very large spread, ranging from 1-10. Thus, it is not accurate to say that they are comparable. Instead, one wonders what this spread is the result of. Is there a vast difference in the b-adrenergic signaling

and thus a difference in the phosphorylation levels or is there a difference in the phosphatase activities in these muscles. A careful assessment of the spread in phosphorylation levels is needed.

We suspect that this is due to limitations in mass spec measurements (Fig 1l). We do not think that an extensive exploration of this point will likely provide a more refined answer than the level of phosphorylation is higher in some samples than others, as already presented.

In addition to altered intramuscular Ca2+ levels linked to DHPR-RyR1, SOCE is also examined. The authors propose that there is reduced Ca2+ fluxes of the t-tubular system in BlaJ muscle as a result of increased oxidative stress in the muscle. However, it is unclear where the oxidative stress originates from which should be clarified. Also, RyR1 is known to be a redox sensitive channel and there is also data suggestion that b-adrenergic signaling may enhance ROS production. If SOCE in t-tubular system is altered by ROS it is likely that RyR is as well, please assess the RyR1's redox state in the WT and BlaJ groups.

The SOCE proteins Orai1 and STIM1 are both subject to oxidation (Johnson et al 2022, Cell Calcium) and it is likely that both proteins are in this state. Any biochemical assay performed to show the most likely outcome that both proteins are oxidized will not conceptually move the manuscript further forward than our functional assays already have.

The source of ROS is expected to be due to raised cytoplasmic and mitochondrial Ca^{2+} levels. We agree that the pathways here can be explored but we feel this is a topic for future studies. We suspected that the RyR of the young psoas Blaj is oxidized and such a trend is observed in Fig. 6g.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have changed the title of manuscript from “Muscle Thermogenic Properties Underlie Limb-Girdle Muscular Dystrophy 2B Progression” to “Muscle-specific Ryanodine Receptor Properties Underlie Limb-Girdle Muscular Dystrophy 2B/R2 Progression”. Such a topic has been reported by other investigators, which reduce the novelty of the current study.

The novelty of our study has not been compromised by older studies on dysferlin and RyR (mostly by Robert Block, focussing on Ca release), nor the paper from Bursac lab that was submitted virtually in parallel with this manuscript.

We are the first to demonstrate the difference in mildly and strongly dystrophic dysferlin-deficient muscle is the activity of the RyRs in their respective muscle populations. Importantly, **we show that this difference is present in the wild-type mouse for the fundamentally important purpose of muscle-based non-shivering thermogenesis (NST) in different parts of the body**.

The difference in RyRs for NST is **further exacerbated** in the strongly dystrophic psoas muscle of Blaj due to the absence of dysferlin. Mechanistically, lack of dysferlin causes a weakening of the DHPR’s capacity to inhibit the resting closed state of the RyR *on top of* the RyR leak induced by phosphorylation for NST to cause these additive effects, which underpin the pathophysiology. This finding is novel and has not been presented before and we believe that this represents a significant advancement of the field both mechanistically and conceptually.

Integration of Thermogenesis Findings

The paper's title initially suggested a primary focus on thermogenesis in the context of Limb-Girdle Muscular Dystrophy 2B (LGMD2B), which demonstrated great novelty. However, the actual content primarily addresses RyR1 Ca2+ leak dynamics and dysferlin's role in muscle pathology, with minimal data supporting the mechanism of thermogenesis. A more detailed investigation into how these thermogenic properties directly influence the progression of LGMD2B and their mechanistic interplay with Ca2+ handling anomalies would significantly strengthen the narrative. The current presentation isolates these findings rather than weaving them into a cohesive story that enhances our understanding of the disease's pathology.

We moved our title to focus on the RyR because it is the factor regulating muscle-based NST and it should be acknowledged as the primary concern with the progression of dysferlinopathy. NST is the reason that the RyR is activated in the psoas muscles. We have provided a concise description of RyRs and NST in the manuscript, and directed readers interested in further details to our review of muscle-based NST (Launikonis & Murphy, 2025, *Annu Rev Physiol*, published online).

Broader Implications for Muscle Pathology and Function

The broader implications of RyR1 Ca2+ leak for muscle pathology and disease progression of LGMD2B are not adequately addressed. If RyR1 leak is a significant driver of LGMD2B,

then mitigating this leak should demonstrably protect against muscle degeneration and disease progression. Demonstrating how interventions that reduce RyR1 leak translate to improved muscle integrity and reduced fibrosis would be critical. For instance, Khodabukus (2024) recently showed that dysferlin-deficient mice treated with the RyR inhibitor dantrolene or the L-type calcium channel blocker diltiazem restore LGMD2B functionality. They also demonstrated that chemically induced chronic RyR leak in healthy myobundles phenocopies LGMD2B contractile and metabolic deficits.

We have cited that the recently published paper by Khodabukus that they were able to show that blocking leak rescued the cultured fibres from pathology, which provides this information. Their basic results on RyR leak are consistent with what we have demonstrated with mechanically skinned fibres, which, importantly, provides consistency through the studies that use different approaches.

However, there are fundamentally important questions that remain - as the cultured fibres in the Bursac study are not fully differentiated. The consequence of this is that DHPR does not provide the resting inhibition of all RyRs; nor does it provide a demonstration of differences between mild and strongly dystrophic dysferlin-deficient cultured fibres. Data we resolve using mature skeletal muscle fibres from different muscles in mice.

The authors need to address these substantial issues through a more integrated methodological approach, provide stronger quantitative evidence for their claims, and directly link their findings to functional outcomes in muscle pathology and thermogenesis regulation.

We have addressed all above issues.

Reviewer #3 (Remarks to the Author):

The authors have responded adequately to many of my earlier comments. The following remain to be considered, however.

We wish to thank the reviewer for their time and positive comments on our manuscript.

Relevance to LGMDR2: Human dysferlinopathies target the limb and limb girdle muscles first, which are not likely to be as thermogenic as the muscles of the chest, abdomen and back. How might the authors reconcile the pattern of pathogenesis in man with their observations?

Thanks for raising this interesting point. Extrapolating muscle-specific conditions from murine models to humans is quite challenging, and limitations as well as key differences between the organisms should be considered. Of great importance are the biomechanical properties of the limb muscles, which differ significantly between rodents and humans. For instance, the extent of muscle excursion during a gait cycle is higher in the majority of limb muscles of humans compared to their murine counterparts. Of particular interest, during a gait cycle, the human gastrocnemius (typically first muscle to show problems in dysferlinopathy patients) has an excursion ~2 times larger than mouse gastrocnemius. We believe this difference is key in providing a constitutively higher basal stress to human limb muscles compared to

mouse muscles, which is likely a pathogenic contributor to the onset and development of the condition in humans. We have now raised this point in the Discussion.

Doesn't this mean that in man, the relationship between thermogenesis and dysferlinopathy is weak, at best? If other factors (the authors mention mechanical stress) play a more important role in pathogenesis, why not simply induce more stress rather than focus on thermogenesis?

The muscle that does not follow the trend in this disease of occurring at the limb girdles is the gastrocnemius. This does not fit with muscle-based thermogenesis occurring near the body core to more efficiently heat the internal organs. Other stresses such was osmotic shock have been used in the past to invoke pathology in otherwise mildly dystrophy muscles in this disease (in mouse and not physiological anyway). This tells us that adding a stress provides the potential to push the condition down a strongly dystrophic pathway in a particular muscle that is otherwise only mildly affected.

We pointed out, probably too abruptly in the revised Discussion, that eccentric contraction probably is one of those stresses that affects the outcome of the muscle in this disease – and could explain what is occurring in the gastrocnemius of man – but this may be interpretated as being the underlying reason for the progression of pathology in man. We have now edited this section of the Discussion so that we do not create confusion that the limb girdle muscle pathology is driven by events other than related to the thermogenic needs.

Mouse strains: How many generations separate the C57Bl/6 mice and the BlaJ mice studied here? The differences in levels of psoas MYH7 (Sup. Fig. 5) are larger than one might expect for BlaJ mice that were rederived from the parent C57Bl/6 line recently.

Mice were purchased from a commercial colony. We cannot easily answer your question. Regardless, we have focussed our work on single fibres, and one fibre type.

This remains a serious question. If there has been genetic drift between the two strains of mice compared in this paper, as the data on MYH7 suggest, then the proteomic data become suspect.

Our supplier has confirmed that the mice they have sent us have not passed 7 generations of inbreeding, which should not result in genetic drift. This is following the guidelines recommended by The Jackson laboratory (https://www.jax.org/jax-mice-and-services/customer-support/technical-support/strain-background-and-genetic-drift).

Fatty infiltration: The Grounds laboratory has reported that dysferlin-null psoas muscle accumulates large amounts of lipid, especially with age. The authors do not mention making a similar observation. Does this not occur in their mice? If it does, how might it alter the interpretation of their results?

We show histology in Fig 1 and note fatty infiltration in older psoas muscle. Yet, this does not alter the interpretation of our results. We have focused on the changes in the physiology within the muscle fibres, which will be a primary event in dysferlinopathy. That is, Ca2+ handling changes in the psoas fibres occur in the young mouse, prior to fat infiltration.

This is certainly true for the physiological studies of Ca handling, but it is unlikely to be true for the proteomic analysis, which should show differences based at least in part on a shift from muscle to fat.
The proteomics data that was presented in our manuscript did not include dysferlin-null psoas muscle.

Proteomics: Fig. 4 shows that the proteome of WT TA and psoas muscles of 8 week old mice are different. Why was a similar analysis not performed with young BlaJ mice?

Yes, it was. However, our focus was on the differences in the wild type, between psoas and TA, which represented the primary deviation between the muscles that provide an explanation for the differences that are exacerbated in the Blaj. We decided these latter results add little to the manuscript.

I suggest including these results as Supplemental Information.

We have now deposited the full proteomic data set of dysferlin-null muscles (and WT) into ProteomeXchange with PRIDE. This information will be available in the Data Availability statement. Project accession: PXD056568.

Fiber type: Although the authors examine the myosin heavy chains in Supp. Fig. 5, there is no discussion of the differences they report (e.g., in Myh7 between BlaJ and WT psoas), which may indicate a higher content of type 1 fibers in the BlaJ muscle. The possibility that the other proteomic differences which they report (in accord with earlier papers) may play a role in the outcome of their experiments would be consistent with their DDA analysis of WT psoas (Fig. 4), which shows the pathway with the highest level of significance to be “metabolic”, rather than “thermogenesis”.

We don’t see any statistically significant difference in the fibre type proportions among the groups that would affect or explain our results as any alternative to what we have presented in the manuscript. The fibre types expressed were 25-30% type IIx; 60-65% type IIb; and ~5% type I fibres across the four groups (Fig S5). Regarding Ca2+ handling, the results are drawn exclusively from type II fibres (we separate out the low probability of selecting any type I fibres with the functional response to high Ca2+ in the presence of myosin-II ATPase inhibitor BTS in the t-system experiments; and the response to Sr2+ in the bapta lysis experiments on the force transducer). We have now made these statements in the Methods.

The proteomic studies point to more significant differences in the “metabolic” than in the “thermogenic” pathways. These can occur even when fiber type analyses do not show significant differences. The authors should address this result, which does not fully support their hypothesis.

This may be the case, but the functional studies show that non-shivering thermogenesis (NST) in the psoas is greater than in the TA or EDL; and the weakening of the inhibitory link

between the DHPR and RyR in the dysferlin-null mouse psoas muscle exacerbates the leak that leads to pathology with time. Furthermore, not only the proteomic data support our finding regarding NST, also our observation of higher norepinephrine amounts in psoas compared to TA, relevant for both cold and diet-induced thermogenesis. In relation to metabolism, it is expected that along thermogenesis, metabolic changes would take place to support thermogenesis-associated energy expenditure, hence the presence of biological processes such as fatty acid beta-oxidation and aerobic respiration in the list of enriched BPs identified in psoas (Fig. 4e), consistent with existing literature (Shabalina et al., 2010).

We make a comment about this in Results next to the related data.

Additional concerns:

Is the difference in Fig. 1i between the psoas muscles of WT and BlaJ mice significant? If not, shouldn't it be?

The p value for this comparison comes to 0.0527. Based on the values of BlaJ and the current spread of the population, our impression is that this trend would likely be significant if more experiments were conducted.

They should be done.

We have a result between psoas WT and Blaj that is not significant. We have stated this. The non-significance of this difference is not important, and indeed the determined difference in RyR leak between these muscles was found to be due to differences related to the absence of dysferlin (DHPR stability and its inhibitory capacity of RyR leak), not the phosphorylation of the channel. Thus, the difference is the summation of these two effects in the Blaj that are not both present in the WT.

Why is the Ca concentration in the t-system in BlaJ mice treated with cAMP lower than the concentration in its absence (compare Fig. 2b and 2e)? Shouldn't it be higher if cAMP promotes
phosphorylation of S2844 to enhance RyR1 Ca leak?

These are different, individual fibre experimental traces that come from different muscles; 2b corresponds to a psoas fiber experiment, where the effect of nifedipine was tested to explore a potential role of DHPR on the RyR1 leak. One the other hand, 2e corresponds to TA fiber experiments, where RyR1 phosphorylation was induced by cAMP exposure (Singh et al. 2023). It is possible that the cAMP treatment followed is not sufficient for the TA fiber to reach the levels observed in psoas, nor alter its physiology to the same extent. In addition, there may be natural variation between fibres that account for the difference in steady state [Ca2+]t-sys. Also, as with any experimental measurement, there is error between calibrations of each fibre. Regardless, we are confident in the summary of data that we present and the conclusions we have drawn. These traces accurately represent the physiology being described.

This point should be discussed in the text or in Supplemental Information.

We have added this information in the Methods (Section: "*Analysis of* $[Ca^{2+}]_{t\text{-}sys}$ *measurements for estimating RyR* Ca^{2+} *leak*"): "We note that there is some variability across the $[Ca^{2+}]_{t\text{-}sys}$ values presented, for example, across Fig. 2. Note that this occurs frequently if comparing

fibres from different muscles. This may reflect differences in populations of t-system membrane Ca^{2+} channels and pumps, which must affect the evolution of $[Ca^{2+}]_{t\text{-}sys}$ and its steady state."

In Fig. 6b, BlaJ psoas +RyR1 leak is depicted as being lower than -RyR1 leak. Is this correct?

Yes.

Unless I am missing something, this result is inconsistent with the argument that Ca leak into the t-system is greater in aged Blaj psoas muscle.

We have provided a section in The Methods that describes how $[Ca^{2+}]_{t\text{-}sys}$ is used to determine leak and how adding a RyR blocker can decrease $[Ca^{2+}]_{t\text{-}sys}$ or raise it. The latter indicates the leakiest RyR, where SOCE fluxes were dominant over PMCA fluxes in the presence of a functional RyR.

In Sup. Fig. 4, there is no significant difference in mitochondrial Ca content of psoas muscle in 8 and 36 week old BlaJ mice. If ROS generation associated with pathology is linked to accumulation of Ca in the mitochondria, shouldn't the older muscle have significantly higher levels?

We suspect that the Ca2+ content of the mitochondria (the steady state) is approaching saturation of the relationship between mitochondrial Ca2+ and cytoplasmic Ca2+ in these old psoas fibres from BlaJ mice. As mitochondrial Ca2+ content is higher, the resolution power decreases, impeding us from resolving mild differences at those levels (Lamboley et al. 2021).

The Lamboley et al paper seems to show that the differences in mitochondrial Ca levels under conditions of high, moderate and low leak are almost aways significant. Thus, this result is unexpected and inconsistent with the hypothesis of the paper. At the least, it needs to be discussed.
We noted the importance of this point in the Lamboley paper and followed-up with a paper on this topic: Seng et al 2022 AJP. The Seng paper is cited in the manuscript to direct readers interested in this point.

Methods:

What is the range of concentrations of BAPTA used? Re BAPTA, the authors may wish to refer to PMID 36406982, which suggests that the triad junction is a privileged space where BAPTA is active when applied at very low concentrations.

Bapta was used in the µM to low mM range.

This should be stated in Methods.

This is now stated in the Methods.

Why was such a high concentration (50 mM) of EGTA chosen?

This was done to heavily buffer the bulk cytoplasmic [Ca2+]. This assists in detecting a difference in [Ca2+]cyto and [Ca2+]JS, which is critical for measuring RyR Ca2+ leak (Cully et al. 2018).

This should be stated in Methods.

This is now stated in Methods.

Reviewer #4 (Remarks to the Author):

In the revised manuscript and the extensive point-by-point responses to the Reviewers, the authors have addressed all of the prior concerns. Important changes include: (1) a shift of emphasis to the well-documented pathologic Ca leak by RyR1 in dysferlin-deficient muscle, rather than emphasizing an inference to thermogenic properties, (2) new additional data in Fig. 4 (NE levels, metabolite ratios, mito membrane potential)to support a role for psoas muscle in thermogenesis.
We wish to thank the reviewer for their time and positive comments on our manuscript.

Reviewer #5 (Remarks to the Author):

The authors have responded to the reviewers comments in an appropriate way. However, the response had not been added into the manuscript which is needed so not only reviewers will benefit from the responses but, more importantly, the potential readers.

We wish to thank the reviewer for their time and positive comments on our manuscript. We have gone back to your previous review and added further comments to the manuscript to improve the manuscript in line with these comments.

Reviewer #6 (Remarks to the Author):

This manuscript provides an interesting finding which has been suspected but not directly shown . Especially the role of back psoas muscle in muscle thermogenesis . Most interesting finding is how an increased Ca2+ leak due to lack of coordination between DHPR and RYR can be detrimental to muscle physiology and metabolism due to increased Ca2+ leak in a dysferlin KO model. Key experiments document that this is the case .Data support the conclusions.

I dont believe that we need additional experiments to prove that Dysferlin is critical foe t-tubular integrity and RYR Ca2 release function . Ryr leak has been associated with thermogenesis ever since malignant Hyperthermia was discovered . Actually I am surprised

that the phenotype is mild . Overall this is a manuscript that provides and establishes a new paradigm to study and understand muscle thermogenesis albeit in a dysferlin deficient model.

We wish to thank the reviewer for their time and positive comments on our manuscript.

We are especially pleased to note that the reviewer recognizes the importance and impact of the work and the completeness of the work in its current form.

REVIEWERS' COMMENTS

Reviewer #7 (Remarks to the Author):

The responses to the reviews are well-handled. However, since the paper contains no actual thermogenesis data, it seems inconsistent to frame some of the findings within a thermogenic context.

We thank the reviewer for their time and positive comments.

In two recent publications we have provided direct evidence that RyR1 Ca^{2+} leak in muscle causes nonshivering thermogenesis (NST) in mammals (Meizoso-Huesca et al 2022, PNAS; Singh et al 2023, PNAS). Demonstration of increased RyR1 leak in skeletal muscle of mammals, as done in the present study, is therefore consistent with increased heat generation (NST) in those muscles.

In this study we demonstrate that there are differences in leak in regions of muscles in the mouse body. At room temperature this difference exists between the core and distal muscles. This is the case in both control and Blaj mice and we propose this fundamental difference within the mice is to keep the core warm, without expending energy in the distal muscle for unnecessary NST. These regional differences are most unlikely to be resolved, and in fact, would be expected to be lost in whole animal measurements of thermogenesis as the editor indicates that reviewer #1 suggests should be done, which led to the recruitment of reviewer #7 to comment on this.

Additionally, our proteomic profiling of the response for the differences between the core and distal muscle showed also that thermogenesis/response to the cold was a major difference.

We make it clear in the manuscript that the measurement of an increase in RyR1 Ca2+ leak in mammalian skeletal muscle is sufficient to indicate that NST has increased.

We have amended the text of the study to make it clear that it is the chronically raised RyR Ca2+ leak that underlies the progression of LGMD and not thermogenesis. However, it is a fundamentally important point of the study that NST is the reason that leak is raised regionally in the first place.