FHL2 anchors mitochondria to actin and adapts mitochondrial dynamics to glucose supply

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Re: JCB manuscript #201912077

Dr. Thomas L Schwarz
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3 Blackfan Circle
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Dear Tom,

Thank you for submitting your manuscript entitled "FHL2 anchors mitochondria to actin and adapts mitochondrial dynamics to glucose supply". The manuscript has been evaluated by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

You will see that, although all three reviewers feel that the premise of the study is potentially interesting, they each raise a number of substantive (and consistent) concerns which preclude publication of the paper in its current form.

Although your manuscript is intriguing, I feel that the points raised by the reviewers are more substantial than can be addressed in a typical revision period. Therefore, if you wish to expedite publication of the current data, it may be best to pursue publication at another journal.

However, given interest in the topic, we would be open to an appeal of our decision and resubmission to JCB of a significantly revised and extended manuscript that fully addresses the reviewers' concerns and is subject to further peer-review. Such a revision would need to include new data to address each of the reviewers' comments in full with the following exception: while we agree with reviewer#1 that a mechanistic extension would enhance the impact of the paper, we do not feel that this would be necessary to support the main conclusions of the paper so we would not require this for resubmission.

If you would like to resubmit this work to JCB, please contact the journal office to discuss an appeal of this decision or you may submit an appeal directly through our manuscript submission system. Please note that priority and novelty would be reassessed at resubmission.

Regardless of how you choose to proceed, we hope that the comments below will prove constructive as your work progresses. You can contact the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Thank you for thinking of JCB as an appropriate place to publish your work.

Sincerely,

Richard Youle, PhD

Monitoring Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Mitochondrial dynamics including division, fusion and motility are important for proper mitochondrial function, and appropriate transport of mitochondria in neuronal axons is important for normal maintainence of neuronal physiology. Previous reports from this lab established that a glucose spike decreases mitochondrial motility in axons though O-GlcNAcylation of the protein Milton. The present study by Basu et al. extends this previous study and demonstrates that actin filament binding by FHL2 is important in this process by binding preferentially to O-GlcNAcylated Milton. They also show data that suggest that FHL2 binding to TRAK causes F-actin accumulation around mitochondria. The combined data suggest that TRAK-bound FHL2 causes mitochondria to stop their microtubule-based movement due to FHL2-mediated actin accumulation and actin binding.

Though the present study is interesting, it is somewhat superficial and lacks key mechanistic details on how actin filaments are formed, and mediate glucose induced arrest of mitochondrial motility. In addition, there are a number of significant technical concerns.

1. The central result in the paper is that GlcNAcylation of TRAK is important for FHL2 binding. Evidence for this is provided in a series of westerns comparing OGA over-expression (which should reduce GlcNAcylation) with OGT expression (which should increase GlcNAcylation). However, the actual western results are not very impressive. In Figure 4B, 4C, and 4E, there is significant pull-down of one protein with the other even in the OGA sample. Figure 4B is not quantified, and the crucial western (of FHL2 in the IP) is not of high quality. Also, there is not a control with neither OGA nor OGT. The ensemble of the data suggest that there is a mild increase in FHL2/TRAK interaction upon TRAK GlcNAcylation, even when the GlcNAcylation is forced.
2. Following on the first point, Figure 4F-G uses the TRAK mutant that cannot be GlcNAcylated to show that it binds less to FHL2 than WT TRAK. Why is there any binding to this mutant, if GlcNAcylation is essential?
3. The evidence that actin accumulates around mitochondria in non-neuronal cells is not impressive (Figure 2C and Figure 7H). In particular, the image in 7H does not really seem to be specific enrichment on mitochondria. It is odd that this is not also shown for neuronal cells. Do they see an increase upon the glucose shift or OGT expression?
4. Related to the last point, do the authors have any idea as to the mechanism that forms the actin structures associated with mitochondria downstream of OGT expression? This is important as this would establish a mechanistic relationship between OGT expression and actin filaments mediated mitochondrial motility arrest. Past studies of mitochondria and actin have implicated several factors as being of possible importance, including Arp2/3 and Spire1C. Are these proteins involved? From the cited work, FHL2 might bind F-actin but is not thought to mediate its polymerization. Even the claim that it binds actin appears to be based on one publication (Coghill 2003) that only seems to use somewhat superficial assays. How confident are the authors on a direct interaction?
5. An important control is to test whether LatA treatment alters TRAK GlcNAcylation itself?

6. For the quantification of mitochondrial motility in Figure 2, there are two technical issues:
a. it is not clear that this method is examining directional motility, or whether it reflects any change of position (directional or not). If this quantification does not measure directional motility, how are we to know that the mitochondria are not simply just 'wiggling' more or less in the different treatments?
b. it would be useful to have more specific details in the methods, rather than stating that the imaging was conducted the same as for neurons. For example, were these images really taken at 0.5 sec intervals for 90 min (which is what one deduces from piecing together from the figure legend and methods)?
7. For the endosomal motility in Figure S2D-E, there should be a control with no treatment (neither OGT nor OGA transfected).
8. For the PEX-miro experiments in Figure 3:
a. It would be useful to know if the OGT effect on PEX motility is inhibited by LatA.
b. It would be useful to know whether these PEX build up more actin around them than control PEX, in the presence or absence of OGT.
c. A recent study has shown that endogenous Miro1 is present on peroxisomes and it modulates the motility of the same (Castro et al., Traffic, 2018). In the light of this previous study, it is interesting to note that in Fig 3E-3F, OGT over-expression did not arrest peroxisomal motility in control cells. Why would this not occur?
9. Since the authors demonstrate a crucial role of FHL2 in actin mediated arrest of mitochondrial motility during glucose stimulation, it is important to have a little more information as to the localization of FHL2. Where does endogenous FHL2 localize in both unstimulated and glucose-stimulated cells? If they cannot examine endogenous FHL2, where does epitope-tagged FHL2 localize?
10. Following on the last point, it would be very good to know whether the FHL2/TRAK interaction is direct, especially since (at the bottom of page 8) the authors state that FHL2 can bind to the Miro/TRAK complex.
11. The FHL2 knock-down experiments performed in neurons (Figure 5) would be useful to perform in culture cells (U2OS) to test two things:
a. Is FHL2 necessary for OGT-mediated mitochondrial slowing?
b. Is FHL2 necessary for OGT-mediate actin accumulation around mitochondria?
12. Miro-Milton mitochondrial motility involves microtubules and the authors demonstrate that FHL2-mediated actin meshwork restricts this movement. It would be good to elaborate on this mechanism a little further. Do they think that the FHL2/actin interaction is sufficient to resist the force generated by microtubule motors?
13. For the FHL2 localization studies in Figure 7, it is important to show the effect of FHL2 expression without the Omp25 mitochondrial tag. Do mitochondria slow down as well? Does HA-FHL2 localize to mitochondria upon the appropriate stimuli?

Minor points:
1. In the results section for Figure 2, it is mentioned that the motility analysis is conducted in both Cos7 and U2OS cells. However, only Cos7 cells appear to be shown.
2. It would be good to have marks of MW for western blot images.
3. Figure 1A legend doesn't match the description in results. Why 6 mM glucose for first 55 hrs?
4. For Fig 1C,1E, 2B,2D etc., along with the p-values, it would be good to indicate which ones are significant.
5. In Figure 6C, there appears to be an overall decrease in mitochondrial motility in FHL2 shRNA cells (in low glucose). This finding might be pointed out.
6. In the first paragraph of the Introduction, is the "2 billion years" of mitochondrial evolution correct?

Reviewer #2 (Comments to the Authors (Required)):

Previous work from this lab provided extensive evidence that the OGT-dependent modification of the OMM microtubule motor protein adaptor Milton with GlcNAc, which is stimulated by elevated glucose, inhibits the translocation of mitochondria along microtubules in neuronal axons. Somewhat surprising, however, was the finding that motility arrest does not involve a disconnect between Milton and kinesin- the motor remains associated with GlcNAc-modified Milton. As a result, the underlying cause of motility arrest was unclear. This new study presents evidence that the motility arrest is caused by the interaction of GlcNAc-modified Milton with an actin binding protein (FHL2). While this study is very interesting and has some strengths, I have a number of significant concerns which are detailed below.

Perhaps my single biggest concern revolves around whether FHL2 really binds F-actin. The authors cite seven papers when they state without providing any evidence of their own that FHL2 binds to F-actin. I looked up these seven papers. In Boateng et al. (2016) FHL2 is shown to bind to the actin binding protein mAbp1, not to F-actin. Johannessen et al (2006) is a review on FHL2. Some of the key take a ways are it is localizes diffusely in the cytoplasm and in the nucleus (no mention of co-localization with F-actin), and that by 2006 it had already been reported to bind 50 different proteins, including "receptors, structural proteins, signal transducers, transcription factors and cofactors, splicing factors, DNA replication and repair enzymes, and metabolic enzymes" (sounds sticky!), but not actin! It goes on to talk about FHL2 function as regards cardiac physiology, bone formation, muscle function, placental development etc, but not a word about actin. Indeed a large fraction of the extensive literature on FHL2 is on its role as a transcriptional co-activator whose dysregulation is implicated in many types of cancers. Li et al (2001) focused on the localization of FHL2 at focal adhesions and provided no substantive data that it binds to F-actin. Tran et al (2016) is another review that focused on the role of FHL2 in apoptosis, cell migration, cardiovascular function, vascular patterning, and regulation of nuclear receptor function. In all of these sections I did not find any mention of F-actin, and actin is not present in the huge table they present of FHL2 interacting proteins. Olson and Nordhein is yet another review. They mention that FHL2 shuttles between focal adhesions and the nucleus, but say nothing about its interaction with F-actin. The paper by Ng et al only identified FHL2-regulated genes in liver by microarray and bioinformatics analysis. Finally, the one paper that seems to support the idea that FHL2 binds F-actin is the Coghill paper on FHL3. But their "evidence" that FHL3 binds to F-actin in Y2H (not sure what that tells us) and IP (find me an IP that does not have actin in it!!). What is required here is the decades-old norm in the actin field for proving interaction with F-actin: a binding isotherm performed by sedimentation assay using purified protein and F-actin at increasing concentrations. So all together, the data in the literature they cite supporting the idea that FHL2 binds to F-actin is in my opinion essentially zero. Of note, it is also not encouraging that expression data bases show FHL2 expression in the brain is very low, especially as compared to heart muscle. Finally is the issue of whether any LIM domains have been shown biochemically to bind to F-actin. While LIM domain-containing proteins have in some cases been linked to the regulation of the actin cytoskeleton, (e.g. LIM-Kinase, which regulates the F-actin depolymerizing protein cofilin), to my knowledge no LIM domain has been shown to bind to F-actin in a standard F-actin sedimentation assay using purified proteins. One well-characterized example is the LIM domain-containing protein zyxin, which has been studied extensively by Mary Beckerle. While she has shown that GFP-zyxin localizes to strained stress fibers transiently and only at sites where the stress fiber is about to break (Dev Cell 2010), she has never been able to show that zyxin binds F-actin in a standard F-actin pelleting assay (published data). Moreover, the mechanism that restricts zyxin recruitment to the immediate vicinity of stress

fiber rupture sites is unknown. Consistent with all this, I could not find a paper in the literature showing that any LIM domain binds to F-actin in standard biochemical assays for this interaction. I assume the authors had the same trouble given the papers they cite supporting the interaction between FHL2 and F-actin. Given all this, and the complete absence of any biochemical data in this paper that FHL2 binds to F-actin, I find it a stretch to conclude that FHL2 links mitochondria to F-actin.

The authors earlier Cell paper describing the role of GlcNAc modification of Milton in regulating mitochondrial transport focused on the microtubule-dependent movement of this organelle in the axons of Hippocampal neurons. This context is great because the fast anterograde and retrograde transport of mitochondria along microtubules in these "2D" structures is so clear and so readily scorable as regards movement versus cessation of movement. What is a lot less satisfying in the considerable amount of data in this current paper where mitochondrial motility is scored in tissue culture cells (Cos, U2OS). First, this scoring is not restricted to the subset of mitochondrial movements in these cells that are likely to be microtubule based (fast, persistent, linear trajectories). This seems like a disconnect with the Cell paper. Instead, the motility that is measured is drastically weighted in the direction of the pervasive "jiggling" that mitochondria exhibit in such cells. The key data regarding this jiggling is that actin disassembly with Latruculin does not alter the extent of jiggling when glucose is low or OGT is not over-expressed, but it does result in increased jiggling (basically to the same values as in the control) when glucose is high or OGT is over-expressed. In other words, Latrunculin reverses the very low jiggling value seen in high glucose or OGT over-expression. Their interpretation of this result (that OGT over expression or glucose elevation stops jiggling because mitochondria get connected to cytoplasmic F-actin via FHL2 recruitment) may be correct. But I wonder if Latrunculin might alter jiggling indirectly based on work from the Weitz lab. In the 2014 Cell paper from this lab (158, p822), the authors showed that actomyosin contractions in the cytoplasm are transmitted across the cytoplasm because it behaves like a viscoelastic gel. One readout of this was fluctuations in mitochondrial shape. While their main tool to show this was myosin inhibition, my guess is they would have blocked mitochondrial shape fluctuations by disassembling actin as well. I get it that the controls in this current paper show the Latrunculin effect is specific to OGT over-expressing/high glucose conditions, but I remain somewhat skeptical that jiggling stops because the mitochondria become bound to F-actin. Is there some other way this later point could be shown (see also my next comment). An acid test of their model would be a KD/replacement using a version of FHL2 that can bind to Glc-NAc modified Milton but not F-actin. Obviously such a mutant cannot be searched for if they cannot first obtain biochemical evidence that FHL2 binds to F-actin.

I found the evidence in Figure 2C that O-GlcNAcylation-mediated motility arrest in tissue culture cells is associated with changes in the association of mitochondria with F-actin to be pretty unconvincing. The "contacts" between mitochondria and actin in these diffraction-limited images are present in both control and OGT-expressing cells. More generally, I was not sure that they are saying OGT expression drives the attachment of mitochondria to actin structures (the way I assume an actin-based anchor would work) or the assembly of F-actin on the OMM (as in the JCB paper from the Karbowski lab following CCCP treatment, although lots of labs have also been weighing in on actin assembly on the OMM recently- Lippincott-Schwartz, Holzbaur etc.) Of note, my confusion regarding their conclusions was compounded by the images in Figure 7, Panel H, which the authors say show actin surrounding mitochondria with FHL2 constitutively bound to their surface (like in CCCP treated cells). To me, the image in Panel H shows dots of actin in the immediate vicinity of these mitochondria, not actin surrounding mitochondria as in the Karbowski paper (moreover, most of these central actin dots are not actually near mitochondria). These are an important issues to clarify, as excess actin assembly on the OMM (as in the over-expression of

dominant actin Spire 1C; Manor et al, 2016) looks like it stops mitochondrial jiggling. In terms of how FHL2-dependent anchoring of mitochondria to cytoplasmic actin structures works, quantitative, dynamic imaging of moving mitochondria encountering such structures plus or minus OGT expression would really help.

In a related issue, no localization data for endogenous FHL2 is presented. Is it present on cortical actin? That would be expected given that it is supposed to be an actin binding protein. Can they see it go on to mitochondria upon shift to high glucose (either dynamically as a FP-tagged protein or by IF)? No such pertinent localization data is presented.

With regard to the experiments on peroxisomes, previous studies of their motility focused primarily on the subset of peroxisomes that undergo fast, directionally-persistent, microtubule-dependent movements. Like my comments above regarding mitochondrial jiggling, I think the authors should show that OGT over expression stops these fast movement by connecting the organelles to F-actin (rather than, or in addition to, the static measurements of peroxisome distribution in the cell that they present). With regard to their distribution data, I am also perplexed by the observation that OGT over expression causes the peroxisomes with modified Milton on their surface to become perinuclear. This observation makes sense in the context of their earlier Cell paper, where OGT over expression was shown to arrest microtubule plus end-directed organelle transport. But it is not consistent with the underlying mechanism of arrest being the attachment of the organelle to F-actin (this paper), given that in most tissue culture cells the bulk of F-actin is in the cortex at the cell periphery. In other words, I would have expected the modified peroxisomes to accumulate in the periphery over time in high glucose/OGT over expression, not in perinuclear regions where there is very little F-actin. To me, this is a significant disconnect as regards their model.

If I did not miss something, the evidence presented here that FHL2 interacts with GlcNAc-modified Milton is based entirely on IPs. IPs cannot prove direct interaction. For example, given that Milton is in a stable complex with OGT, what is the evidence that Milton's "interaction" with FHL2 is not through OGT bound to Milton in the immunoprecipitate? This possibility (and other possible interactions) cannot be excluded without performing direct binding experiments using purified Milton and FHL2 to show that they interact directly (and the interaction is attenuated when Milton has Glc-NAc on it). This is a significant issue that requires clarification before publication.

Reviewer #3 (Comments to the Authors (Required)):

Control of mitochondrial motility is critical for cell function, especially in highly polarized cells, such as neurons. Thus, understanding of the mechanisms controlling mitochondria motility is important.
In this manuscript, by Basu et al., the authors provide some evidence linking the actin-binding protein FHL2 with mitochondrial adaptation to glucose supply. They developed a protocol in which hippocampal neurons adapted to grow on 1mM glucose are shifted to 5mM glucose followed by observation of mitochondrial motility. These cell culture conditions are thought to approximate the glucose fluctuations in the brain (although the 1mM glucose is far from physiologically relevant!). The data clearly show that a switch in glucose concentration reduces mitochondrial motility. This process was suppressed by treatment with actin-depolymerizing Latrunculin, and overexpression of OGT, indicating roles for the actin cytoskeleton and O-GlcNAcylation.
The authors also propose that FHL2 can regulate mitochondrial motility by anchoring them to the actin cytoskeleton. The immunoprecipitation experiments revealed that FHL2 is enriched in Milton (TRAK) immunoprecipitate from cells overexpressing OGT. Thus, in response to varying glucose

concentrations, FHL2 could be recruited to the mitochondria by O-GlcNAcylated Milton protein. Importantly, the role of FHL2 in control of mitochondrial motility was also confirmed with shRNA experiments.
Overall this is a potentially interesting work that provides new insights on the regulation of mitochondrial motility in neurons, and non-neuronal cells. Most of the conclusions are supported by strong evidence. However, the direct role of FHL2-mediated recruitment of the actin cytoskeleton to the mitochondria is not supported to the same degree as other parts of this manuscript (see below). This important issue should be addressed, before the manuscript can be recommended for publication in the high caliber journal, such as The Journal of Cell Biology.

Specific comments:

1. The immunoprecipitation studies (Figure 4) indicate that in OGT transfected cells FHL2 interaction with the mitochondria (through binding to Milton) is increased, as compared to OGA-expressing cells. However, knowing the subcellular localization of FHL2 in control, OGT-transfected and preferably under different glucose concentrations could strengthen a direct mitochondrial role of this protein in the regulation of mitochondrial motility. Indeed, as the authors acknowledge, the modulation of FHL2 expression can affect the overall organization of the actin cytoskeleton. Cell fractionation and imaging experiments would make this point stronger.
2. In line with the above comment, the authors could further verify the role of Milton in the mitochondrial assembly of actin. If their hypothesis is correct then Milton knockout or knockdown would result in the reduction of mitochondria-associated FHL2 and as a result lower levels of mitochondria-associated actin, especially under 5mM glucose or upon OGT-expression. For example, if the proposed mechanism is valid then Milton downregulation could reduce mitochondrial actin levels shown to be higher in OGT-expressing, Cos-7 cells (Figure 2C).
3. The levels of mitochondria-associate actin in Cos-7 cells (Figure 2C) should be also analyzed using fluorescent phalloidin. Otherwise, it is not clear whether the apparent effects are due to OGT activity or differences in life Act-RFP expression or assembly levels.
4. Generally, additional imaging and/or biochemical approaches could be used to further strengthen the role of FHL2 in the mitochondria: actin cytoskeleton interactions, including cell fractionation and analyses of actin and FHL2 localization under distinct conditions (e.g. Figures 2C, 4, and 5). These experiments could be performed in non-neuronal cells (e.g. Cos-7) and preferably supplemented with actin imaging under similar conditions. Without such data, the premise that mitochondrial interaction with actin cytoskeleton per se, but not other non-specific changes in the activity of relevant proteins, are important for glucose-dependent changes in mitochondrial motility is not strongly established.
5. Figure 3. The experiments shown in Figure 3 provide some evidence that OGT overexpression can also control organelle motility when anchored to peroxisomes (Pex-Miro expression). However, the role of actin in this system was not investigated.

We thank the reviewers and Dr. Youle in taking the time to read through our manuscript and give us in-depth feedback. Dr. Youle was particularly nice to thoroughly read our initial plans on responding and to clarify what he expected. The experiments done to address these recommendations have increased the quality of our paper in an efficient and directed manner.

In this re-submission, we have addressed all the technical concerns raised by the reviewers and have made appropriate changes to the figures, results and discussions. While we agree that there is more to learn about the mechanisms of FHL2 mediated F-actin recruitment, our present study is focused on this novel role of FHL2 and, as the editor suggested, we will leave additional mechanistic studies (especially those including protein purification) for the future.

Reviewer #1 (Comments to the Authors (Required)):

Mitochondrial dynamics including division, fusion and motility are important for proper mitochondrial function, and appropriate transport of mitochondria in neuronal axons is important for normal maintainence of neuronal physiology. Previous reports from this lab established that a glucose spike decreases mitochondrial motility in axons though O-GlcNAcylation of the protein Milton. The present study by Basu et al. extends this previous study and demonstrates that actin filament binding by FHL2 is important in this process by binding preferentially to O-GlcNAcylated Milton. They also show data that suggest that FHL2 binding to TRAK causes F-actin accumulation around mitochondria. The combined data suggest that TRAK-bound FHL2 causes mitochondria to stop their microtubule-based movement due to FHL2-mediated actin accumulation and actin binding.

Though the present study is interesting, it is somewhat superficial and lacks key mechanistic details on how actin filaments are formed, and mediate glucose induced arrest of mitochondrial motility. In addition, there are a number of significant technical concerns.

The central result in the paper is that GlcNAcylation of TRAK is important for FHL2 binding. Evidence for this is provided in a series of westerns comparing OGA over-expression (which should reduce GlcNAcylation) with OGT expression (which should increase GlcNAcylation). However, the actual western results are not very impressive. In Figure 4B, 4C, and 4E, there is significant pull-down of one protein with the other even in the OGA sample.

Response: The reviewer correctly notes that OGA does not completely prevent the binding of FHL2 to TRAK1 in these co-IP experiments. This, however, is consistent with the fact that OGA expression does not abolish all GlcNAcylation of TRAK (see for example bottom panels in 4D). This may not be the only reason for FHL2 association in the presence of OGA: although significant FHL2 recruitment in response to glucose is mediated by O-GlcNAcylation, FHL2 may also be recruited to the complex by other GlcNAc-independent mechanisms that also anchor mitochondria and indeed we suspect this is true. We have now included a discussion about this possibility in the revision.

Figure 4B is not quantified, and the crucial western (of FHL2 in the IP) is not of high quality. Also, there is not a control with neither OGA nor OGT. The ensemble of the data suggest that there is a mild increase in FHL2/TRAK interaction upon TRAK GlcNAcylation, even when the GlcNAcylation is forced.

Response: We have now added the quantification of these blots in the revision, along with the condition lacking both OGA and OGT (Figure 4B-C). We include a blot with higher quality. Note, however, that Figure 4A-C represents immunoprecipitations of *endogenous* TRAK1 and FHL2. The signal to noise ratio in the western is reflective of the low levels of FHL2 in HEK293T cells. Both the mass-spec and the western show about a 60% increase in FHL2 binding to TRAK1 in response to OGT. Figures 4D to 4G, on the other hand, represent westerns from HEK293T cells expressing MYC tagged TRAK1, and FLAG tagged FHL2 and, not surprisingly have cleaner signals and as quantified in 4D, show a 5-fold change in FHL2 association. Thus, the data strongly support the ability of TRAK O-GlcNAcylation to induce association with FHL2 while not excluding other mechanisms as additional paths to the association.

2. Following on the first point, Figure 4F-G uses the TRAK mutant that cannot be GlcNAcylated to show that it binds less to FHL2 than WT TRAK. Why is there any binding to this mutant, if GlcNAcylation is essential?

Response: This is indeed an important point. As mentioned above, we do not argue that O-GlcNAcylation is the only driver for the association. We show here that O-GlcNAcylation is the means by which the shift in glucose triggers the change, which results in more FHL2 binding to TRAK. The Discussion should now make this clear.

3. The evidence that actin accumulates around mitochondria in non-neuronal cells is not impressive (Figure 2C and Figure 7H). In particular, the image in 7H does not really seem to be specific enrichment on mitochondria.

Response: We have improved the images to make the actin-association clearer. In particular, we include a close-up of the region around mitochondria so that the signal is not dominated by cortical F-actin and stress fibers. These structures contain significant amounts of F-actin, and naturally we would not anticipate that ALL the actin in the cell would be recruited to mitochondria; abundant cortical F-actin would persist. The new images with higher resolution are especially helpful for the former Figure 7 (now Figure 8); they make the recruitment more obvious.

Most importantly, we have not depended on a subjective evaluation of this association. We have quantified the amount of F-actin on and around the mitochondria and expressed it as a density. Similarly, we have quantified the density of cytosolic F-actin (excluding the mitochondria) and the total density of F-actin in the cell. This method of F-actin quantification on mitochondria normalizes for cell shape, mitochondrial volume and cytosolic actin redistribution. We have expanded the discussion of this method in the paper.

Specifically:

$$\boldsymbol{Actin\ enrichment} = \frac{\textbf{Actin density on mitochondria} - \textbf{ actin density in the cytosol (excluding mitochondria)}}{\textbf{total actin density}}$$

This quantification clearly shows that in both cases (i.e., in Figure 2C and 8H), there is a significant enrichment of F-actin specifically on and around the mitochondria when FHL2 is there.

It is odd that this is not also shown for neuronal cells. Do they see an increase upon the glucose shift or OGT expression?

Response: We of course have also asked if actin in neurons would be detectably increased around mitochondria. Axonal F-actin however is always densely packed and quite stable (D'Este et al., 2015; Han et al., 2017; Xu et al., 2013). The resulting high intensity of F-actin staining in axons makes it difficult to determine if any further accumulation happens. F-actin accumulation is also unnecessary to invoke anchoring (especially in areas with high F-actin density); FHL2 may simply anchor mitochondria to the preexisting filaments that are always close-by in an axon. The rearrangement of the actin cytoskeleton in neurons therefore may not occur to the same degree as in non-neuronal cells. Thus, although the mitochondrial anchoring in neurons clearly depends on F-actin (as it is rescued by latrunculin A treatment) it does not involve a *clearly detectable* increase of F-actin around mitochondria in response to OGT. We have now made these points explicitly in the revision and have included a new supplementary figure S8. There is a trend towards a slight increase in actin around mitochondria when OGT is expressed (p=0.15). Regardless, the mechanism we propose does not require additional actin to form around the anchored mitochondria.

4. Related to the last point, do the authors have any idea as to the mechanism that forms the actin structures associated with mitochondria downstream of OGT expression? This is important as this would establish a mechanistic relationship between OGT expression and actin filaments mediated mitochondrial motility arrest. Past studies of mitochondria and actin have implicated several factors as being of possible importance, including Arp2/3 and Spire1C. Are these proteins involved? From the cited work, FHL2 might bind F-actin but is not thought to mediate its polymerization. Even the claim that it binds actin appears to be based on one publication (Coghill 2003) that only seems to use somewhat superficial assays. How confident are the authors on a direct interaction?

Response: This is an important point and an important next step for our lab; examining Arp2/3 and Spire1C are very much in our plans, so, we thank the reviewer for this comment. Because we have shown that FHL2 expression is necessary and sufficient to initiate the actin-dependent arrest, that is the focus of the present manuscript and we are deferring the downstream mechanisms of F-actin re-arrangement for future study.

5. An important control is to test whether LatA treatment alters TRAK GlcNAcylation itself?

Response: We thank the reviewer for this suggestion. The requested data appear as a new panel (Figure S4C). This data, along with the data shown in Figure 4F, makes it clear that LatA treatment affects neither TRAK O-GlcNAcylation nor FHL2 association, thus indicating that the F-actin anchoring must instead occur downstream.

6. For the quantification of mitochondrial motility in Figure 2, there are two technical issues:

a. it is not clear that this method is examining directional motility, or whether it reflects any change of position (directional or not). If this quantification does not measure directional motility, how are we to know that the mitochondria are not simply just 'wiggling' more or less in the different treatments?

Response: We thank the reviewer for this comment because it was an important consideration in the design of our algorithm. We have increased our discussion about this method and have published a detailed protocols paper on QuoVadoPro (Basu and Schwarz, 2020). Briefly, our quantifications are heavily weighted towards processive movements. We determine the variance in pixel occupancy of each pixel and divide the variance by the summed occupancy of that pixel. In the case of "wiggling" mitochondria, although the variance in pixel occupancy containing the mitochondria will be high over time (especially at the edges), the total occupancy of the pixels will also be high (due to the same pixels being occupied by the mitochondria repeatedly). Conversely, when the mitochondria show processive motion (i.e., move from occupying one set of pixels to a different set of pixels), the total occupancy of each subset of pixels is low while the variances for each pixel subset are high. Our quantification normalizes the variance to the sum, and thus weighs the processive motion of the mitochondria more heavily.

Nevertheless, as we have stated in the paper (and have now made it more explicit), this is not an absolute measure of processive movement but a proxy for quantifying mitochondrial movement in non-neuronal cells. We present these data to demonstrate that the mechanism of OGT induced mitochondrial movement arrest is comparable across the cell types used in our study. All our conclusions about processive mitochondrial movement are drawn from the experiments in neuronal axons using conventional kymography.

it would be useful to have more specific details in the methods, rather than stating that the imaging was conducted the same as for neurons. For example, were these images really taken at 0.5 sec intervals for 90 min (which is what one deduces from piecing together from the figure legend and methods)?

Response: We have increased the details of this section in the methods. All time-lapse imaging experiments (for neurons and non-neuronal cells) of mitochondria or other organelles were imaged at a frame rate of 2Hz for 3-5 mins.

7. For the endosomal motility in Figure S2D-E, there should be a control with no treatment (neither OGT nor OGA transfected).

Response: We have incorporated a GFP-expressing condition and re-done all the related quantifications (Figure S2).

8. For the PEX-miro experiments in Figure 3:

It would be useful to know if the OGT effect on PEX motility is inhibited by LatA.

Response: This was a particularly nice suggestion. Our new Figure 3 shows that LatA indeed can reverse the motility arrest of Pex-miro brought about by OGT.

It would be useful to know whether these PEX build up more actin around them than control PEX, in the presence or absence of OGT.

Response: Though peroxisomes are a minor aspect of the study, we share the reviewer's interest in this question. We tried this experiment a number of times. The small size of peroxisomes, however, has made it challenging to reliably quantify changes in the actin intensity around peroxisomes. Therefore, we have relied on the effects of latrunculin A treatment to show that the arrest of Pex-miro positive peroxisomes is indeed F-actin dependent.

A recent study has shown that endogenous Miro1 is present on peroxisomes and it modulates the motility of the same (Castro et al., Traffic, 2018). In the light of this previous study, it is interesting to note that in Fig 3E-3F, OGT over-expression did not arrest peroxisomal motility in control cells. Why would this not occur?

Response: This is a valid point and one that had also given us much thought. The literature is complicated on this point. In a recent paper from the Kittler lab, (Covill-Cooke et al., 2020) for example, they see Miro on peroxisomes (but only with massive over-expression) and say that it does not mediate peroxisomal motility, but instead regulates peroxisomal fission. Moreover, when Miro was knocked out, they did not see a resulting change in peroxisomal motility implying that it was not mediated by a Miro-based motor complex. A paper that just appeared from the DeCamilli lab (Guillén-Samander et al., 2021) also over-expresses the mitochondrial isoform of Miro and finds it alters peroxisome contacts with ER. They do not look at motility, but their data imply that Miro is not normally on those peroxisomes enough to maximize ER contacts. For our purposes, it was similarly sufficient to find that, in our hands (Fig. 3 E-F) the COS-7 cell peroxisomes are normally not very motile at all until we place additional Miro on them with the Pex-Miro constructs. Thus, if the endogenous Miro is present on the organelle, it is not sufficient to mediate much movement and the movement we study is overwhelmingly attributable to the Pex-Miro we have directed to the peroxisomes. Because the peroxisomal motility is low without Pex-Miro, we can't say whether or not normal motility is inhibited by OGT (p=0.33). But the inhibition of movement by OGT is very evident when Pex-Miro has transferred the motor-adaptor complex onto the peroxisomes and that is, for us, the key point. Because we expect other readers to also wonder about this, we have added a discussion of this issue in the revised manuscript, including references to the papers mentioned above.

9. Since the authors demonstrate a crucial role of FHL2 in actin mediated arrest of mitochondrial motility during glucose stimulation, it is important to have a little more information as to the localization of FHL2. Where does endogenous FHL2 localize in both unstimulated and glucose-stimulated cells? If they cannot examine endogenous FHL2, where does epitope tagged FHL2 localize?

Response: We have now validated an antibody for staining endogenous FHL2 (Figure S5) and looked at the localization of endogenous FHL2 in response to OGT vs CNTRL (Figure 5). As reported by others, FHL2 is often concentrated at focal adhesion sites and we see patches at the cell periphery that are likely to be those sites. When OGT is expressed, in good agreement with our immunoprecipitation and Mass-spec studies, the intensity of endogenous FHL2 increases on mitochondria in presence of OGT as compared to control, but this does not require the removal of FHL2 from the cell periphery. More likely, it is recruited from a cytosolic pool.

10. Following on the last point, it would be very good to know whether the FHL2/TRAK interaction is direct, especially since (at the bottom of page 8) the authors state that FHL2 can bind to the Miro/TRAK complex.

Response: The reviewer is correct to point out that we were careless on p. 8 in our use of the word "bind". We do not know whether FHL2 binds TRAK directly. Our only evidence of interaction is by immunoprecipitations from cell lysates and the interaction may indeed be mediated by intermediate factors. We tried to say "interact" or "associate" throughout so as not to imply direct binding and hope there aren't any slip ups that we missed. Showing a direct interaction of FHL2 and TRAK will need purified proteins, and though we have been able to express some segments of TRAK, we cannot obtain soluble full-length TRAK and feel strongly that the protein chemistry is beyond the scope of this paper.

11. The FHL2 knock-down experiments performed in neurons (Figure 5) would be useful to perform in culture cells (U2OS) to test two things:

a. Is FHL2 necessary for OGT-mediated mitochondrial slowing?

b. Is FHL2 necessary for OGT-mediate actin accumulation around mitochondria?

Response: We have done these suggested experiments in COS-7 cells (since we mostly show COS-7 data in the other Figures) and added multiple relevant figure panels and related discussion (Figures 5 and 6).

a. **We used a validated shRNA sequence to knock down FHL2 in COS-7 cells. As in neurons, knockdown of FHL2 rescues the OGT mediated mitochondrial arrest (Figure 6).**

b. **FHL2 was also necessary for the OGT-mediated F-actin re-distribution around mitochondria, we monitored F-actin morphology in COS-7 cells expressing the shRNA against FHL2, with and without OGT (Figure 5).**

These results are nice support for our model and we thank the reviewer for suggesting these experiments.

12. Miro-Milton mitochondrial motility involves microtubules and the authors demonstrate that FHL2-mediated actin meshwork restricts this movement. It would be good to elaborate on this mechanism a little further. Do they think that the FHL2/actin interaction is sufficient to resist the force generated by microtubule motors?

Response: We have discussed this in more detail in the manuscript. It is clear that the motors have not somehow been switched into an off state by the presence of FHL2 in the complex, because latrunculin restores their activity without dissociating FHL2 from the complex (figure 1 and 8). Also, Kif5 remains in the complex in the mass spec data (figure 4). It is most likely, therefore, that the anchoring does indeed resist the force of the motors. An exception may be in those cases where actin is massively surrounding a mitochondrion – if a large "cocoon" of actin has formed, it may prevent the mito-associated motors from contacting the microtubules. In neurons, however, we do not see such an extreme cocoon and so a simple force-resisting anchor model is most likely.

This is also consistent with our previously published work in which we forced a kinesin motor onto mitochondria (Gutnick et al., 2019). We found that some mitochondria were mobilized by acutely recruiting the kinesin, but some were immoveable (i.e. anchored in place despite having kinesin). More mitochondria were mobilized when latrunculin disrupted actin. Thus, some mitochondria can be anchored by actin and indeed cannot be moved by microtubule-based motors.

13. For the FHL2 localization studies in Figure 7, it is important to show the effect of FHL2 expression without the Omp25 mitochondrial tag. Do mitochondria slow down as well? Does HA-FHL2 localize to mitochondria upon the appropriate stimuli?

Response: We have now included this data (Figure S7) showing that cytosolic FHL2 (expressed without the OMP25 tag) does not slow down the mitochondria. We have also probed the localization of endogenous (and untagged) FHL2 in COS-7 cells with and without OGT. Untagged FHL2, as reported before, is concentrated at the focal adhesions and the cell periphery. Upon OGT expression, FHL2 becomes distinctly concentrated on mitochondria (Figure 5). Thus, both predictions of our model are borne out.

Minor points:

1. In the results section for Figure 2, it is mentioned that the motility analysis is conducted in both Cos7 and U2OS cells. However, only Cos7 cells appear to be shown.

Response: We have added a figure to show the effects of OGT on mitochondrial motility and endosomal motility (control) in U2OS cells. U2OS cells respond to OGT in a similar manner as COS-7 cells (Figure S2F-I).

2. It would be good to have marks of MW for western blot images.

Response: They have now been added throughout.

3. Figure 1A legend doesn't match the description in results. Why 6 mM glucose for first 55 hrs?

Response: Sorry, this was indeed confusing - we have now clarified this point in the legend and methods. The neurons were dissected in medium containing the typical 25 mM glucose, washed once and plated with 5mM glucose medium. We maintained the neuronal cultures by feeding them with 5 mM glucose till day in vitro (DIV) 6. The measured value of 6mM at the first time point reflects the incomplete exchange of the 25mM glucose dissection medium because one can never draw off all the medium. Consequently, the initial measured concentration is actually 6mM, until glucose consumption and subsequent feedings bring it down to 5mM. More importantly, at DIV6 we reduce the levels of glucose to 1mM. We then increase it to 5mM on DIV9. This shift from 1mM to 5mM glucose is our experimental trigger, something that we have tried to control tightly.

4. For Fig 1C,1E, 2B,2D etc., along with the p-values, it would be good to indicate which ones are significant.

Response: We agree that indicating significance is often helpful to the reader. However, we are trying to follow the NIH guidelines and what many journals now consider to be best practice, which is to display the p value. We have mentioned all the results which we have considered to be significant in the results section.

5. In Figure 6C, there appears to be an overall decrease in mitochondrial motility in FHL2 shRNA cells (in low glucose). This finding might be pointed out.

Response: We hesitated to say much about this trend as the p value was 0.14. However, we have now pointed it out in the results. If this decrease is a bona fide effect of FHL2 knockdown, for the purpose of our experiment, it is all the more impressive that the glucose shift does not decrease motility in presence of the FHL2 shRNA – confirming that FHL2 is needed for the glucose-driven arrest.

6. In the first paragraph of the Introduction, is the "2 billion years" of mitochondrial evolution correct?

Response: We would probably be on safer ground if we said "a billion or more years" – that seems to be the ballpark of most estimates. Just to be safe, we have now removed this sentence altogether.

Reviewer #2 (Comments to the Authors (Required)):

Previous work from this lab provided extensive evidence that the OGT-dependent modification of the OMM microtubule motor protein adaptor Milton with GlcNAc, which is stimulated by elevated glucose, inhibits the translocation of mitochondria along microtubules in neuronal axons. Somewhat surprising, however, was the finding that motility arrest does not involve a disconnect between Milton and kinesin-the motor remains associated with GlcNAc-modified Milton. As a result, the underlying cause of motility arrest was unclear. This new study presents evidence that the motility arrest is caused by the interaction of GlcNAc-modified Milton with an actin binding protein (FHL2). While this study is very interesting and has some strengths, I have a number of significant concerns which are detailed below.

Perhaps my single biggest concern revolves around whether FHL2 really binds F-actin. The authors cite seven papers when they state without providing any evidence of their own that FHL2 binds to F-actin. I looked up these seven papers. In Boateng et al. (2016) FHL2 is shown to bind to the actin binding protein mAbp1, not to F-actin. Johannessen et al (2006) is a review on FHL2. Some of the key take a ways are it is localizes diffusely in the cytoplasm and in the nucleus (no mention of co-localization with F-actin), and that by 2006 it had already been reported to bind 50 different proteins, including "receptors, structural proteins, signal transducers, transcription factors and cofactors, splicing factors, DNA replication and repair enzymes, and metabolic enzymes" (sounds sticky!), but not actin! It goes on to talk about FHL2 function as regards cardiac physiology, bone formation, muscle function, placental development etc, but not a word about actin. Indeed a large fraction of the extensive literature on FHL2 is on its role as a transcriptional co-activator whose dysregulation is implicated in many types of cancers. Li et al (2001) focused on the localization of FHL2 at focal adhesions and provided no substantive data that it binds to F-

actin. Tran et al (2016) is another review that focused on the role of FHL2 in apoptosis, cell migration, cardiovascular function, vascular patterning, and regulation of nuclear receptor function. In all of these sections I did not find any mention of F-actin, and actin is not present in the huge table they present of FHL2 interacting proteins. Olson and Nordhein is yet another review. They mention that FHL2 shuttles between focal adhesions and the nucleus, but say nothing about its interaction with F-actin. The paper by Ng et al only identified FHL2-regulated genes in liver by microarray and bioinformatics analysis. Finally, the one paper that seems to support the idea that FHL2 binds F-actin is the Coghill paper on FHL3. But their "evidence" that FHL3 binds to F-actin in Y2H (not sure what that tells us) and IP (find me an IP that does not have actin in it!!). What is required here is the decades-old norm in the actin field for proving interaction with F-actin: a binding isotherm performed by sedimentation assay using purified protein and F-actin at increasing concentrations. So all together, the data in the literature they cite supporting the idea that FHL2 binds to F-actin is in my opinion essentially zero. Of note, it is also not encouraging that expression data bases show FHL2 expression in the brain is very low, especially as compared to heart muscle. Finally is the issue of whether any LIM domains have been shown biochemically to bind to F-actin. While LIM domain-containing proteins have in some cases been linked to the regulation of the actin cytoskeleton, (e.g. LIM-Kinase, which regulates the F-actin depolymerizing protein cofilin), to my knowledge no LIM domain has been shown to bind to F-actin in a standard F-actin sedimentation assay using purified proteins. One well-characterized example is the LIM domain-containing protein zyxin, which has been studied extensively by Mary Beckerle. While she has shown that GFP-zyxin localizes to strained stress fibers transiently and only at sites where the stress fiber is about to break (Dev Cell 2010), she has never been able to show that zyxin binds F-actin in a standard F-actin pelleting assay (published data). Moreover, the mechanism that restricts zyxin recruitment to the immediate vicinity of stress fiber rupture sites is unknown. Consistent with all this, I could not find a paper in the literature showing that any LIM domain binds to F-actin in standard biochemical assays for this interaction. I assume the authors had the same trouble given the papers they cite supporting the interaction between FHL2 and F-actin. Given all this, and the complete absence of any biochemical data in this paper that FHL2 binds to F-actin, I find it a stretch to conclude that FHL2 links mitochondria to F-actin.

Response: We appreciate the reviewer's detailed discussion about FHL2 and F-actin binding. It is indeed true that evidence for the direct binding of FHL2 to F-actin is scarce. It was not our intention to suggest such a direct interaction and we have carefully gone through the manuscript to be sure we always say "actin associated" or "actin-interacting" and to make explicit that we do not know whether or not it binds directly to actin. As the reviewer points out, FHL2 does bind to several F-actin binding proteins as we have referenced (for example FHL3 and mAbp1). Thus, the actin-dependent mitochondrial anchoring downstream of FHL2 recruitment may occur as a result of FHL2 directly binding to F-actin or by recruiting other actin-binding proteins. The details of how that interaction occurs are for future study. We will embark on that without prejudice as to whether it will prove to be direct, as suggested by the cited yeast two-hybrid interaction, or indirect.

For the purpose of this manuscript our goal was to determine whether or not FHL2 was a critical part of the pathway that linked TRAK O-GlcNAcylation to actin-dependent arrest. To that end we show that that FHL2 localizes to mitochondria and is both necessary (Figure 6) and sufficient (Figure 8) to mediate the F-actin dependent arrest.

With regard to the relative levels in brain vs heart, we offer no opinion as to why it is very abundant in heart, but that is not relevant here. We show FHL2 is present in neurons and its knockdown prevents

the arrest; thus, it is clearly present in sufficient quantity to function in our pathway. In addition, all the published databases we examined find FHL2 expressed in neurons (Zeisel et al., 2018; Zhang et al., 2014; Zhang et al., 2016).

The authors earlier Cell paper describing the role of GlcNAc modification of Milton in regulating mitochondrial transport focused on the microtubule-dependent movement of this organelle in the axons of Hippocampal neurons. This context is great because the fast anterograde and retrograde transport of mitochondria along microtubules in these "2D" structures is so clear and so readily scorable as regards movement versus cessation of movement. What is a lot less satisfying in the considerable amount of data in this current paper where mitochondrial motility is scored in tissue culture cells (Cos, U2OS). First, this scoring is not restricted to the subset of mitochondrial movements in these cells that are likely to be microtubule based (fast, persistent, linear trajectories). This seems like a disconnect with the Cell paper. Instead, the motility that is measured is drastically weighted in the direction of the pervasive "jiggling" that mitochondria exhibit in such cells. The key data regarding this jiggling is that actin disassembly with Latruculin does not alter the extent of jiggling when glucose is low or OGT is not over-expressed, but it does result in increased jiggling (basically to the same values as in the control) when glucose is high or OGT is over-expressed. In other words, Latrunculin reverses the very low jiggling value seen in high glucose or OGT over-expression. Their interpretation of this result (that OGT over expression or glucose elevation stops jiggling because mitochondria get connected to cytoplasmic F-actin via FHL2 recruitment) may be correct. But I wonder if Latrunculin might alter jiggling indirectly based on work from the Weitz lab. In the 2014 Cell paper from this lab (158, p822), the authors showed that actomyosin contractions in the cytoplasm are transmitted across the cytoplasm because it behaves like a viscoelastic gel. One readout of this was fluctuations in mitochondrial shape. While their main tool to show this was myosin inhibition, my guess is they would have blocked mitochondrial shape fluctuations by disassembling actin as well. I get it that the controls in this current paper show the Latrunculin effect is specific to OGT over-expressing/high glucose conditions, but I remain somewhat skeptical that jiggling stops because the mitochondria become bound to F-actin. Is there some other way this later point could be shown (see also my next comment). An acid test of their model would be a KD/replacement using a version of FHL2 that can bind to Glc-NAc modified Milton but not F-actin. Obviously such a mutant cannot be searched for if they cannot first obtain biochemical evidence that FHL2 binds to F-actin.

Response: We appreciate the reviewer's concern. We love studying motility in neurons and turned to the cell lines for experiments (such as the mass spec) where the cell lines had clear advantages. In whatever ways possible we aimed to show that the phenomenon in the cell line was the same as that in neurons. As the reviewer notes, we are looking at an arrest in COS-7 cells that is dependent on O-GlcNAcylation and it is also, as in the neurons, dependent on FHL2 and actin. We hope the following three points will address the technical concerns:

1. The method of quantification that we have used was designed to weigh processive motion of mitochondria more heavily than jiggling movement (as pointed out in the response to reviewer 1, item #6). We took this approach since we wanted to look at the motility most likely to be due to microtubule-based motors. The algorithm we used does so because, the pixels containing the "jiggling" mitochondria exhibit a high variance over time and also a HIGH sum of intensity over time. By contrast, processive movement allows the mitochondria to occupy different sets of pixels, each of which exhibit a high variance of intensity over time but LOW sum. As our quantification normalizes the variance to the sum, it weighs the processive motion of the mitochondria more heavily. Thus, it is the processive movement

of mitochondria rather than "jiggling" that is primarily susceptible to OGT dependent arrest. Further details and clarifications can be found in our published protocols paper (Basu and Schwarz, 2020).

2. In each experiment concerning non-neuronal cells and F-actin disruption, we have tried to include the best possible controls, to show that our observations are indeed due to arrest of processive mitochondrial movement (Figure 2), and not due to any visco-elastic changes in the cytosol due to the disruption of F-actin. Indeed, we have titrated the amount of LatA that we use to treat our COS-7 cells (0.05µM) to a dose, that does not grossly affect the F-actin morphology (which will also cause the cytosol to collapse), but still is sufficient to mobilize the arrested mitochondria. The Weitz lab work is indeed interesting but the lab uses LatA at a concentration of 1uM (20x ours) which depletes the F-actin to a far greater extent. We are looking at a distinct phenomenon.

3. We are careful not to presume everything we see in COS-7 cells as processive movement; neurons are the proper system for that. As we had stated in the manuscript (and have made more evident now) we use the variance as a proxy for mitochondrial movement – it is not a direct measure – and it allows us to demonstrate that the mechanism of OGT induced mitochondrial movement arrest is comparable across the cell types used in our study.

4. Finding a mutant version of FHL2 that cannot trigger the F-actin mediated mitochondrial arrest would be great, of course. We plan to undertake more structure/function studies on FHL2 but this is beyond the scope of this paper (as discussed above).

I found the evidence in Figure 2C that O-GlcNAcylation-mediated motility arrest in tissue culture cells is associated with changes in the association of mitochondria with F-actin to be pretty unconvincing. The "contacts" between mitochondria and actin in these diffraction-limited images are present in both control and OGT-expressing cells.

Response: We have improved the images. As mentioned in the response #3 to Reviewer 1 we include now better resolved images of the region of the cell where the mitochondria are so that the increase in associated actin is quite obvious. There are a lot of F-actin structures in cells, and it is not surprising that some mitochondria are adjacent to F-actin even in the control conditions in COS-7 cells. That is why we have carefully quantified the extent of the association so as not to judge subjectively from the images. We have now extended our methods detailing our approach to quantifying F-actin enrichment around mitochondria. Whether by eye or by quantification, we do not see in the control cells the encirclement of the mitochondria by actin rings that is very apparent after OGT expression.

More generally, I was not sure that they are saying OGT expression drives the attachment of mitochondria to actin structures (the way I assume an actin-based anchor would work) or the assembly of F-actin on the OMM (as in the JCB paper from the Karbowski lab following CCCP treatment, although lots of labs have also been weighing in on actin assembly on the OMM recently- Lippincott-Schwartz, Holzbaur etc.)

Response: This is correct. We think both (attachment to F-actin and accumulation of F-actin) are possible and even likely and have made that explicit in the discussion. In neurons, where we do not detect a massive rearrangement of the F-actin (Figure S8), it may be a case of anchoring to pre-existing structures. In COS-7 cells, where there is a large accumulation of F-actin surrounding the mitochondrion, the assembly of large actin structures on the OMM seems more likely and may (see response to reviewer 1 above) involve Arp2/3 or Spire1C.

Of note, my confusion regarding their conclusions was compounded by the images in Figure 7, Panel H, which the authors say show actin surrounding mitochondria with FHL2 constitutively bound to their surface (like in CCCP treated cells). To me, the image in Panel H shows dots of actin in the immediate vicinity of these mitochondria, not actin surrounding mitochondria as in the Karbowski paper (moreover, most of these central actin dots are not actually near mitochondria). These are an important issues to clarify, as excess actin assembly on the OMM (as in the over-expression of dominant actin Spire 1C; Manor et al, 2016) looks like it stops mitochondrial jiggling. In terms of how FHL2-dependent anchoring of mitochondria to cytoplasmic actin structures works, quantitative, dynamic imaging of moving mitochondria encountering such structures plus or minus OGT expression would really help.

Response: We had done a disservice to the data in Fig 7H by showing the entire cell (because the entire cell was imaged in order to do the quantification). We have now updated Figure 7 (Figure 8 in the revised manuscript) by adding a high-resolution image of the mitochondria, which will make the association with actin clear. The dots of actin that are prominent in both the control and OGT cells are possibly an artifact of fixation, but in any case, quite distinct from the actin filaments that align with or encircle mitochondria.

In a related issue, no localization data for endogenous FHL2 is presented. Is it present on cortical actin? That would be expected given that it is supposed to be an actin binding protein. Can they see it go on to mitochondria upon shift to high glucose (either dynamically as a FP-tagged protein or by IF)? No such pertinent localization data is presented.

Response: This is a great suggestion that we got from multiple reviewers. To address this suggestion, we validated an antibody that could be used to stain endogenous FHL2 (Figure S5). Using that anti-FHL2 antibody, we have now added the requested localization data for FHL2 in control and OGT expressing cells (Figure 5) and included appropriate discussions. As predicted by the reviewer, FHL2 indeed enriches on the mitochondria upon OGT expression. In all cells, there are bright patches of FHL2 toward the periphery that likely correspond to focal adhesions (a few can be seen in the whole cell image of Figure 5), but we have not tried to characterize them. These bright patches do not seem to be affected with OGT expression. It is likely that the mitochondrial enrichment occurs from the cytosolic pool of FHL2.

With regard to the experiments on peroxisomes, previous studies of their motility focused primarily on the subset of peroxisomes that undergo fast, directionally-persistent, microtubule-dependent movements. Like my comments above regarding mitochondrial jiggling, I think the authors should show that OGT over expression stops these fast movement by connecting the organelles to F-actin (rather than, or in addition to, the static measurements of peroxisome distribution in the cell that they present). With regard to their distribution data, I am also perplexed by the observation that OGT over expression causes the peroxisomes with modified Milton on their surface to become perinuclear. This observation makes sense in the context of their earlier Cell paper, where OGT over expression was shown to arrest microtubule plus end-directed organelle transport. But it is not consistent with the underlying mechanism of arrest being the attachment of the organelle to F-actin (this paper), given that in most tissue culture cells the bulk of F-actin is in the cortex at the cell periphery. In other words, I would have expected the modified peroxisomes to accumulate in the periphery over time in high glucose/OGT over expression, not

in perinuclear regions where there is very little F-actin. To me, this is a significant disconnect as regards their model.

Response: We appreciate this comment.

1. **Our reply above about jiggling vs processivity in mitochondrial movement pertains to peroxisomes as well: the variance analysis emphasizes processivity.**
2. **We did not intend this to be a study of peroxisomal motility – we are using a completely artificial system of forced direction of Miro onto the peroxisomes to make the point that Miro and its associated proteins are sufficient to convey the OGT response.**
3. **To show that the phenomenon with peroxisomes really is the same as that that occurs on mitochondria, i.e. that OGT indeed causes the peroxisomes with pex-miro1 to be stopped by the F-actin network, we have extended the peroxisome experiments (Figure 3). In addition to being stopped by OGT, we now show that the movement of Miro-bearing peroxisomes can be rescued by disrupting the F-actin network (using LatA). We analyze both movement and distribution as we had done for mitochondria.**
4. **The reviewer notices that the Miro-bearing peroxisomes are more perinuclear when arrested by OGT than when free to move. In fact, they are distributed very much the way they are in control cells that don't express pex-Miro1. We suspect that this is the case, since the pex-Miro and OGT are co-expressed, and under this condition, the pex-miro never induces peroxisomal motility (to disperse them) to begin with. As soon as miro goes onto the peroxisomes, O-GlcNAcylated TRAK goes along with it, causing the peroxisomes to be anchored in place. Following the arrest, as with mitochondria, they don't move to preexisting concentrations of cortical actin.**

If I did not miss something, the evidence presented here that FHL2 interacts with GlcNAc-modified Milton is based entirely on IPs. IPs cannot prove direct interaction. For example, given that Milton is in a stable complex with OGT, what is the evidence that Milton's "interaction" with FHL2 is not through OGT bound to Milton in the immunoprecipitate? This possibility (and other possible interactions) cannot be excluded without performing direct binding experiments using purified Milton and FHL2 to show that they interact directly (and the interaction is attenuated when Milton has Glc-NAc on it). This is a significant issue that requires clarification before publication.

Response: We appreciate this comment by the reviewer:

1. It was not our intention to suggest that FHL2 and TRAK1 interact directly. Our IP data show that TRAK1 can "recruit" FHL2 upon being O-GlcNAcylated and we trust there are no places now where we got careless and said "bind" instead of "associates with" or immuno-precipitates with". As the reviewer mentioned, it is a natural follow up for our lab to purify TRAK1, FHL2 and potentially undiscovered intermediates to show direct and indirect binding affinities. However, as Dr. Youle has pointed out, this is beyond the scope of this manuscript and does not affect the integrity of our model.

2. As an aside, the particular intermediate the reviewer mentions as an example, binding of FHL2 to OGT instead, is probably excluded by our experiment with TRAK1^{QMut} (Figure 4). TRAK1^{QMut} is fully able to bind OGT but immuno-precipitates poorly with FHL2. Thus, the TRAK1-FHL2 association is influenced to a greater degree by O-GlcNAcylations than by OGT binding. Since OGT was just one of any number of possible intermediates, we don't single it out in the text for exclusion.

Reviewer #3 (Comments to the Authors (Required)):

Control of mitochondrial motility is critical for cell function, especially in highly polarized cells, such as neurons. Thus, understanding of the mechanisms controlling mitochondria motility is important.

In this manuscript, by Basu et al., the authors provide some evidence linking the actin-binding protein FHL2 with mitochondrial adaptation to glucose supply. They developed a protocol in which hippocampal neurons adapted to grow on 1mM glucose are shifted to 5mM glucose followed by observation of mitochondrial motility. These cell culture conditions are thought to approximate the glucose fluctuations in the brain (although the 1mM glucose is far from physiologically relevant!). The data clearly show that a switch in glucose concentration reduces mitochondrial motility. This process was suppressed by treatment with actin-depolymerizing Latrunculin, and overexpression of OGT, indicating roles for the actin cytoskeleton and O-GlcNAcylation.

The authors also propose that FHL2 can regulate mitochondrial motility by anchoring them to the actin cytoskeleton. The immunoprecipitation experiments revealed that FHL2 is enriched in Milton (TRAK) immunoprecipitate from cells overexpressing OGT. Thus, in response to varying glucose concentrations, FHL2 could be recruited to the mitochondria by O-GlcNAcylated Milton protein. Importantly, the role of FHL2 in control of mitochondrial motility was also confirmed with shRNA experiments.

Overall this is a potentially interesting work that provides new insights on the regulation of mitochondrial motility in neurons, and non-neuronal cells. Most of the conclusions are supported by strong evidence. However, the direct role of FHL2-mediated recruitment of the actin cytoskeleton to the mitochondria is not supported to the same degree as other parts of this manuscript (see below). This important issue should be addressed, before the manuscript can be recommended for publication in the high caliber journal, such as The Journal of Cell Biology.

Specific comments:

1. The immunoprecipitation studies (Figure 4) indicate that in OGT transfected cells FHL2 interaction with the mitochondria (through binding to Milton) is increased, as compared to OGA-expressing cells. However, knowing the subcellular localization of FHL2 in control, OGT-transfected and preferably under different glucose concentrations could strengthen a direct mitochondrial role of this protein in the regulation of mitochondrial motility. Indeed, as the authors acknowledge, the modulation of FHL2 expression can affect the overall organization of the actin cytoskeleton. Cell fractionation and imaging experiments would make this point stronger.

Response: This is a very valid point. Once we had found an acceptable antibody that we could validate (Figure S5), we took the imaging approach, and it did indeed validate our conclusions from the immunoprecipitations. Figure 5A-B now shows the localization of endogenous FHL2 and its concentration on mitochondria in response to OGT expression (as also requested by Reviewers 1 (point

9) and 2). Regarding expression levels, we have confirmed that the levels of FHL2 do not change with OGT or OGA expression (Figure S5A-B and Figure 4B). But the reviewer was more likely concerned about what happens to FHL2 distribution with OGT expression which the immunocytochemistry resolved. We had also performed a fractionation which also showed enrichment on mitochondria upon OGT expression (a Western is shown below for the interest of the reviewer) but have not characterized the fractionation so thoroughly as to exclude other contaminating membranes, and so decided to use the immunocytochemistry in the manuscript.

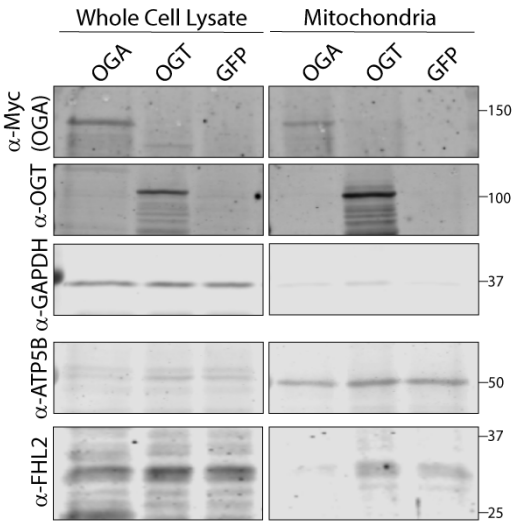

Figure in response to reviewer 3, comment #1. HEK293T cells expressing OGT, OGA or GFP (control) were lyzed and their mitochondria were enriched by centrifugation through a sucrose cushion as described in (Chung et al., 2016). Mitochondrial fractions and whole cell lysates were immunoblotted to probe for OGA, OGT, GAPDH, ATP5A and FHL2. GAPDH, a predominantly cytosolic protein is depleted from the mitochondrial fractions while ATP5A, a mitochondrial membrane protein is enriched, thus showing a successful mitochondrial enrichment. In the enriched mitochondria, the levels of FHL2 on mitochondria from cells expressing OGT are higher than the levels of FHL2 on mitochondria enriched from cells expressing OGA.

2. In line with the above comment, the authors could further verify the role of Milton in the mitochondrial assembly of actin. If their hypothesis is correct then Milton knockout or knockdown would result in the reduction of mitochondria-associated FHL2 and as a result lower levels of mitochondria-associated actin, especially under 5mM glucose or upon OGT-expression. For example, if the proposed mechanism is valid then Milton downregulation could reduce mitochondrial actin levels shown to be higher in OGT-expressing, Cos-7 cells (Figure 2C).

Response: This was indeed a good suggestion, one that greatly strengthens the proposed model. In response to this comment, we knocked down TRAK1 in COS-7 cells with two independent shRNAs. As predicted by the model, TRAK was necessary for the OGT-mediated accumulation of F-actin around mitochondria. This is now shown in Figure 2E and F.

3. The levels of mitochondria-associate actin in COS7 cells (Figure 2C) should be also analyzed using fluorescent phalloidin. Otherwise, it is not clear whether the apparent effects are due to OGT activity or differences in life Act-RFP expression or assembly levels.

Response: We have reproduced the phenotype of F-actin associated with mitochondria in presence of OGT using Phalloidin and F-tractin (another live actin marker). We include this data as a part of the supplementary Figure 2 and Figure 3.

4. Generally, additional imaging and/or biochemical approaches could be used to further strengthen the role of FHL2 in the mitochondria: actin cytoskeleton interactions, including cell fractionation and analyses of actin and FHL2 localization under distinct conditions (e.g. Figures 2C, 4, and 5). These experiments could be performed in non-neuronal cells (e.g. Cos-7) and preferably supplemented with actin imaging under similar conditions. Without such data, the premise that mitochondrial interaction with actin cytoskeleton per se, but not other non-specific changes in the activity of relevant proteins, are important for glucose-dependent changes in mitochondrial motility is not strongly established.

Response: We have added immunolocalization of FHL2 in COS-7 cells, as mentioned in response #1. This experiment employed the same conditions used to image the actin accumulation in Figure 2C. The immunolocalization is also consistent with the fractionation experiment shown above. If the reviewer is concerned that Miro, TRAK, or motors might be lost from the mitochondria upon OGT activation, we know from IP experiments and the mass spec that the complex has not dissociated. Moreover, the actin depolymerization with latrunculin restores motility, which indicates that the motors remain active.

5. Figure 3. The experiments shown in Figure 3 provide some evidence that OGT overexpression can also control organelle motility when anchored to peroxisomes (Pex-Miro expression). However, the role of actin in this system was not investigated.

Response: We have now rescued the peroxisome motility in the presence of OGT with F-actin depolymerization, as requested. This nicely confirms that the arrest mechanism normally operating on mitochondria is transferred to peroxisomes when Miro is targeted there (Figure 3, E-G).

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June 23, 2021

RE: JCB Manuscript #201912077R-A

Dr. Thomas L Schwarz
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Boston, Massachusetts 02115

Dear Tom:

Thank you for submitting your revised manuscript entitled "FHL2 anchors mitochondria to actin and adapts mitochondrial dynamics to glucose supply". Your manuscript has now been seen again by two of the original reviewers (unfortunately, reviewer #3 was not able to re-review your paper so we assessed your responses to reviewer #3's comments editorially). As you will see, the two reviewers now recommend acceptance (and we agree) and, therefore, we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

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Thank you for this interesting contribution, we look forward to publishing your paper in Journal of Cell Biology.

Sincerely,

Richard Youle, PhD
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Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

The authors have done an excellent job on the revisions, and this paper is ready to go.

Reviewer #2 (Comments to the Authors (Required)):

The revised manuscript is acceptable.