nature portfolio

Peer Review File

Protein Kinase 2 of the Giant Sarcomeric Protein UNC-89 Regulates Mitochondrial Morphology and Function

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors have investigated the effect of a mutation in the C. elegans gene unc-89. The protein is in the M-line of the muscle sarcomere and is essential for the assembly of the correct structure. UNC-89 is a large titin-like protein with tandem immunoglobulin domains and two kinase domains near the C-terminus. The kinase domain, PK2, is predicted to be enzymatically active, although the substrate is not known. With the aim of inactivating PK2, a lysine residue in the catalytic domain was substituted with alanine. The structure and function of muscles was nearly normal, but mitochondria were fragmented. The effect on mitochondrial function is described. Mitochondrial fragmentation depends on the GTPase, DRP-1, and it is suggested that this may be a substrate of PK2.
This is not the first study of the interaction between muscle sarcomeres and mitochondria. Avellaneda et al (Nat.comm 2021) described mechanical feedback between myofibrils and mitochondria in Drosophila. The elongated shape of mitochondria in Drosophila flight muscle is determined by an interaction with myofibrils. Decreased expression of DRP-1 in Drosophila flight muscle resulted in a decrease in fission of mitochondria, which is consistent with the finding that the fragmentation of mitochondria in C. elegans PK2 mutants is associated with an increase in the amount of DRP-1 in the mitochondria. A mechanical effect similar to that in Drosophila could be happening in C. elegans. If the PK2 mutant directly or indirectly affects the stiffness of the sarcomere, this could influence the shape and fragmentation of mitochondria. This possibility should be considered.
The finding that a mutation in an UNC-89 kinase domain affects the shape of mitochondria is unexpected and is the first clue to the function of obscurin kinases. The identification of the substrate of PK2 would be an important sequel.

Detailed comments
1. The Introduction and Discussion have extensive descriptions of various mutants not directly relevant to UNC-89. This detracts from the focus of the work and distracts the reader. I suggest cutting out all that is not essential to the argument.
2. There is comparison of C. elegans obscurin with the protein in vertebrates, but nothing about Drosophila. Previous findings on the function of Drosophila obscurin could usefully be compared with UNC-89. What is the phenotype of the Drosophila obscuirn knock out? The recent work on Drosophila PK1 (Zacharchenko et al Open Biol 2023) should be mentioned. Has any substrate of the kinases in Drosophila obscurin been identified?
3. p.1 The obscurin gene in Drosophila is now called obsc (Flybase).
4. p.8 2nd paragraph. This repeats the introduction on p. 5. UNC-68 is a distraction and

best omitted.
5. Figure 8 B,C,D,E There does not appear to be a difference between unc-89(sf22) and WT. I would leave out this figure.
6. Figure 9B. Why is the effect greater in unc-89(sf22) than unc-89(syb1360)?
7. p.16 2nd paragraph. Is this a description of DRP-1 function in vertebrates, or in C. elegans? The mitochondrial complexes differ in Drosophila and vertebrates and it is likely C. elegans will be more like Drosophila.
8. p. 18. The effect of the PK2 mutation could be mechanical (see above), not necessarily involving a signalling pathway. The interaction between sarcomeres and mitochondria was first shown in Drosophila.

Reviewer #2 (Remarks to the Author):

The Benian lab analyzes a novel obscurin mutant with mitochondrial phenotypes. Obcurin has two C-terminal kinase domains PK1 and PK2. PK1 is likely a pseudokinase in C.elegans and it certainly is one in Drosophila (Zacharchenko et al. 2023, Open Biology; this paper should be discussed). PK2 is potentially an active kinase, although no functions or substrates are known. They made a putative kinase dead KtoA mutant in PK2 which shows a strong mitochondrial phenotype.
Myofibers and mitochondria are closely apposed and strongly interdependent, but have largely been analyzed separately; therefore the idea to identify a structural sarcomere component that regulates mitochondria is highly attractive and made me inclined to like the paper.
The paper starts out very strongly with the initial generation and analysis of the KtoA mutant. I am impressed by the rigour of the analysis, the generation of multiple independent alleles, all of which have mitochondrial defects and no apparent muscle defects.
One major weakness is that it is unclear if the mitochondrial phenotype is a direct or indirect effect of the KtoA mutation. It is also unclear if there is really no muscle phenotype. To state that with certainty would require electron microscopy of muscles and mitochondria in wt and mutants, as well as aged wt and aged mutants (maybe the phenotype is contractile load-dependent and shows up only later).
Another major weakness is that there is no evidence that obscurin PK2 is a kinase at all.

The paper talks a lot about kinase aspects of PK2 throughout the paper, but presents no evidence in that direction. For example, the authors show an exciting enrichment of Drp-1 at mitochondria, and an enrichment of UCP-4. Both proteins are rather small (80 kD for Drp-1: note that the MW marker is wrong in Fig 7C; 38 kD for Ucp-4), meaning that a phosphorylation should show up as a band shift in wt conditions compared to the PK2 mutants. The absence of any change in size suggests these proteins are not phosphorylated by PK2. The observed enrichment could be far downstream of the KtoA phenotype or the PK2 mutant may not act as a kinase mutant at all.
The remainder of the phenotypic description is somewhat unhelpful in clarifying the mechanism of obscurin function. There are various changes in electron transport chain complexes, in glycolysis, and in ATP/ADP ratio, but whether these are owing to complicated feedback loops or downstream events or direct effects of PK2, remains unclear.
It might also be helpful to analyze a PK2 overexpression mutant or a tissue-specific RNAi knockdown of the PK2-containing isoforms to learn more about the phenotype.
Minor points are some inconsistencies:
Drp1 localization to mitochondria is increased, but respiration is increased.
Complex 1-induced respiration is increased (Fig. 9), even though complex IV protein is reduced (Fig. 10). How is that possible when the measured oxygen requires complex IV to be made?
Overall, the manuscript may be more successful if split in two parts: an initial more detailed analysis of the novel allele with electron microscopy, followed by a later paper once a mechanism or substrate of PK2 (if it exists) has been identified.

Reviewer #3 (Remarks to the Author):

Unc-89 PK2 mutant affects mitochondria
Summary
The results in this paper strongly suggest that the giant M-line protein UNC-89 is a key player in signaling between the contractile apparatus and the metabolic activity centers of the cell. This is a novel and important finding and I highly recommend acceptance. UNC-89 contains several different types of conserved domains, including multiple immunoglobulin domains and fibronectin type III repeats, and is important for assembly of M-line proteins, organization of the sarcoplasmic reticulum, and optimal muscle function. In this report, the authors convincingly demonstrate that it is the catalytic activity of PK2, one of the two protein kinase domains in UNC-89, that plays a key role in proper mitochondrial morphology and function. This hypothesis is in keeping with the effects on metabolic

pathways and the electron transport chain proteins in the mouse double knock out of OBSC and obsl1, which are two of the three mammalian UNC-89 homologs expressed in striated muscle. Loss of PK2 catalytic activity in C. elegans leads to fragmented mitochondria. The authors provide genetic and cell biological data consistent with a molecular mechanism in which the lack of PK2 activity increases the fission activity of the DRP-1 protein by increasing DRP-1 association with mitochondrial membranes. The authors also identify alterations in several aspects of mitochondrial function, including apparent uncoupling of electron transport chains, which correlates with increased expression of the uncoupling protein UCP-4.

Results

The authors used CRISPR/Cas9 to introduce a canonical kinase-dead lysine to alanine (KtoA) mutation into one of the two kinase domains in the giant M-line protein UNC-89. In obtaining four independent CRISPR isolates of the same amino acid change in the PK2 kinase domain, in using two different guide RNAs to make the mutations, and subsequently outcrossing all four isolates to wild type, the authors went well beyond the typical standards in controlling for possible off-target mutations. Therefore, the mitochondrial and metabolic phenotypes they describe can be attributed to the single missense change in the PK2 domain.

Additional strong evidence linking PK2 kinase activity to the mitochondrial organization function of UNC-89 comes from other unc-89 alleles that affect a subset of the isoforms produced from the locus: The strains that lack some large isoforms but express small isoforms with a PK2 domain do not have the mitochondrial fragmentation, whereas mutant that specifically lack kinase-containing isoforms have fragmented mitochondria.

The KtoA mutant protein has normal expression levels (western blot) and shows normal localization to the M-line (the thick-filament attachment structure). The authors investigated the cellular structures whose organization is dependent on UNC-89 and found that, unlike all previously studied unc-89 mutations, the CRISPR KtoA alleles had no detectable effect on these structures. The structure of the muscle contractile apparatus in these mutants is normal, as assessed by antibody markers for thick filaments, thin filaments, and dense bodies (thin filament attachment structure). Similarly, markers for microtubules and for the sarcoplasmic reticulum, which are affected in other unc-89 mutants, showed normal distribution in the KtoA strains.

The authors investigated the mechanism underlying the fragmented mitochondria phenotype of unc-89(sf22) by making a double mutant with an allele affecting the DRP-1 protein, which is required for fission of mitochondria. The drp-1 mutant has large, clumped mitochondria. The authors found that the double mutant resembled drp-1 [large, clumped mitochondria] and argue that this suggests drp-1 may be a target of UNC-89 kinase activity (line 270). I think the authors should first state that the epistasis of drp-1 over sf22 indicates that the fragmentation of mitochondria in unc-89(sf22) requires DRP-1 protein. Once that statement is made, then broach the possibility that UNC-89 may be a regulator of DRP-1 activity, and DRP-1 may be more active when PK2 has no catalytic activity. The authors made an anti-DRP-1 antibody and showed that KtoA mutants have normal levels of DRP-1 protein but have a higher level of DRP-1 associated with mitochondria. This is a plausible explanation for the fragmentation phenotype.

The authors also identify alterations in several aspects of mitochondrial function, including apparent uncoupling of electron transport chains, which correlates with increased expression of the uncoupling protein UCP-4. For the general reader, I suggest the authors explain what coupling is earlier in the paper, since this phenomenon is mentioned very early in the paper. They should also include how uncoupling leads to higher ATP levels. This is a straight forward concept for glycolysis, but less so for respiration.

Minor points and wording suggestions:
Line 63 This pathway might help
Line 82 gene, which utilizes … splicing to generate
Line 91 with segments of
Line 95 co-immunolocalization (noun), and analysis (noun) of muscle …
Line 96 Replace colon with a period
Line 97 one of the kinase domains [or either of the kinase domains]
Line 108 obscurin (OBSC), obscurin-like 1 (obsl1), and …
Line 118 types
Line 128 expressed, the global KO is embryonic lethal, and
Line 149 chain (ETC)
Line 171 remove space between Cas and 9
Line 172 mutation that likely inactivates the PK2
Line 182 lysine to alanine
Line 184 in which the homologous lysine in UNC-89 …
Line 185 to an alanine. The strain was then outcrossed 4 times to wild type to remove potential off-target mutations induced by CRISPR.
Line 222 study of the mouse double knock out for

Line 228 UNC-95, we show that mitochondria in wild-type muscle are organized …
Line 231 2017). In comparison, mitochondria in unc-89(sf22) appear fragmented.
Line 241 isoforms, but do express other isoforms (Small …

Line 258 says CRISPR alleles were crossed 4X to wild type whereas lines 464-466 say 3X.
Line 262 fusion and fission are primarily controlled by 3 …
Line 273 DRP-1 shuttles
Line 287 Mammalian cells that have null mutations in the two mitochondrial fusion genes Mfn1 and Mfn2 have fragmented mitochondria and also show decreased cellular respiration.
Lines 294, 303, 317, 318 the myo-3 promoter is not specific to body-wall muscle since it drives expression in single-sarcomere muscles and in the gonad sheath.

We thank the three reviewers for their interest in our original manuscript and their very insightful comments and suggestions. We tried to seriously address all of them. Our responses are shown below in italics blue font.

Reviewers' comments:

"Reviewer #1 (Remarks to the Author):

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This is not the first study of the interaction between muscle sarcomeres and mitochondria. Avellaneda et al (Nat.comm 2021) described mechanical feedback between myofibrils and mitochondria in Drosophila. The elongated shape of mitochondria in Drosophila flight muscle is determined by an interaction with myofibrils. Decreased expression of DRP-1 in Drosophila flight muscle resulted in a decrease in fission of mitochondria, which is consistent with the finding that the fragmentation of mitochondria in C. elegans PK2 mutants is associated with an increase in the amount of DRP-1 in the mitochondria. A mechanical effect similar to that in Drosophila could be happening in C. elegans. If the PK2 mutant directly or indirectly affects the stiffness of the sarcomere, this could influence the shape and fragmentation of mitochondria. This possibility should be considered."

We apologize for overlooking this important paper! In the Discussion, we state on page 22:

"Although our studies provide a possible molecular mechanism by which sarcomeres communicate with mitochondria, our study is not the first to provide evidence of communication between these organelles. In a remarkably sophisticated study using Drosophila, Avellaneda et al. (2021) demonstrated that there is communication between mitochondria and myofibrils during muscle development. Drosophila have two types of muscle, fibrillar, exemplified by indirect flight muscle in which individual myofibrils are not laterally aligned with their neighbors, and body muscles such as in the legs in which myofibrils are aligned laterally to form a tube. The morphology and organization of mitochondria are different in these muscle types. In indirect flight muscle the mitochondria are elongated and lie between the myofibrils, whereas in leg muscle the mitochondria lie mostly within the tube and peripherally. When Avellaneda et al. overexpressed a mitofusin homolog required for mitochondrial fusion, or expressed a dominant negative Drp1 required for fission, the morphology of indirect flight muscle resembled leg

muscle with myofibrils aligned laterally and often forming tubes and mitochondria located inside."

Also, there is a distinction between this previous work and our work. The Drosophila study suggests communication from mitochondria to sarcomere, whereas our work suggests communication from sarcomere to mitochondria.

A mechanical feedback mechanism certainly seems attractive. However, currently, we have no way to measure the stiffness of body wall muscle in C. elegans.

"The finding that a mutation in an UNC-89 kinase domain affects the shape of mitochondria is unexpected and is the first clue to the function of obscurin kinases. The identification of the substrate of PK2 would be an important sequel."

We thank the reviewer for appreciating the significance of our work and pointing out that our results are the first clue to the function of UNC-89/obscurin kinase domains. We would very much like to identify the substrate for worm PK2, but this will require a significant amount of extra work. Hopefully, we can describe it in a future manuscript.

"Detailed comments
1. The Introduction and Discussion have extensive descriptions of various mutants not directly relevant to UNC-89. This detracts from the focus of the work and distracts the reader. I suggest cutting out all that is not essential to the argument."

In the Introduction we have eliminated mentioning mutants in SPEG, and eliminated a whole paragraph on the known or suspected substrates for SPEG kinase domains. We have also removed unnecessary verbiage throughout the manuscript, but also needed to add 2 paragraphs to the Introduction on the Drosophila obscurin mutant and PK1 to PK2 interaction, a section on overexpression of PK2 to the Results, and a paragraph in the Discussion on the impressive study by Avellaneda et al. Despite these necessary additions, we have reduced the Introduction-Results-Discussion from 5841 to 5198 words.

"2. There is comparison of C. elegans obscurin with the protein in vertebrates, but nothing about Drosophila. Previous findings on the function of Drosophila obscurin could usefully be compared with UNC-89. What is the phenotype of the Drosophila obscuirn knock out? The recent work on Drosophila PK1 (Zacharchenko et al Open Biol 2023) should be mentioned. Has any substrate of the kinases in Drosophila obscurin been identified?"

Thank you for pointing this out. As suggested we have added in the Introduction the phenotype of obscurin deficiency in flies (Katzemich et al., 2012)(page 4), and the work by Zacharchenko et al., (2023) which demonstrates that Drosophila PK1 is an inactive pseudokinase, and that PK1 interacts with PK2 (page 6). To our knowledge, no substrates for the obscurin kinases from Drosophila have been identified to date.

“3. p.1 The obscurin gene in Drosophila is now called obsc (Flybase).”

This has been corrected.

“4. p.8 2nd paragraph. This repeats the introduction on p. 5. UNC-68 is a distraction and best omitted.”

Thank you for pointing out the reiteration of information in the Introduction and in the Results regarding the myofibril to SR linkage function of obscurin/UNC-89. In accord, we have simplified that paragraph in the Results, by stating at the top of page 11, “Because deficiency of mouse obscurin and worm UNC-89 result in disorganized SR, we examined SR organization using the marker UNC-68 (Ryanodine receptor).” Although we have now moved the UNC-68 imaging to Supplementary Fig S4, we would like to retain this data because sf22 does not affect the organization of the SR in contrast to other mutant alleles of unc-89.

“5. Figure 8 B,C,D,E There does not appear to be a difference between unc-89(sf22) and WT. I would leave out this figure.”

In agreement, we have eliminated the Perceval data from the original Figure 8 (now Figure 7), but have moved it to Supplementary Fig S9. We would like to retain this data, however, since we have noted in the Results (top of page 16) that “Although most of these results do not reach the level of statistical significance, they do show trends consistent with our tentative conclusions.”

“6. Figure 9B. Why is the effect greater in unc-89(sf22) than unc-89(syb1360)?”

That is a good question. We cannot explain this, however, it might be due to background mutations. Each strain was independently generated by CRISPR and although outcrossed 3-4X to wild type, it is likely that there are still some CRISPR induced background mutations which are different in the two strains.

“7. p.16 2nd paragraph. Is this a description of DRP-1 function in vertebrates, or in C. elegans? The mitochondrial complexes differ in Drosophila and vertebrates and it is likely C. elegans will be more like Drosophila.”

Sorry for that ambiguity. We have now clarified that the Tilokani et al. reference is talking about vertebrates. We now write on the top of page 20, “The conserved GTPase Drp1 is required for mitochondrial fragmentation, and in mammals, this occurs by recruitment of Drp1 to the outer membrane by adaptors, then oligomerization, recruitment of dynamin 2 and membrane scission (60).”

“8. p. 18. The effect of the PK2 mutation could be mechanical (see above), not necessarily

involving a signalling pathway. The interaction between sarcomeres and mitochondria was first shown in Drosophila."

Points well-taken. We address this above, and unfortunately, we have no way to measure the stiffness of body wall muscle in C. elegans. However, we would like to point out that a mechanical effect is not necessarily contradictory, or even independent, to a signaling pathway based on PK2 phosphotransfer.

Zacharchenko et al., 2023 have shown a direct interaction of the inactive pseudokinase PK1 with the predictably active PK2 in Drosophila, speculating that PK1 is likely to act as an allosteric regulator of PK2 (which is frequently observed in dual kinase systems). PK1 and PK2 kinases are linked by a long, instrinsically disordered sequence. The equivalently disordered inter-kinase sequence from C. elegans has been shown (by us) to behave as an elastic spring, and that in-frame deletion results in a significant phenotype (Qadota et al., 2020). Such domain arrangement in the form of the PK1-spring-PK2 is conserved throughout UNC-89/obscurin proteins of all phyla and Zacharchenko et al proposed that the PK1-spring-PK2 conserved segment acts as an integrated functional unit. The authors speculated that the mechanical response of the interkinase region could provide mechanical regulation to the complex formed by PK1-PK2, permitting either its formation or causing its disassembly upon filament stretch (caused by shearing forces during muscle function). If these principles applied to UNC-89 from C. elegans, then mechanical forces could regulate the phosphotransfer output of PK2. Thereby, a mechanical effect might be directly connected to PK2 activity. This interpretation based on a conciliation of Drosophila data (Zacharchenko et al., 2013) and our data in this study is, however, highly speculative at this time. In order not to provide readers with a hypothetical view that is currently unsupported by experimental evidence, we prefer not to include this interpretation in our manuscript. We provide here these thoughts just to point out the possibility that mechanical signals and PK2 activity might coalesce into a same signaling pathway.

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One major weakness is that it is unclear if the mitochondrial phenotype is a direct or indirect effect of the KtoA mutation. It is also unclear if there is really no muscle phenotype. To state

that with certainty would require electron microscopy of muscles and mitochondria in wt and mutants, as well as aged wt and aged mutants (maybe the phenotype is contractile load-dependent and shows up only later)."

We thank the reviewer for his/her generally positive reception to our paper. However, in the manuscript, we do not claim that the mitochondrial phenotype is either a direct or indirect effect of the PK2 KtoA mutation. We simply describe the phenotype in detail. In several places we are careful to say that the effect results from phosphorylation of a substrate (like DRP-1) directly by PK2 or through a kinase cascade.

There is no muscle sarcomere phenotype using a widely accepted way of assessing sarcomere structure, immunostaining and confocal microscopy using antibodies to various sarcomere components. However, to be certain, we have conducted EM to examine the sarcomere structure at higher resolution, and this is presented in a new part d for Fig. 2. We now state, on the bottom of page 10, "To examine the myofilament lattice at higher resolution we used transmission EM. As shown in ***Fig 2d****, the ultrastructure of the sarcomeres in unc-89(sf22) and in unc-89(syb1360), a second, independently isolated PK2 KtoA mutant allele (see below), appear as in wild type; there are normally organized A- and I-bands, normal numbers of thick and thin filaments, and normally organized dense bodies, M-lines and membranous sacs of the SR."*

To explore a possible muscle phenotype even further, we provide a new Supplemental Fig S6 which examines the structure of pharyngeal muscle since UNC-89 is known to be expressed in these muscles and some unc-89 mutants are known to have a structural defect. Similar to what we found for body wall muscle, the PK2 KtoA mutants show normal pharyngeal muscle structure.

A study of these particular mutants on aging muscle was not undertaken as it would require considerable effort and we have not yet studied the effect of any of the numerous unc-89 mutants on aging. Please allow this work to be described perhaps in the future in a separate paper.

"Another major weakness is that there is no evidence that obscurin PK2 is a kinase at all. The paper talks a lot about kinase aspects of PK2 throughout the paper, but presents no evidence in that direction. For example, the authors show an exciting enrichment of Drp-1 at mitochondria, and an enrichment of UCP-4. Both proteins are rather small (80 kD for Drp-1: note that the MW marker is wrong in Fig 7C; 38 kD for Ucp-4), meaning that a phosphorylation should show up as a band shift in wt conditions compared to the PK2 mutants. The absence of any change in size suggests these proteins are not phosphorylated by PK2. The observed enrichment could be far downstream of the KtoA phenotype or the PK2 mutant may not act as a kinase mutant at all."

We agree with the reviewer that the lack of experimental demonstration of PK2 phosphotransfer activity is a weakness in our manuscript. However, despite very substantial efforts we have not been able to obtain recombinant samples of the PK2 kinase suitable to

perform the required experimentation. In fact, pursuing further this aim has been the main reason for delaying our re-submission of this paper.

Specifically, the laboratory of one of our co-authors (Mayans) has ample expertise on the recombinant production of muscle kinases. Prior to the initial submission of this manuscript, they explored extensively the production of PK2 in bacteria, testing a multitude of bacterial strains, solubility tags, production protocols and PK2 constructs of varied composition. The latter is known to be a critical parameter in the production of kinases from muscle filaments, as these kinase domains exist within the context of a long polypeptidic chain and are flanked by additional N- and C-terminal domains that often contribute to their stability (Bogomolovas et al. & Mayans, 2022 Methods in Enzymol). These efforts, however, resulted in samples of moderate yield and quality. Independent efforts by the Benian lab to produce the sample recombinantly in bacteria had a similar outcome. This agrees with previous experiences with UNC-89/Obsc PK2 from Drosophila that could not be produced successfully either in bacteria, or in eukaryotic insect cells, and had to be finally produced recombinantly in Drosophila muscle (Zacharchenko et al. & Mayans, 2023). In an effort to address reviewer comments, we attempted next to produce PK2 recombinantly in C. elegans to overcome any possible concerns on the integrity of the kinase native fold. For this, we created transgenic worms that expressed the PK2 domain with an HA tag and IP-ed it using anti-HA-magnetic beads. The material so obtained contained not only a protein of the size expected for PK2-HA, but also an equally abundant protein of about 200 kDa, which we cannot explain. Despite the modest purity of the samples and since no known substrate is available to validate catalysis (!), we tested this material against known generic kinase substrates—casein and myelin basic protein. We observed no obvious activity by looking for a shift in PhosTag gel, or by western blot using anti-phospho-MBP, this highlighting the very significant challenge of confirming phosphotransfer activity when the substrate is not known (!). The low amount of PK2 samples so produced and the moderate sample purity did not make it realistic to pursue other experimental approaches using sample so produced.
Given these circumstances, we used our best bacterially-produced recombinant PK2 samples to attempt a range of experiments. The Mayans laboratory tested phosphotransfer by incubating PK2 with C. elegans lysates as well as explored autophosphorylation, in both cases using radioactive ATP. The Benian lab provided independently produced recombinant sample of both wild type and KtoA PK2 versions to a company that performed phosphotransfer assays commercially by testing PK2 against 83 substrates known to be phosphorylated by protein kinases. Unfortunately, apparent hits were also present in the KtoA control sample suggesting that the observed activity might originate from contaminating bacterial kinases (e.g. arginine kinases), which seems to agree with the fact that the positive hit peptides were rich in arginines. In summary, two of our laboratories have devoted significant efforts during a prolonged period of time to identify the PK2 substrate and have been unsuccessful. Achieving this goal will require methodologies not relying on recombinant samples and will involve a significant additional effort and resources. We regret that we cannot provide experimental evidence at this time.

However, because of their physiological and biomedical relevance, kinases are one of the most thoroughly studied protein classes. The kinase fold is, in fact, highly conserved and the groups involved in phosphotransfer are well characterized and strictly conserved in canonical kinases.

Thus, we have performed a thorough bioinformatics analysis of PK2 by analyzing the conservation of their catalytic sequence motifs, predicting its 3D-fold, and performing a comparison to established active kinases within its phylogenetic class. This analysis is now contributed to the manuscript (pages 7-10; Fig. 1a-c; Supplementary Fig S1-S3). The results confirm that PK2 shares the hallmarks of canonical active kinases, displaying a typical active site and ATP binding pocket. 3D-modelling confirms that PK2 can adopt a catalytically-committed conformational state compatible with the delivery of phosphotransfer. In our view, all features indicate that PK2 is an active kinase.

The reviewer is correct in that there does not seem to be an expected band shift for DRP-1 (which in mammals is known to be phosphorylated) or for UCP-4. However, it should be pointed out that DRP in mammals undergoes multiple phosphorylations, some of which promote and some of which inhibit association with mitochondria, and so the net effect might not be discernable by a gel. Also, if only a small percent of a protein is phosphorylated there will not be discernable shift of mobility on a gel

"The remainder of the phenotypic description is somewhat unhelpful in clarifying the mechanism of obscurin function. There are various changes in electron transport chain complexes, in glycolysis, and in ATP/ADP ratio, but whether these are owing to complicated feedback loops or downstream events or direct effects of PK2, remains unclear."

This criticism is certainly valid, but it is still remarkable that these changes, direct or indirect, result from changing a single amino acid in one domain of a giant protein. Moreover, they illuminate a role for UNC-89/obscurin in sarcomere-to-mitochondria communication whereby signaling from the sarcomere regulates both mitochondrial structural organization and bioenergetics to maintain muscle function.

"It might also be helpful to analyze a PK2 overexpression mutant or a tissue-specific RNAi knockdown of the PK2-containing isoforms to learn more about the phenotype."

Following this excellent suggestion by the reviewer, we have examined PK2 overexpression using a transgene that expresses a M-line-localizing segment of UNC-89 that contains PK2 under control of a heat shock promoter. As shown in a new Supplementary Fig S10 and described on pages 18-19), overexpression of PK2 results in mitochondrial clumping and a decrease in the association of DRP-1 with mitochondria. This result is opposite from inactivation of PK2 by the KtoA mutation.

The reviewer should note that we present in the paper the phenotype of unc-89(tm752) which lacks all PK2-containing isoforms—at least for mitochondria, it is similar to the PK2 KtoA mutants.

"Minor points are some inconsistencies:
Drp1 localization to mitochondria is increased, but respiration is increased."

We had expected that respiration would be decreased given that mitochondrial fragmentation is usually associated with decreased mitochondrial function. This paradox was considered in the last paragraph of the Discussion, beginning at the bottom of page 22: "However, some studies suggest that mitochondrial fragmentation is not always associated with decreased mitochondrial function and may be beneficial… definitive study was reported on heart muscle (67). After exercising mice for 1 hour, the authors that mitochondria were fragmented, total Drp1 and activated (serine 616) Drp1 was increased, oxidative stress was not increased, mitochondrial membrane potential was maintained, pro-mitophagy proteins decreased, and mitochondrial respiration was increased. Their conclusion is that mitochondrial fragmentation associated with increased mitochondrial function is part of the normal cardiac response to increase energetic demand encountered during normal levels of exercise."

"Complex 1-induced respiration is increased (Fig. 9), even though complex IV protein is reduced (Fig. 10). How is that possible when the measured oxygen requires complex IV to be made?"

We agree that our finding that MTCO1 (complex IV) levels were surprising, especially in light of the increased complex I-supported respiration. Our interpretation of the data is that even if MTCO1 levels are reduced with the PK2 KtoA mutation, this reduction is not rate limiting for respiration. Additionally, it is important to note that MTCO1 is just one subunit of complex IV, and while MTCO1 reduction might result in impaired complex IV assembly, this does not have to be the case (the reduction in one subunit does not automatically lead to an overall decrease in the assembled holocomplex). We were unable to test the impact of our PK2 KtoA mutation on complex IV assembly, as the most appropriate method for such analyses would be blue native PAGE followed by immunoblotting for complex IV. This method requires an antibody that recognizes native forms of C. elegans complex IV subunits, and unfortunately, we have not identified an antibody that works under these conditions. However, given our complex I respiration results, we would predict that in the context of PK2 KtoA, complex IV assembly would be preserved.

"Overall, the manuscript may be more successful if split in two parts: an initial more detailed analysis of the novel allele with electron microscopy, followed by a later paper once a mechanism or substrate of PK2 (if it exists) has been identified."

We acknowledge that alternative presentations of our work are possible. However, we are not confident at this time that we will be able to identify the substrate targeted by PK2 since our (very extensive!) efforts to this effect have been unsuccessful to this date. Given this circumstance, we feel that the publication of our work in its current form is the most realistic option and we ask that the work is kindly considered as here presented.

"Reviewer #3 (Remarks to the Author):

Unc-89 PK2 mutant affects mitochondria

Summary
The results in this paper strongly suggest that the giant M-line protein UNC-89 is a key player in signaling between the contractile apparatus and the metabolic activity centers of the cell. This is a novel and important finding and I highly recommend acceptance. UNC-89 contains several different types of conserved domains, including multiple immunoglobulin domains and fibronectin type III repeats, and is important for assembly of M-line proteins, organization of the sarcoplasmic reticulum, and optimal muscle function. In this report, the authors convincingly demonstrate that it is the catalytic activity of PK2, one of the two protein kinase domains in UNC-89, that plays a key role in proper mitochondrial morphology and function. This hypothesis is in keeping with the effects on metabolic pathways and the electron transport chain proteins in the mouse double knock out of OBSC and obsl1, which are two of the three mammalian UNC-89 homologs expressed in striated muscle. Loss of PK2 catalytic activity in C. elegans leads to fragmented mitochondria. The authors provide genetic and cell biological data consistent with a molecular mechanism in which the lack of PK2 activity increases the fission activity of the DRP-1 protein by increasing DRP-1 association with mitochondrial membranes. The authors also identify alterations in several aspects of mitochondrial function, including apparent uncoupling of electron transport chains, which correlates with increased expression of the uncoupling protein UCP-4."

We very much appreciate this reviewer's positive reception to our paper.

"Results

The authors used CRISPR/Cas9 to introduce a canonical kinase-dead lysine to alanine (KtoA) mutation into one of the two kinase domains in the giant M-line protein UNC-89. In obtaining four independent CRISPR isolates of the same amino acid change in the PK2 kinase domain, in using two different guide RNAs to make the mutations, and subsequently outcrossing all four isolates to wild type, the authors went well beyond the typical standards in controlling for possible off-target mutations. Therefore, the mitochondrial and metabolic phenotypes they describe can be attributed to the single missense change in the PK2 domain.

Additional strong evidence linking PK2 kinase activity to the mitochondrial organization function of UNC-89 comes from other unc-89 alleles that affect a subset of the isoforms produced from the locus: The strains that lack some large isoforms but express small isoforms with a PK2 domain do not have the mitochondrial fragmentation, whereas mutant that specifically lack kinase-containing isoforms have fragmented mitochondria.

The KtoA mutant protein has normal expression levels (western blot) and shows normal localization to the M-line (the thick-filament attachment structure). The authors investigated the cellular structures whose organization is dependent on UNC-89 and found that, unlike all previously studied unc-89 mutations, the CRISPR KtoA alleles had no detectable effect on these structures. The structure of the muscle contractile apparatus in these mutants is normal, as assessed by antibody markers for thick filaments, thin filaments, and dense bodies (thin filament attachment structure). Similarly, markers for microtubules and for the sarcoplasmic

reticulum, which are affected in other unc-89 mutants, showed normal distribution in the KtoA strains.

The authors investigated the mechanism underlying the fragmented mitochondria phenotype of unc-89(sf22) by making a double mutant with an allele affecting the DRP-1 protein, which is required for fission of mitochondria. The drp-1 mutant has large, clumped mitochondria. The authors found that the double mutant resembled drp-1 [large, clumped mitochondria] and argue that this suggests drp-1 may be a target of UNC-89 kinase activity (line 270).

I think the authors should first state that the epistasis of drp-1 over sf22 indicates that the fragmentation of mitochondria in unc-89(sf22) requires DRP-1 protein. Once that statement is made, then broach the possibility that UNC-89 may be a regulator of DRP-1 activity, and DRP-1 may be more active when PK2 has no catalytic activity."

This is an excellent point. In response, we now write on page 14: "The epistasis of drp-1 over sf22 indicates that the fragmentation of mitochondria in unc-89(sf22) requires DRP-1 protein. One possibility is that UNC-89 PK2 may be an inhibitor of DRP-1 activity, and DRP-1 may be more active when PK2 has no catalytic activity. This further suggests that DRP-1 protein could be a target of the protein kinase activity of UNC-89 PK2, directly or indirectly through a kinase cascade."

"The authors made an anti-DRP-1 antibody and showed that KtoA mutants have normal levels of DRP-1 protein but have a higher level of DRP-1 associated with mitochondria. This is a plausible explanation for the fragmentation phenotype.

The authors also identify alterations in several aspects of mitochondrial function, including apparent uncoupling of electron transport chains, which correlates with increased expression of the uncoupling protein UCP-4. For the general reader, I suggest the authors explain what coupling is earlier in the paper, since this phenomenon is mentioned very early in the paper. They should also include how uncoupling leads to higher ATP levels. This is a straight forward concept for glycolysis, but less so for respiration."

*To not disrupt the flow of the paper, we decided to leave an explanation of what uncoupling is until where we had it, about 3 paragraphs after the result was given. Thus, uncoupling should lead to lower, not higher ATP levels, and we now point out, near the top of page 18: "Thus, uncoupling should result in less ATP production, and the observed increase in ATP is likely due to increased glycolysis (**Fig 9**).."*

"Minor points and wording suggestions:

Line 63 This pathway might help
Line 82 gene, which utilizes … splicing to generate
Line 91 with segments of
Line 95 co-immunolocalization (noun), and analysis (noun) of muscle …

Line 96 Replace colon with a period
Line 97 one of the kinase domains [or either of the kinase domains]
Line 108 obscurin (OBSC), obscurin-like 1 (obsl1), and …
Line 118 types
Line 128 expressed, the global KO is embryonic lethal, and
Line 149 chain (ETC)
Line 171 remove space between Cas and 9
Line 172 mutation that likely inactivates the PK2
Line 182 lysine to alanine
Line 184 in which the homologous lysine in UNC-89 …
Line 185 to an alanine. The strain was then outcrossed 4 times to wild type to remove potential off-target mutations induced by CRISPR.
Line 222 study of the mouse double knock out for
Line 228 UNC-95, we show that mitochondria in wild-type muscle are organized …
Line 231 2017). In comparison, mitochondria in unc-89(sf22) appear fragmented.
Line 241 isoforms, but do express other isoforms (Small …
Line 258 says CRISPR alleles were crossed 4X to wild type whereas lines 464-466 say 3X.
Line 262 fusion and fission are primarily controlled by 3 …
Line 273 DRP-1 shuttles
Line 287 Mammalian cells that have null mutations in the two mitochondrial fusion genes Mfn1 and Mfn2 have fragmented mitochondria and also show decreased cellular respiration.
Lines 294, 303, 317, 318 the myo-3 promoter is not specific to body-wall muscle since it drives expression in single-sarcomere muscles and in the gonad sheath."

Thank you for pointing our mistakes and making excellent word changes. All your suggestions have been used.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have answered my concerns. They are right to point out that in Drosophila the communication is from mitochondria to sarcomere and vice versa in C. elegans.

The Alpha-fold findings convincingly support the suggestion that PK2 is an active kinase like other DMT kinases.

Line 220 The mutation K to A renders kinases inactive. Are the mutants in a catalytically-primed state? Is the PK2 mutant in this state? Are other kinases made unstable by the mutation, although PK2 is not?

L412 It is not the mitochondria that are mutant, but Unc-89.

The manuscript is greatly improved with the added detail.

You could try co-expressing PK2 with chaperones. We obtained soluble Drosophila PK2 with this approach, but were unable to separate the chaperones from the PK2. That would need more work.

Belinda Bullard

Reviewer #2 (Remarks to the Author):

The manuscript has been much improved: especially the EM of muscles make the absence of muscle phenotypes more convincing. Then, the overexpression phenotype being opposite to the knockdown make the phenotype much more likely to be a direct effect of obscurin. Lastly, the alphafold modeling of the kinase domains provides a stronger background to the entire story. I therefore am satisfied with the revisions.

Reviewer #3 (Remarks to the Author):

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Summary

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localization to the M-line (the thick-filament attachment structure). The authors investigated the cellular structures whose organization is dependent on UNC-89 and found that, unlike all previously studied unc-89 mutations, the CRISPR KtoA alleles had no detectable effect on these structures when analyzed by electon microscopy as well as antibody markers for thick filaments, thin filaments, and dense bodies (thin filament attachment structure). Similarly, markers for microtubules and for the sarcoplasmic reticulum, which are affected in other unc-89 mutants, showed normal distribution in the KtoA strains.

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The authors have satisfactorily answered suggestions and criticisms of the manuscript.