THE EMBO JOURNAL

Manuscript EMBO-2014-89039

The small GTPase Arf1 modulates mitochondrial morphology and function

Karin B. Ackema, Jürgen Hench, Stefan Böckler, Shyi Chyi Wang, Ursula Sauder, Heidi Mergentaler, Benedikt Westermann, Frédéric Bard, Stephan Frank and Anne Spang

Corresponding author: Anne Spang, University of Basel

Review timeline:

Submission date:	25 September 2013
Editorial Decision:	30 October 2013
Resubmission:	20 May 2014
Editorial Decision:	25 June 2014
Revision received:	29 July 2014
Editorial Decision:	01 August 2014
Accepted:	07 August 2014

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Andrea Leibfried

1st Editorial Decision 30 October 2013

Thank you for submitting your manuscript entitled 'Small GTPases of the early secretory pathway modulate mitochondrial morphology and function'. Please excuse the slight delay in getting back to you, I have gotten the third review on your manuscript later than expected.

As you will see in the specific comments below, all referees agree that you observe an interesting phenotype. However, they think that the current manuscript does not provide sufficient mechanistic insight into the process that leads to changes in mitochondrial architecture upon loss of Arf1/Gbf1 function. Furthermore, they point out that the paper needs major amendments and restructuring as well as further experiments to address technical concerns. Because of the numerous concerns raised by the referees, I am very sorry to say that I cannot offer publication in The EMBO Journal.

Having said that, I still think that your observations are potentially interesting. Therefore, should you be able to address the referees' concerns, I will be happy to reconsider your manuscript as a resubmission. I would try to go back to the same set of referees in this case, however, I would have to take novelty into account at the time of resubmission. Especially an extension on the data on MIRO and VDAC depletions and on MADs as well as on the analysis of rescue by overexpression of cdc48 along the lines as suggested by referee 2, point 8 and in the general remarks stated by this referee (2nd paragraph) would be required for reconsideration here. Furthermore, the technical concerns would have to be fully addressed. A lot of work is hence required to address the comments

raised by the referees, so it might be in your own interest to seek publication elsewhere at this point. In this case a restructuring to a short-format/single message paper might be advisable.
I am very sorry to disappoint you on this occasion, but I hope that you will find the referees' comments helpful.

REFEREE COMMENTS

Referee #1

General summary and opinion about the principle significance of the study, its questions and findings:

-This manuscript reports a new role of ARF1 and the ARF1-GEF, GBF1 in mitochondria architecture. When ARF1 and GBF1 are depleted by RNAi in C.elegans, the mitochondria that are tubular in WT becomes hyperconnected and form a branched network
The authors show that this is not due to a cytoskeleton issue because treatment of the worms with drugs driving actin and microtubule depolymerisation does not recapitulates the mitochondria phenotype
-The authors also rule out a role for transport in the early secretory pathway (in which, of course, ARF1 and GBF1 plays a critical role). They show this in two ways. The first is that other C.elegans ARF1 GEF, AGEF does not lead to the same mitochondrial phenotype when depleted by RNAi. Furthermore, a mitochondrial phenotype is also observed in yeast mutant for ARF1 and Gea1 (GBF1) but not for COPB mutant, that is a COPI subunit.
-They show that ARF1 is present on mitochondria
-The authors then embark upon showing that ARF1 or GBF1 are not needed for the localization/function of the dynamin-like molecule DRP1involved in mitochondrial fission or the mitofusin Fzo involved in mitochondrial fusion. Of course, depletion of DRP1 also causes hyperconnected mitochondria but the phenotype is the same when both DRP1 and ARF1 or DRP1 and GBF1 are depleted. There is seemingly no enhancement that would suggest that there are in the same pathway. Same thing goes for Fzo.
The authors then investigate whether affecting ER morphology could in turn affect mito organization but this does not seem the case.
-Also they investigate the relative organization of the two organelles and found that in ARF1 and GBF1 loss of function, it is not overtly changed.
-They pursue testing their hypothesis that ARf1 and GBf1 would be somehow involved in ER/Mito contacts. Depleting MIRO and VDAC, two Calcium sensors specifically localised to MAMs but distinct to the yeast ERMES, leads to the same phenotype as ARF1 and GBF1, that is the hyperconnected mitochondria.
Overall, the authors suggest that the observed mitochondrial hyperconnectivity in ARF1, GBF1, Miro and VDAC RNAi is a read-out for he loss of ER-mitochondria contact sites. This suggests that these contacts prevent somehow the fusion of mitochondria, in line with Friedman's data.
-Last, by IP, they show that yeast ARf1 binds CDC48, a key triple A ATPase required for ERAD and recently shown to also function in MAD (mitochondrial associated degradation).
Overexpression of CDC48 partly rescues the ARf1 mito hyperconnected phenotype, suggesting that this hyperconnectivity (due to the loss of ER/Mito contact) is also the site of increased MAD, where redox damaged mitochondrial proteins are extracted and degraded, thus saving the mitochondria.

I have purposively left Sar1 out this summary and I will come back to it at the end of the review (point 9)

This is a very interesting story, very clearly written, showing a novel role for Arf1 and GBf1.
Although the manuscript contains a lot of work, the last portion of the paper (that swings the story toward mitochondrial repair and degradation) appears somehow undeveloped.
The bulk of the paper is toward negative results (albeit extremely well documented), namely that the arf1/gbf1 depletion phenotype is not due to defects in the actin dynamics, in trafficking in the early secretory pathway, in the transport/delivery of Drp1 or Fzo to mitochondria and to ER to Mito contact.
Only the last figure is proposing the beginning of mechanism and this should be expanded (see my point 7-8)

It is also a long manuscript with 9 figures and I would suggest that some of these negative data are put in Suppl figures to give room to more documentation on the last part
Once this is fixed, the manuscript should be published in the EMBO J.

Specific major concerns essential to be addressed to support the conclusions:

I apologize that my specific comments are so numerous but this is because the manuscript contains a lot of data.

1) I have a hard time to relate the C.elegans phenotype to the yeast phenotype. Would globular be equivalent to hyperconnected? In this regard, Figure 4 is oddly organized. Why not show first the major mitochondrial phenotype observed in all the different mutants at restrictive temperature before starting the quantitation. For instance, is yeast gea1 mutant phenotype the same as arf1? The pictures are too small to see this clearly.

2) Regarding the non-additivity of the DRP1 and ARF1 or GBF1 phenotype, it would be good to have a picture of it. What is displayed in the graph is the % of cells with a hyperconnected phenotype, not the degree of hyperconnectivity? Is it possible to measure the size of the "net" and score for this?
In yeast (Figure 6), it appears that mitochondria appears way more compact in the double mutants dnm1/arf1, so it seems there that DNM1 and ARF1 act in the same pathway (leading to the bead on the string phenotype). How can the authors reconcile this discrepancy with the C.elegans model. The same is true for fzo mutation.

3) This impact of this result is also slightly minimized by the fact that overexpression of Dnm1 in the arf1 yeast mutant does not lead to restoration of the WT phenotype?
Also is it possible to overexpress DRP1 in GBF1 RNAi worms and observed the potential restoration of the WT phenotype?

4) The authors claim that "unlike in oocytes (Ackema et al., 2013), knockdown of ARF-1.2 or GBF-1 did not lead to a gross morphological change in the ER
of the body wall muscle". This seems indeed the case in Fig 8A but not 8B. In the ARF1 depleted animals, the ER network is much smaller than in the WT (at the nuclear section). I don't understand the discrepancy. In this case at least, it seems that the hyperconnected ER morphology could drive the mitochondria hyperconnectivity.

How does a gbf1 depleted worm look in this type of analysis, especially in nuclear confocal sections?

5) I am confused by the conclusions of Figure 8 (that ER to mito contacts do not seem affected by depleting arf1 or gbf1)?
I am especially puzzled considering what I think the overall conclusions of the paper is, that is that the mitochondrial hyperconnectivity driven by arf1 and gbf1 RNAi is a read-out for an impairment of the ER to Mito contacts (as shown in Figure 9).
SO, all in all, I was expected to see fewer contacts. However, at the resolution and size of the pictures, it might be difficult to assess and score. Is it possible to establish a counting method scoring the pixel overlap between ER and Mito in the ARf1 and GBF1 RNAi worms? I feel that it is an important part to sort out.

6) The argument of the CHIMERA expression (FigS3-not S6 as mentioned in text) in yeast does not rescue the mitochondria phenotype could be powerful to indicate that ER to mito contact are not at stake in the observed hyperconnected mitochondrial phenotype, if only the authors would describe the tool a bit better. What type of contacts does it form or would it rescue?

However, if indeed ER to mito contacts are not at stake, I don't understand the author's reasoning to test Miro/Gem in the mito phenotype. Indeed, the Rho-like GTPase Miro1/Gem1 is reported to regulate connections between ER and mitochondria.
Do the authors mean that ER to mito contacts are different from ER to mito connections??? I don't think so. This transition needs to be made clearer and as mentioned above, this part needs to become more prominent.

7) It is essential to describe the phenotype of MIRO and VDAC depletions in more details. What does the ER look like in the depleted worms?
What is the extent of ER to mito contacts (or connections?).
Does double depletion miro/arf1 or vdac/arf1 accentuate the phenotype in the worm? Please show pictures
Why is it not accentuated in miro/gbf1 double depleted worms? This is not what is expected (so far arf1 and gbf1 loss of function have shown identical phenotypes) and shed a shadow of doubt on the results obtained with drp1/gbf1 double depleted worms (that also shows not additivity).

8) The CDC48 part is very interesting and needs to be extended.
The yeast Arf1 IP pulling
Does C.elegans ARF1 interact biochemically with p97 (or equivalent)

The rescue by CDC48 overexpression (unfortunately a single panel in the last figure) is also very interesting. Does p97 also rescue the Arf1/Gbf1 depletion in worms?

From this, it is tempting to conclude that mitochondrial hyperconnectivity means more mitochondrial damages and more need for MAD, this should be tested.
Can the authors trigger a redox damage in mitochondrial protein and see whether mitochondria become hyperconnected?
Can depletion of CDC48 also lead to hyperconnected mito?
Can CDC48 be localized to these connections?
What about VMS1, the protein that is part of the complex with CDC48 in mediating MAD?
What about ERAD instead of MAD?
What could be the role of Arf1 and Gbf1 in this? Are they involved in the delivery of CDC48/p97 to the sites? Could the same analysis shown in Figure 7 be repeated for CDC48?

9) The Sar1 story.
In my opinion, and given the huge amount of data regarding Arf1 and Gbf1, it might be wise to publish the Sar1 data somewhere else. It only complicates the analysis.
The role of ARf1 and GBF1 in this novel phenotype is not understood. Sar1 depletion or mutation phenotype is opposite? Does Sec12 loss of function lead to the same phenotype as Sar1? Does Sar1 compete with Arf1 for binding to mitochondria?
Does a double Sar1/Arf1 mutant or depletion rescue the Mito phenotype?
Does overexpression of Sar1 in an Arf1 depleted animal rescue the mito phenotype?
This would add another figure to this already huge paper.

Overall, this paper reports novel and interesting data and will be completely suitable for publication in the EMBO Journal if the part on Miro and CDC48 is significantly extended and solidified.

Minor concerns that should be addressed:

10) Affecting actin and MT polymerization with the drug used clearly does not lead to hyperconnected mitochondria. What about preventing their dynamics by blocking their depolymerisation, using taxol for MT and jasplakinolide for actin? How does this affect mitochondria architecture?

11) I would argue that depleting AGEF would affect trans Golgi to plasma membrane trafficking, not trafficking through the early secretory pathway. It remains an adequate control to say that secretion per se is not affected but not really to challenge ARF1 and GBF1 function in the early secretory pathway.
The COPB KD in C.elegans and mutation in yeast Sec21 (COPI), none leading to a mito phenotype is good evidence that transport is not involved.

12) The display of the redox reaction in Fig 3C and D is weird. What is the need to cut out very small and odd-shaped pieces of the staining?

13) The worm phenotype is very cute but cannot be attributed solely to the mito phenotype. Of course secretion will also be affected.

How does a COPI depleted worm perform in this test?

Referee #2

In an earlier paper, these authors showed that levels of the ArfGEF GBF-1 govern ER morphology and different vesicular transport pathways. Here, they describe some interesting effects of Arf1 and Sar1 manipulations on mitochondrial morphology. They show that sar-1 RNAi collapses the mitochondrial network while RNAi for arf-1.2 and gbf-1 result in mitochondrial hyper-connectivity. They test a number of parameters, including the effects of cytoskeleton inhibitors and overexpression of CDC48. Surprisingly, this last treatment suppresses the connectivity caused by ARF RNAi. The authors conclude that ARF1 and SAR1 regulate mitochondrial dynamics and they suggest that this regulation is independent of fission and fusion machineries.

The basic observations suggesting cross talk between the secretory pathway and mitochondria are interesting. However, the effects are likely indirect and many of the conclusions are tentative. The paper would be strengthened by showing more directly that cdc48 influences mitochondrial fission or fusion. One possible connection would be controlling the turnover of Fzo1, since that was shown to be regulated by cdc48 in other systems. The authors could determine the levels of fzo-1 protein. Additionally, the authors should test how cdc48 function might be disrupted in arf-1.2 and gbf-1 RNAi worms. Does cdc48 RNAi increase connectivity? Is CDC48 sequestered by the Golgi? Is ERAD induced to such an extent that no CDC48 is left for mitochondrial functions?

As another general comment, the paper is weakened by the many comparisons with yeast, particularly when the underlying processes are known to use different proteins, as is the case for mitochondrial transport, cytoskeletal anchoring, ER tethers and the recruitment factors for Drp1/Dnm1.

Some more specific comments:

1. Why would gbf-1 RNAi affect cytoskeleton? Can think of other reasons for why disruptions in the secretory pathway would affect muscle contraction. This part seems more suitable as supplemental data.

2. Section on cox enzymatic activity is confusing; enzymatic activity not decreased in arf-1.2 but mitochondrial morphology is affected. The authors should test whether the numbers of bends are affected by arf-1.2 RNAi.

3. Yeast and mammalian Sar and Arf homologues clearly also affect mitochondrial morphologies but the effects are quite different from those in worms. Not sure how helpful this is.

4. Fractionation results do not necessarily show mitochondrial localization of Sar1 and Arf1. Sec61 is also present, suggesting that these fractions still contain substantial amounts of ER. Better fractionation data is needed to make this point more clearly. The effects of Arf1 and Sar1 are more likely indirect. Displacement of CDC48 to a different fraction might be more relevant. The authors should test this.

5. The tests of genetic interactions with fission and fusion proteins are OK, but I don't think that the authors can conclude that arf-1.2 effects on mitochondrial morphology are independent of fission and fusion proteins. More likely, those override the effects of arf-1.2.

6. The tethering part also suffers from parallels with yeast. The yeast mitochondrial Arf phenotype is different and the tethers are different, so how can you draw a conclusion about the effects of tethers on Arf induced hyperconnectivity in worms?

7. Knock down of Miro and VDAC also lead to hyperconnectivity but these proteins have other functions too, so they also do not show that ER tethers are involved.

8. Rescue of mitochondrial morphology by overexpression of cdc48 fits earlier studies of fzo-1 turnover and is therefore the most plausible explanation for the hyperconnected phenotype. This part is, however, only shown with a single overexpression experiment. RNAi and other tests are needed.

Referee #3:

In this manuscript, Spang and coworkers address the role of the small GTPases of the Arf family in mitochondrial morphology and function. While the discovery could be potentially interesting, the manuscript is purely observational and it unfortunately contains factual and experimental mistakes that make it a weak candidate for publication at all.
The manuscript is largely observational, the approach is lacking any quantitative analysis and the reader is left with the conclusion that ablation of some small GTPases causes changes in mitochondrial morphology maybe by cooperating with Miro1, but as it will be clearer later, this is unlikely.

Major points
Gbf-1 increases the numbers of connected mitochondria, and a similar phenotype is observed when arf1-2 is downregulated. Author state that they observe a similar phenotype with sar1 knockdown, but the phenotype is actually rather different. The Sar1 part is confusing and does not really add much to the paper. This reviewer understands that it is an important control in the secretory pathway, but different mitochondrial morphologies mean that the mechanism shall be different and thus these data are actually counterproductive and shall be removed.
The authors state that they measure mitochondrial activity by measuring the response to paraquat. Unfortunately, this is not at all a way to measure mitochondrial function, but just a measurement of the sensitivity to an e- cycler that induces a ROS production loop. In addition, the authors conclude from this experiment and from a COX staining that the Gbf1 and arf1-2 cause also mitochondrial dysfunction. It is unclear if the knockdown primarily affects COX or mitochondrial morphology; if the respiratory defect is confined to COX (whose functional reserve is anyway very high, see the papers by Attardi on the topic) or it is widespread. A better mitochondrial functional characterization is required.
The analysis of the mechanism by which the small GTPases knockdown affects mitochondrial morphology is not satisfactory. Apparently, none of the known mediators of fusion/fission seems to alter the mitochondrial dysmorphology, and the mitochondrial purification is unsatisfactory (many protocols to purify mitochondria from MAMs have been published and shall be used, see for example Wiechowski et al, Nat Prot). Besides the technicalities that lower the strength of the mitochondrial localization of Gbf and Arf, the lack of a genetic interaction with the known players of fusion/fission of course raises the question of how Gbf and Arf regulate mitochondrial morphology. The analysis of the role of mitochondria-ER contact in Arf/Gbf regulation of mitochondrial morphology is inconclusive. Knocking down Miro1 has not been reported to affect ER-mitochondria tethering (which shall be anyway measured for each treatment supposed by the authors to affect ER-mito contact): it regulates mitochondrial movement and in plants also morphology. No surprise that it causes mitochondrial elongation, which is not further increased by Gbf knockdown. Rather surprisingly, the authors conclude that Miro and Gbf are in the same pathway, but by analogy the same conclusion shall have been drawn for Drp1. Thus, to assign any role to Miro is premature and simple genetics as performed here is not sufficient. Evidence of interaction and of modulation of the other functions of Miro (i.e., mitochondrial transport) must be presented; similarly, Miro can't be equated to an ER-mitochondria tether. Indeed, even in yeast the ERMES is not a tether, but a protein complex whose knockdown can rescue yeast mutants that overexpress an artificial ER-mito tether. In conclusion, this paper is not suitable for publication: vis-à-vis an interesting topic, the authors fall short to provide a mechanism for the observed mitochondrial shape and possibly functional changes. I suggest that they explore in depth how these GTPases work; alternatively, the paper is best suited for a journal that publishes short reports with one focused observation and less mechanistic details. In any case, the paper must be revised and the odd part on mitochondria-ER "Miro-mediated" contacts shall be dropped.

Minor points
The manuscript shows a lack of proficiency in mitochondrial science that is at times embarrassing. Just as one example, the authors claim in the abstract that VDAC (the acronym of voltage dependent anion channel, aka as porin, a channel with a cutoff of 5kDa) is a calcium channel and that its ablation causes mitochondrial elongation. This is just one of the many mistakes that shall be revised maybe by asking a colleague proficient in mitochondrial science to read the paper
Some references are not formatted.

Resubmission 20 May 2014

Referee #1

…
This is a very interesting story, very clearly written, showing a novel role for Arf1 and GBf1.
Although the manuscript contains a lot of work, the last portion of the paper (that swings the story toward mitochondrial repair and degradation) appears somehow undeveloped.
The bulk of the paper is toward negative results (albeit extremely well documented), namely that the arf1/gbf1 depletion phenotype is not due to defects in the actin dynamics, in trafficking in the early secretory pathway, in the transport/delivery of Drp1 or Fzo to mitochondria and to ER to Mito contact.
Only the last figure is proposing the beginning of mechanism and this should be expanded (see my point 7-8)
It is also a long manuscript with 9 figures and I would suggest that some of these negative data are put in Suppl figures to give room to more documentation on the last part
Once this is fixed, the manuscript should be published in the EMBO J.

Thank you!

Specific major concerns essential to be addressed to support the conclusions:

I apologize that my specific comments are so numerous but this is because the manuscript contains a lot of data.

1) I have a hard time to relate the C.elegans phenotype to the yeast phenotype. Would globular be equivalent to hyperconnected? In this regard, Figure 4 is oddly organized. Why not show first the major mitochondrial phenotype observed in all the different mutant at restrictive temperature before starting the quantitation. For instance, is yeast gea1 mutant phenotype the same as arf1? The pictures are too small to see this clearly.

We changed the organization of Figure 4 (now Figure 3). The image for the *gea1/2* mutant has now been included and the size of the images was increased. The yeast phenotypes as well the *C. elegans* phenotypes are indistinguishable between the gea1/2 (*gbf-1(RNAi)*) and the arf1 mutants (*arf-1.2(RNAi)*).

In *C. elegans* we observe a hyper-connected phenotype, while in yeast mitochondria appears to be more collapsed into globular structures. We believe nevertheless this is due to the same defect as Fzo1 and Arf1 appear both to have a role in mitophagy.
In addition we think that Arf1 is required for the functionality of ER-mitochondria contact sites. When Arf1 function is compromised, mitochondria become damaged and defective, however due to the defect in mitophagy, the defective compartments cannot be removed. In *C. elegans* the consequences might be hyper-connection, which we also observed for miro-1 and vdac-1 knockdowns, while the outcome in yeast might be somewhat different. Still we would argue that the underlying mechanism remains the same.

2) Regarding the non-additivity of the DRP1 and ARF1 or GBF1 phenotype, it would be good to have a picture of it. What is displayed in the graph is the % of cells with a hyperconnected phenotype, not the degree of hyperconnectivity? Is it possible to measure the size of the "net" and

score for this?
In yeast (Figure 6), it appears that mitochondria appears way more compact in the double mutants dnm1/arf1, so it seems there that DNM1 and ARF1 act in the same pathway (leading to the bead on the string phenotype). How can the authors reconcile this discrepancy with the C.elegans model. The same is true for fzo mutation.

An alternative explanation to the one given above for the different over all phenotype in yeast and *C. elegans* could be due to the severity of the knockdown versus the temperature-sensitive mutation in yeast. In *C. elegans* we knockdown one Arf: arf-1.2, and it is conceivable that other Arf proteins would be partially redundant to arf-1.2, and thus could take over some of the function when arf-1.2 is knocked down. In yeast, we use a temperature-sensitive (ts) mutant that is ts because both Arf1 activity providing genes *ARF1* and *ARF2* are affected (mind that we use a background in which *ARF2* is deleted).

We tried to measure the compactness of the mitochondria net, but were unable to do it in a reliable way. We rephrased the section and toned down our conclusion about Dnm1/DRP-1.

3) This impact of this result is also slightly minimized by the fact that overexpression of Dnm1 in the arf1 yeast mutant does not lead to restoration of the WT phenotype?
Also is it possible to overexpress DRP1 in GBF1 RNAi worms and observed the potential restoration of the WT phenotype?

Given that we now provide evidence that loss of Arf1 function causes Fzo1 clustering/aggregation and partial degradation, and hence more affecting Fzo1, it appears no longer surprising that *DNM1* overexpression did not rescue arf1-11Δarf2 at the restrictive temperature. We nevertheless obtained a DRP-1 overexpressing strain from Alex van der Bliek, and *gbf-1(RNAi)* in this strain background still resulted in hyper-connected mitochondria, and DRP-1 was still present on the mitochondria. Since this is yet another negative result, we did not include it in the manuscript.

4) The authors claim that "unlike in oocytes (Ackema et al., 2013), knockdown of ARF-1.2 or GBF-1 did not lead to a gross morphological change in the ER
of the body wall muscle". This seems indeed the case in Fig 8A but not 8B. In the ARF1 depleted animals, the ER network is much smaller than in the WT (at the nuclear section). I don't understand the discrepancy. In this case at least, it seems that the hyperconnected ER morphology could drive the mitochondria hyperconnectivity.

We really did not observe a gross morphology change in the ER in body wall muscle and replaced the image for a better one. We would have very much liked to see an ER morphological defect, as it would have made our live much easier….

How does a gbf1 depleted worm look in this type of analysis, especially in nuclear confocal sections?

This is obviously a very good suggestion, and we had attempted to do this experiment before. Unfortunately, this analysis did not work in the *gbf-1(RNAi)* animals because of a defect in endocytosis in these worms (Ackema et al, 2013). The uptake of mitotracker is insufficient. In addition the worms tend to die in the staining solution because they cannot swim as well as wild-type worms.

5) I am confused by the conclusions of Figure 8 (that ER to mito contacts do not seem affected by depleting arf1 or gbf1)?
I am especially puzzled considering what I think the overall conclusions of the paper is, that is that the mitochondrial hyperconnectivity driven by arf1 and gbf1 RNAi is a read-out for an impairment of the ER to Mito contacts (as shown in Figure 9).
SO, all in all, I was expecting to see fewer contacts. However, at the resolution and size of the pictures, it might be difficult to assess and score. Is it possible to establish a counting method scoring the pixel overlap between ER and Mito in the ARf1 and GBF1 RNAi worms? I feel that it is an important part to sort out.

We included data on Mdm34-GFP localization (Fig. S6B), which was not affected in the *arf1-*

11Δarf2 mutant, indicating that ER-mitochondria contact sites still exist. However we cannot score functionality. We believe that the tethering at the contact sites might still be OK, but exchange of lipids may be impaired.

6) The argument of the CHIMERA expression (FigS3-not S6 as mentioned in text) in yeast does not rescue the mitochondria phenotype could be powerful to indicate that ER to mito contact are not at stake in the observed hyperconnected mitochondrial phenotype, if only the authors would describe the tool a bit better. What type of contacts does it form or would it rescue?

We apologize for the mistake in referring to the correct figure. We added more information about he ChiMERA

However, if indeed ER to mito contacts are not at stake, I don't understand the author's reasoning to test Miro/Gem in the mito phenotype. Indeed, the Rho-like GTPase Miro1/Gem1 is reported to regulate connections between ER and mitochondria.
Do the authors mean that ER to mito contacts are different from ER to mito connections??? I don't think so. This transition needs to be made clearer and as mentioned above, this part needs to become more prominent.

We apologize for the confusion. We did not mean to give the impression that contact sites are different form connections. However, the contact site serves more functions than just tethering the two organelles together (i.e. lipid exchange). We modified the text to make our rational for the experiment clearer. We performed a number of experiments with VDAC and MIRO (see also below).

7) It is essential to describe the phenotype of MIRO and VDAC depletions in more details. What does the ER look like in the depleted worms?

We performed the experiment and knockdown of either protein did not affect ER morphology. We did not include the negative data in the manuscript. Neither MIRO nor VDAC have been localized to the ER, and given their suggested function a defect on ER structure was not expected.

What is the extent of ER to mito contacts (or connections?).

We are not sure how we should investigate this in the absence of suitable markers in *C. elegans*. We however determined the localization of Mdm34-GFP in *arf1-11Δarf2* cells, and did not detect any difference.

Does double depletion miro/arf1 or vdac/arf1 accentuate the phenotype in the worm? Please show pictures
Why is it not accentuated in miro/gbf1 double depleted worms? This is not what is expected (so far arf1 and gbf1 loss of function have shown identical phenotypes) and shed a shadow of doubt on the results obtained with drp1/gbf1 double depleted worms (that also shows not additivity).

Double gbf-1/miro did not enhance the phenotype. Images have been added to figure. gbf-1/VDAC has also been included into the graph.
We are not sure if a lack of enhancement is indeed 'unexpected'. Perhaps there is a limit to how much the network can be connected.

8) The CDC48 part is very interesting and needs to be extended.
The yeast Arf1 IP pulling
Does C.elegans ARF1 interact biochemically with p97 (or equivalent)
The rescue by CDC48 overexpression (unfortunately a single panel in the last figure) is also very interesting. Does p97 also rescue the Arf1/Gbf1 depletion in worms?

We obtained worm strains from Thorsten Hoppe, in which the two closest Cdc48 homologues would be individually expressed from a chromosomal array, thus leading to overexpression, although in a mosaic fashion. We were unable to retrieve CDC48.1 OE from the plate that was sent, but we have two strains that should OE CDC48.2. We also received CDC48.1::GFP from the same lab. The

expression is very mosaic, we do not trust a non-integrated overexpressing line if it comes to a rescue phenotype. Importantly, we had problems getting mitotracker into the CDC48.2 OE lines as it seems to get stuck in the gut (granules). Therefore, we could not perform the requested analysis.

From this, it is tempting to conclude that mitochondrial hyperconnectivity means more mitochondrial damages and more need for MAD, this should be tested.
Can the authors trigger a redox damage in mitochondrial protein and see whether mitochondria become hyperconnected?

The literature would rather suggest that redox damage would lead to fragmented mitochondria in mammalian cells. We did not attempt this experiment in body wall muscle cells. Because we were worried how specific the redox damage would be to a mitochondrial proteins compared to any other cellular protein. In addition, *eat-3* deletion in *C. elegans* is supposed to cause oxidative stress; under these conditions mitochondria were fragmented (Kanazawa et al., PLoS Genetics 2008).

Can depletion of CDC48 also lead to hyperconnected mito?

We depleted CDC-48.1 together with CDC-48.2. This did not yield in hyper-connected mitochondria in worm muscle cells.

We also determined the mitochondrial phenotype of *cdc48-3* and *cdc48-6* ts-mutants, however we did not observe a severe alteration in mitochondrial morphology. These data are shown in Fig. S5B. Our observations are in agreement with data published by Nunnari et al (MBoC,1997). Esaki and Ogura reported that expresssion of cdc48-E315Q or cdc48-E588Q results in fragmented mitochondria (J. Struc. Biol, 2012). These phenotypes were observed after 14 hr after endogenous *CDC48* shut off. Certainly our 1 hr temperature-shift experiments are on a very different time scale and may not be comparable.

Can CDC48 be localized to these connections?
What about VMS1, the protein that is part of the complex with CDC48 in mediating MAD?

We tagged Cdc48 with GFP and could not detect any enrichment on mitochondria over the cytoplasmic localization. In addition we probed the strongly enriched mitochondria fraction with antibodies against Cdc48. Again we could not find any binding there. In this context it worthwhile to note, that we also failed to detect Cdc48 in the ER fraction. One possible explanation for this observation is that during the enrichment procedure and the gradient fractionations peripherally associated Cdc48 is lost from the membrane fraction. Since the cytoplasmic localization of Cdc48 was reported before, we did not include these data into the manuscript.

We obtained a strain expressing CDC48.1::GFP I from the Hoppe lab. Similar to the yeast Cdc48, CDC-48.1::GFP was not localized to a specific structure.

The role of Vms1 is debated in the literature. We performed experiments in yeast and in *C. elegans*. Our results would indicate that the Cdc48 partial rescue could be independent of Vms1, but we do see slight phenotypes when Vms1 function is compromised. Thus, Vms1 might be involved, but not as the major player. The data are shown in Fig. S5D

What about ERAD instead of MAD?
What could be the role of Arf1 and Gbf1 in this? Are they involved in the delivery of CDC48/p97 to the sites? Could the same analysis shown in Figure 7 be repeated for CDC48?

We appended Cdc48 chromosomally with GFP in the yeast strains. We could not detect any clear enrichment on mitochondria. It seemed as if Cdc48 was present more on the ER in *arf1-11*, most likely due to the up-regulated UPR in this mutant. To test this hypothesis, we expressed the spliced version of HAC1, the transcriptional activator of UPR in the yeast. This results in a constitutively activated UPR. Under these conditions, Cdc48-GFP was recruited to the ER in all strains tested yet the mitochondrial defect in *arf1-11*Δ*arf2* was not rescued (Fig. 7C) .

9) The Sar1 story.
In my opinion, and given the huge amount of data regarding Arf1 and Gbf1, it might be wise to

publish the Sar1 data somewhere else. It only complicates the analysis.

We agree with the reviewer and removed all the data about Sar1 from the manuscript. We will seek publication of these data in another journal.

The role of ARf1 and GBF1 in this novel phenotype is not understood. Sar1 depletion or mutation phenotype is opposite? Does Sec12 loss of function lead to the same phenotype as Sar1? Does Sar1 compete with Arf1 for binding to mitochondria?
Does a double Sar1/Arf1 mutant or depletion rescue the Mito phenotype?
Does overexpression of Sar1 in an Arf1 depleted animal rescue the mito phenotype?
This would add another figure to this already huge paper.

Since we removed the Sar1 data, we decided not to include the data for these suggestions. As the reviewer recognizes, it would make a big paper only bigger.

Overall, this paper reports novel and interesting data and will be completely suitable for publication in the EMBO Journal if the part on Miro and CDC48 is significantly extended and solidified.

Thank you for the positive conclusion!

Minor concerns that should be addressed:

10) Affecting actin and MT polymerization with the drug used clearly does not lead to hyperconnected mitochondria. What about preventing their dynamics by blocking their depolymerisation, using taxol for MT and jasplakinolide for actin? How does this affect mitochondria architecture?

We don't know how stabilizing cytoskeletal elements would affect mitochondrial dynamics in *C. elegans*. But since we shortened this part drastically and moved the data into supplemental information, we did not see the point in doing extra experiments on the cytoskeleton.

11) I would argue that depleting AGEF would affect trans Golgi to plasma membrane trafficking, not trafficking through the early secretory pathway. It remains an adequate control to say that secretion per se is not affected but not really to challenge ARF1 and GBF1 function in the early secretory pathway.
The COPB KD in C.elegans and mutation in yeast Sec21 (COPI), none leading to a mito phenotype is good evidence that transport is not involved.

The main AGEF responsible for TGN-PM transport in *C.elegans* appears to be AGEF-1 (Ackema et al., PLoS One 2013); Sec7 would be the yeast one. However *agef-1(RNAi)* did not affect mitochondrial dynamics. We have and other have shown that GBF-1 has a role early in the secretory pathway (Witte et al., NCB 2011, Ackema et al., PLoS One 2013), consistent with Gea1/2 having overlapping functions in retrograde transport from the Golgi to the ER in yeast (Peyroche et al., JCS 2001, Spang et al., MBoC 2001). In addition, we performed initially also experiments in with exocyst mutants in yeast, which did not show the same mitochondrial phenotype. Moreover, the mitochondrial phenotype of srf1 mutants is allele specific. Thus we are convinced that this is not a function connected to the secretory pathway per se.
In addition, we tested whether the *arf1-11Δarf2* phenotype could be explained by defects in mitophagy. Indeed this mutant has impaired mitophagy. However this phenotype is linked to a general defect in autophagy, as demonstrated by using another *arf1* allele, *arf1-18Δarf2*, in which we do not observe a strong mitochondrial defect. These data have been included in Fig. S2 and Fig. 9.

12) The display of the redox reaction in Fig 3C and D is weird. What is the need to cut out very small and odd-shaped pieces of the staining?

Unfortunately there is nothing we can do about this, because this is part of the staining method. During embedding the worms take a random position in space, and can be later cut on the microtome in any angle.

13) The worm phenotype is very cute but cannot be attributed solely to the mito phenotype. Of course secretion will also be affected.
How does a COPI depleted worm perform in this test?

We agree, we cannot entirely be sure that coatomer depleted worms would not also have less body bends. We just used this as an indication for further experiments. In addition, this part has been moved to the supplements already. We decided to concentrate our efforts on more pressing experiments.

Referee #2

In an earlier paper, these authors showed that levels of the ArfGEF GBF-1 govern ER morphology and different vesicular transport pathways. Here, they describe some interesting effects of Arf1 and Sar1 manipulations on mitochondrial morphology. They show that sar-1 RNAi collapses the mitochondrial network while RNAi for arf-1.2 and gbf-1 result in mitochondrial hyper-connectivity. They test a number of parameters, including the effects of cytoskeleton inhibitors and overexpression of CDC48. Surprisingly, this last treatment suppresses the connectivity caused by ARF RNAi. The authors conclude that ARF1 and SAR1 regulate mitochondrial dynamics and they suggest that this regulation is independent of fission and fusion machineries.

The basic observations suggesting cross talk between the secretory pathway and mitochondria are interesting. However, the effects are likely indirect and many of the conclusions are tentative. The paper would be strengthened by showing more directly that cdc48 influences mitochondrial fission or fusion. One possible connection would be controlling the turnover of Fzo1, since that was shown to be regulated by cdc48 in other systems.

Thank you for pointing this out!

The authors could determine the levels of fzo-1 protein.

We determined the level of Fzo1 in yeast and find that overexpression of Cdc48 reduces Fzo1 levels in both wild type and *arf1-11*Δ*arf2* mutant strains. Moreover, Fzo1 levels were somewhat, but not as drastically reduced in *arf1-11*Δ*arf2* cells.
We attempted to use *C. elegans CDC-48* overexpressor strains. The Hoppe lab generously supplied us with strains. We were unable to retrieve CDC48.1 OE from the plate that was sent, but we have two strains that should OE CDC48.2. We also received CDC48.1::GFP from the same lab. The expression is very mosaic, we do not trust a non-integrated overexpressing line if it comes to a rescue phenotype. Importantly, we had problems getting mitotracker into the CDC48.2 OE lines as it seems to get stuck in the gut (granules). Therefore we did not use these strains for analysis (except to determine the CDC-48.1::GFP localization, which was cytoplasmic). (see also response to point 8 of reviewer 1)

Additionally, the authors should test how cdc48 function might be disrupted in arf-1.2 and gbf-1 RNAi worms.

We don't think that CDC-48 function is disrupted. Our overexpression experiments in yeast would rather suggest that Cdc48 is rate-limiting in the turnover of Fzo1.

Does cdc48 RNAi increase connectivity?

Concomitant knockdown of both CDC-48.1 and CDC-48.2 did not induce significantly hyperconnected mitochondria. Fig. S5C

Is CDC48 sequestered by the Golgi?

No, we could not find any Cdc48 enriched at Golgi structures in *arf1-11*Δ*arf2* mutants.

Is ERAD induced to such an extent that no CDC48 is left for mitochondrial functions?

Even though UPR is already turned on upon shift to the non-permissive temperature in *arf1-11*Δ*arf2* (Kilchert et al,. MBoC 2010), this does not seem the critical factor. Expression of spliced HAC1, which results in a constitutively active UPR did not induced mitochondrial fragmentation nor did it rescue the *arf1-11*Δ*arf2* phenotype. These data are presented in Fig. 7C. (see also response to point 8 of reviewer 1)

As another general comment, the paper is weakened by the many comparisons with yeast, particularly when the underlying processes are known to use different proteins, as is the case for mitochondrial transport, cytoskeletal anchoring, ER tethers and the recruitment factors for Drp1/Dnm1.

We do think that one of the strength of our analysis is the comparison between yeast and *C. elegans*. Some of the analysis is much easier done in yeast than in worms (and perhaps only feasible in yeast currently). However in worms, we can look at different cell types and developmental processes. This combination has worked well in the past to elucidate cellular mechanisms (see also: Poteryaev et al., Cell 2010). We aim to take advantage of these two model systems, which each has its own strength to understand cellular processes. We apologize if the reviewer finds this confusing.

Nevertheless we took the reviewer's concern seriously, and improved the writing to make the transition between the different model systems easier to follow and to understand. We hope the reviewer is satisfied by these changes.

Some more specific comments:

1. Why would gbf-1 RNAi affect cytoskeleton? Can think of other reasons for why disruptions in the secretory pathway would affect muscle contraction. This part seems more suitable as supplemental data.

This part was moved to supplementary data. We needed to test this possibility to exclude a potential role in cytoskeletal dynamics. It was a useful control.

2. Section on cox enzymatic activity is confusing; enzymatic activity not decreased in arf-1.2 but mitochondrial morphology is affected. The authors should test whether the numbers of bends are affected by arf-1.2 RNAi.

The body bend part has also been moved to supplements, as it only served as entry point in the further analysis. We take this data to indicate that Arf1.2 has a function mainly in mitochondrial morphology. This hypothesis is strengthened by the data that we now included into the manuscript showing that *arf1* mutant cells affect the level and the localization, and presumably the function, of Fzo1 (Fig. 7). Similarly to yeast, we would argue that Arf1 function is provided by more than one Arf protein: in yeast Arf1 and Arf2; in *C. elegans* ARF-1.2 and ARF-3. Only loss of both would presumably give the strongest phenotype.

3. Yeast and mammalian Sar and Arf homologues clearly also affect mitochondrial morphologies but the effects are quite different from those in worms. Not sure how helpful this is.

Well, we just want to make the point that loss of Arf1 function affects mitochondria morphology in multiple systems. We think this is a valid point and therefore we kept the data in the manuscript.

4. Fractionation results do not necessarily show mitochondrial localization of Sar1 and Arf1. Sec61 is also present, suggesting that these fractions still contain substantial amounts of ER. Better fractionation data is needed to make this point more clearly. The effects of Arf1 and Sar1 are more likely indirect. Displacement of CDC48 to a different fraction might be more relevant. The authors should test this.

We aimed to detect Cdc48 on mitochondrial fractions, but we could not detect any. This might be due to the fact that we do multiple centrifugation steps and Cdc48 is a soluble protein, which could easily be lost during the preparation. Next we aimed to detect Cdc48-GFP on mitochondria. However, as reported by others before, Cdc48-GFP is cytoplasmic. In the *arf1* mutant some Cdc48 can be found on the ER, presumably due to the UPR, which is activated in this mutant (Kilchert et

al., MBoC 2010). These data have been included into the manuscript (Fig. 7).

5. The tests of genetic interactions with fission and fusion proteins are OK, but I don't think that the authors can conclude that arf-1.2 effects on mitochondrial morphology are independent of fission and fusion proteins. More likely, those override the effects of arf-1.2.

We agree with the reviewer, we revised this part and rephrased the conclusions. We still think that Arf1 does not act directly through DRP-1/Dnm1. In addition we show now that in *arf1* mutant Fzo1 levels and localization on mitochondria are affected. However since we cannot show that this caused by direct interaction between Arf1 and Fzo1 or the lack thereof, we would like to cautious with our statement about how direct the role of Arf1 is in this process.

6. The tethering part also suffers from parallels with yeast. The yeast mitochondrial Arf phenotype is different and the tethers are different, so how can you draw a conclusion about the effects of tethers on Arf induced hyperconnectivity in worms?

We can do these experiments only in yeast because the identity of the physical tethers is to our knowledge unkown in *C. elegans*. Therefore, this is actually the perfect situation to use yeast as a model. At any rate, the manuscript contains equal, if not by now more data that had been generated in yeast. We admit that our knowledge is largely based on yeast but the correlations are pretty striking.
The hyperconnectivity could be a consequence of altered mitochondria through impaired lipid exchange or defects in Ca^{2+} homeostasis. This would cause the clustering/aggregation of Fzo1 and impair its function. It is likely that if the lipid composition of the outer membrane was affected that also other proteins may become functionally compromised. Perhaps in yeast the phenotype would be more severe and Fzo1 aggregation would cause of mitochondrial fragmentation. The removal of non-functional Fzo1 may leave en because it would act as a sink for also functional Fzo2 molecules. If Cdc48 removes erroneous Fzo1, enough functional Fzo1 ma behind to partially rescue the phenotype. So why would the phenotype be more severe in yeast than in *C.elegans*. For one, the knockdown is certainly not as dramatic as the ts mutations in yeast. The other possibility is the that kinetics of mitochondrial dynamics are different in *C. elegans* muscle cells than in yeast and that the equilibrium state between fission and fusion is different between the two cell types.

7. Knock down of Miro and VDAC also lead to hyperconnectivity but these proteins have other functions too, so they also do not show that ER tethers are involved.

We agree with the reviewer. Nevertheless, there is a correlation. Also pertaining to the point above: knockdown of MIRO resulted in a hyperconnected network in *C.elegans*, while in yeast the knockout caused fragmented mitochondria. We do not claim that the physical contact structure between ER and mitochondria are absent. In fact we detected the ERMES component Mdm34-GFP in yeast. The localization was unaltered in *arf1-11*Δ*arf2* mutant cells. Moreover, if the encounter structure would have been lost entirely, we should have observed a partial rescue by expressing the artificial tether, the ChiMERA construct. But this had no effect. However, we believe that the transport of lipids and/or ions at theses contact sites might be less functional and as a consequence Fzo1 would aggregate.

8. Rescue of mitochondrial morphology by overexpression of cdc48 fits earlier studies of fzo-1 turnover and is therefore the most plausible explanation for the hyperconnected phenotype. This part is, however, only shown with a single overexpression experiment. RNAi and other tests are needed.

Inspired by the reviewer's comment, we determined the fate of Fzo1 in *arf1-11*Δ*arf2* in dependence of *CDC48* overexpression. Fzo1 levels were somewhat reduced in the arf1 mutant, and further reduced when Cdc48 was overexpressed. However more pronounced than the reduction of Fzo1 in the *arf1* mutant, was the clustering/aggregation of Fzo1 in bright foci on mitochondria. Implying that Fzo1 might be partially misfolded/non-functional. Removing the misfolded Fzo1 would then alleviate the phenotype. As a side, we found it surprising that Cdc48 appeared to be rate-limiting for the stability of Fzo1, as even in wild-type Fzo1 levels were strongly reduced just by increasing Cdc48 levels. What this finding also suggests, is that Fzo1may intrinsically somewhat unstable/misfolded and therefore a prime substrate for MAD.

RNAi of CDC-48.1 and CDC-48.2 in *C. elegans* nor shifting the yeast ts-strains *cdc48-3* and *cdc48-6* to restrictive temperature for an hour (some treatment as for the arf1 mutant) did not cause mitochondrial aberrations. The overexperession in *C.elegans* was unfortunately not feasible in a controlled way as there are two CDC-48 genes with at least overlapping functions. Since they would be expressed extrachromosomally, only a mosaic analysis could be done, but we would have no control of the expression levels. In addition, strong overexpression of CDC48 in yeast has been shown to be lethal. Given all these caveats, we decided not to do this experiment. (see also response to point 8 of reviewer 1)

Referee #3:

In this manuscript, Spang and coworkers address the role of the small GTPases of the Arf family in mitochondrial morphology and function. While the discovery could be potentially interesting, the manuscript is purely observational and it unfortunately contains factual and experimental mistakes that make it a weak candidate for publication at all.

We are not aware of any experimental mistakes, and the reviewer does not provide any precise case for such a mistake below. We therefore do no understand the basis of his/her claims.

The manuscript is largely observational, the approach is lacking any quantitative analysis and the reader is left with the conclusion that ablation of some small GTPases causes changes in mitochondrial morphology maybe by cooperating with Miro1, but as it will be clearer later, this is unlikely.

We respectfully disagree with the reviewer. We quantified essentially every phenotype in yeast and in worms with large numbers and always at least three independent experiments. In addition, we performed for example COX activity assays and qPCR. The reviewer will surely agree that these are quantitative measures. Again, we are surprised by the harsh statements of this reviewer, and fail to understand the rational for these statements.

Major points
Gbf-1 increases the numbers of connected mitochondria, and a similar phenotype is observed when arf1-2 is downregulated. Author state that they observe a similar phenotype with sar1 knockdown, but the phenotype is actually rather different. The Sar1 part is confusing and does not really add much to the paper. This reviewer understands that it is an important control in the secretory pathway, but different mitochondrial morphologies mean that the mechanism shall be different and thus these data are actually counterproductive and shall be removed.

We removed the Sar1 data from the manuscript. Just for the record, we claimed in the previous version of the manuscript that the mitochondrial phenotypes caused by loss of function of either Arf1 or Sar1 are different.

The authors state that they measure mitochondrial activity by measuring the response to paraquat. Unfortunately, this is not at all a way to measure mitochondrial function, but just a measurement of the sensitivity to an e- cycler that induces a ROS production loop. In addition, the authors conclude from this experiment and from a COX staining that the Gbf1 and arf1-2 cause also mitochondrial dysfunction. It is unclear if the knockdown primarily affects COX or mitochondrial morphology; if the respiratory defect is confined to COX (whose functional reserve is anyway very high, see the papers by Attardi on the topic) or it is widespread. A better mitochondrial functional characterization is required.

As written in the manuscript, we used paraquat to check for ROS sensitivity. We believe this is the appropriate reagent to do this.
We also stated in the manuscript that *gbf-1(RNAi)* primarly affects morphology and only as a consequence the function. This is also the explanation why *arf-1.2(RNAi)* causes morphological alterations but does not significantly reduce COX activity. In addition, the COX and paraquat data are not the only evidence we provide for a defect in mitochondrial function: the yeast mutants are unable to grow on glycerol. Finally, we have also used SDH activity and saw a similar reduction in

gbf-1(RNAi) compared to *agef-1(RNAi)* or mock treated worms.

The analysis of the mechanism by which the small GTPases knockdown affect mitochondrial morphology is not satisfactory. Apparently, none of the known mediators of fusion/fission seems to alter the mitochondrial dysmorphology, and the mitochondrial purification is unsatisfactory (many protocols to purify mitochondria from MAMs have been published and shall be used, see for example Wiechowski et al, Nat Prot).

There is no protocol to purify MAMs from yeast or from *C. elegans*. In fact we contacted Chris Meisinger (University of Freiburg) who is undoubtedly an expert on yeast mitochondria and their purification. He confirmed us that there is no protocol for the purification of yeast MAMs and that in fact they had tried to develop one without much success. Email conversation, albeit in German, can be provided upon request. Therefore, we kept the mitochondrial enrichment procedure.

Besides the technicalities that lower the strength of the mitochondrial localization of Gbf and Arf, the lack of a genetic interaction with the known players of fusion/fission of course raises the question of how Gbf and Arf regulate mitochondrial morphology. The analysis of the role of mitochondria-ER contact in Arf/Gbf regulation of mitochondrial morphology is inconclusive. Knocking down Miro1 has not been reported to affect ER-mitochondria tethering (which shall be anyway measured for each treatment supposed by the authors to affect ER-mito contact): it regulates mitochondrial movement and in plants also morphology. No surprise that it causes mitochondrial elongation, which is not further increased by Gbf knockdown. Rather surprisingly, the authors conclude that Miro and Gbf are in the same pathway, but by analogy the same conclusion shall have been drawn for Drp1. Thus, to assign any role to Miro is premature and simple genetics as performed here is not sufficient. Evidence of interaction and of modulation of the other functions of Miro (i.e., mitochondrial transport) must be presented; similarly, Miro can't be equated to an ER-mitochondria tether. Indeed, even in yeast the ERMES is not a tether, but a protein complex whose knockdown can rescue yeast mutants that overexpress an artificial ER-mito tether.

We do not want to get into an argument with the reviewer on how to interpret the paper of Kornmann et al., 2009 entitled ‘An ER-mitochondria tethering complex revealed by a synthetic biology screen.‘ We want to point out that we never said in the previous manuscript that ERMES is the tether. Moreover, we stated also that the function of Gem1 in phospholipid transfer from the ER to mitochondria is controversial. Thus we do not feel that this comment is justified. Nevertheless we included now references pointing out MIROs role in mitochondrial transport.

In conclusion, this paper is not suitable for publication: vis-à-vis an interesting topic, the authors fall short to provide a mechanism for the observed mitochondrial shape and possibly functional changes. I suggest that they explore in depth how these GTPases work; alternatively, the paper is best suited for a journal that publishes short reports with one focused observation and less mechanistic details. In any case, the paper must be revised and the odd part on mitochondria-ER "Miro-mediated" contacts shall be dropped.

We added more data towards a possible mechanism on how Arf1 is involved in mitochondrial morphology. We now show that the *arf1-11* Δ*arf2* mutant in yeast causes the aggregation and clustering of Fzo1 (and partial degradation). This phenotype is rescued by the overexpression of Cdc48 because Cdc48 causes the degradation of the defective Fzo1. This effect of Cdc48 is independent of its function in UPR because constitutive activation of UPR did not cause mitochondrial fragmentation nor did it rescue the *arf1-11*Δ*arf2* induced mitochondria phenotype. How does *arf1-11*Δ*arf2* cause the clustering of Fzo1. We propose that this happens because through defective ER-mitochondrial contact sites. Structurally the tethering is still intact but the either lipid or Ca^{2+} is compromised (or both).

Minor points
The manuscript shows a lack of proficiency in mitochondrial science that is at times embarrassing. Just as one example, the authors claim in the abstract that VDAC (the acronym of voltage dependent anion channel, aka as porin, a channel with a cutoff of 5kDa) is a calcium channel and that its ablation causes mitochondrial elongation. This is just one of the many mistakes that shall be revised maybe by asking a colleague proficient in mitochondrial science to read the paper

We apologize for the mistake in the abstract. However, the reviewer might have missed that we actually do refer to VDAC as voltage dependent anion channel on p. 17 and p. 21 in the previous version of the manuscript. Also there are apparently a lot of dispute about the function of mitochondrial proteins, as far as we are aware, the involvement of VDAC in Ca^{2+} homeostasis is not the basis of such a dispute. We fail to understand why this reviewer deems it necessary to insult at least two of our co-authors on this manuscript who are experts on mitochondria and who have contributed to the writing of this manuscript.

Some references are not formatted.

References are formatted.

2nd Editorial Decision 25 June 2014

Thank you for resubmitting your manuscript entitled 'The small GTPase Arf1 modulates mitochondrial morphology and function'. I have now received the reports from all referees. Additionally, I also sought advice on your manuscript, please excuse the delay in sending our decision that was caused by this.

The referees still appreciate your main findings. However, as you will see below, the current manuscript is very difficult to read and the data from the different model systems used are difficult to reconcile and apprehend. Thus, it is currently not easy to extract a clear message and firm conclusions from the manuscript. Therefore, the current manuscript is not suitable for publication in The EMBO Journal.

I discussed your manuscript in light of the referees' comments with my colleagues and I decided as mentioned above to consult an advisor to have a fresh opinion on the data. Based on this additional input, I can offer to consider a revised version of your manuscript for publication here. However, the advisor (similar to the referees) also pointed out that significant restructuring and re-writing would be required to allow further proceedings here. I would thus like to invite you to submit a revised version of the manuscript, also addressing the remaining concerns of the referees. It would be good if you could run an outline on how you are planning to restructure the paper by me upfront. Please let me know in case you have further questions.
Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Referee #1:

The manuscript has been extensively revised. The Sar1 part has been removed, the negative results put in the supplementary data and the CDC48 part has been developed.
The added results give a welcome mechanistic flavor to the mitochondria phenotype in arf/gbf mutant.

In short, independently of Arf function in the secretory pathway/COPI, in Arf mutant, the mitofusin Fzo is mislocalised on clusters along mitochondria perhaps due to the impaired function of ER/mitochondria contact sites (even though those sites still exist). This is and this has two consequences: impaired mitochondria dynamics and function and initiation of mitophagy.
There is no doubt that the results and conclusions are new and very interesting. Because there is so much new data, the reviewer's comments are again long, but after fixing the writing and point 6.3, I definitely advise for publication to the EMBO Journal.

1) Unlike the first version, the manuscript is not written as neatly. In particular some transitions are abrupt and parts miss conclusions.
Furthermore, although I have found the back-and-forth between C.elegans and yeast powerful in the original version, it is more confusing this time. This is not helped by the fact that the authors do not

always specify which systems they are dealing with, and the reader is left to guess with RNAi equating C.elegans and real mutations equating yeast. This should be fixed. I also strongly advised the authors to improve the writing by reading their manuscript calmly and carefully and provide a model.

About the scientific message:
2) The first part is about the C.elegans gbf and arf mutations leading to hyperconnected mitochondria phenotype both in term morphology (hyperconnected) and function.

3) The second part is to recapitulate this phenotype in yeast. Arf and gfp yeats mutant have disrupted mitochondria and the same disruption, showing a conserved role.
However, (and I have asked this question in my original review without getting an adequate answer), I am not sure how the yeast phenotype in Arf and gea mutant (fragmented, round mitochondria) relates to c.elegans of hyperconnected mitochondria.
There is clearly a phenotype in both model organisms but there seem to be almost the opposite. THIS needs to be discussed. In light of the new data on the mitofusin Fzo (that is less abundant in arf mutant), it seems logical that the phenotype should be toward more fragmented (as in yeast). Yet, in C.elegans, the mitochondria appear hyperconnected.
Could the difference between the two model organisms be at least discussed? Could the mislocalised fzo in one case leads to its activation and in other, its inhibition (see point 5)?

4) Arf is found in mitochondria with the caveat that it could come from the ER and be located to ER/mitochondria contact sites.

5) Arf mutant phenotype is not enhanced by loss or overexpression of Drp1 or Fzo. However, Fzo level is decreased in Arf mutant.

6) Arf is found to biochemically interact with CDC48, and O/e of CDC48 rescues Arf loss of function in yeast.
6.1: I found this result difficult to understand at least in the way it is presented. The reasoning is CDC48 would lead to a greater Fzo degradation, so it should enhance the Arf mutant phenotype (since Fzo level are also found to be reduced in this background). How can almost total loss of Fzo rescue the fragmented mitochondrial phenotype?
In support of this, CDC48 overexpressing yeast have fragmented mitochondria (Fig.6A).
What happens to CDC48 level in Arf mutant yeast and C.elegans? I guess nothing since combined mutations of Arf and CDC48 do not affect the mitochondria phenotype.

What Arf1 and 2 do is to mislocalise Fzo to specific spots as shown in Figure 7. This is irrespective of CDC48 (even though this aspect has not been addressed in a combined arf/CDC48 double mutant yeast). These spots are the origin of the problem. Again, the mitochondria phenotype is not due to the decreased Fzo level but to its mislocalisation
The subtitle on page 15 should not be "Fzo levels are reduced in arf mutant cells" but "Fzo is mislocalised in arf mutant cells in deleterious clusters along mitochondria" followed by "CDC48 in MAD removes these deleterious clusters".
This is really not clearly written, and the conclusion comes too late (a page later) at the end of the section on UPR (that is not involved) and MAD.
It is imperative that the authors should provide more mechanistic explanations on how they think Arf/Gbf works with regard with CDC48 and Fzo. A model would be welcome. Also, an explanation on how these Fzo clusters could lead to hyperconnected mito in C elegans and little round mito in yeast should be provided.

6.2: Since Fzo mislocalisation is critical for the phenotype, is this also true in C.elegans? Again, what is the speculation of Fzo clustering on its function? Do the clusters co-localise with ERMES in yeast?

6.3: I am also a little puzzled by the statement that CDC48 acts in MAD and UPR. I was also under the impression that it also acts in ERAD (at least from Jeff Brodsky' s work and others). Surprisingly, this is mentioned in the discussion but nowhere in the result section.

6.4: Vms1 is not introduced. What is this? This paragraph is not concluded.

7) The authors then show that ER/mitochondria contacts (tethering) are not seemingly impaired using morphological evidences but also overexpressing the CHIMERA construct that have not effect on the arf mutant phenotype.

8) Then comes the MIRO/VDAC-1 part. Again, I fail to understand how the authors move from saying the ERMES is normally localized to actually investigate the function of ERMES components.
A sentence such as " although ER to mitochondria connections are seemingly non-affected, we addressed their functionality as it is possible that they are affected in line with defects in mitochondrial function".
The data are convincing but again, what is the relationship between Arf and Miro? How can miro function be affected by Arf?
How does this relate to Fzo mislocalisation?
Again, an integrative model would be welcome.

9) the mitophagy part is short but convincing.

10) Can the title be more specific, perhaps more mechanistic mentioning Fzo mislocalisation and CDC48/MAD and the function of ER/Mito sites.

11) The figures were either way too small or way too big. Difficult to print and/or to look at.

Referee #2:

The manuscript by Ackema et al. is improved by the renewed focus on Arf1 and its GEF. The interplay with cdc48 in the regulation of fzo1 helps some, while cofractionation with the MAM and the effects on mitophagy in yeast make this paper very interesting. A lot of the observations are repeated in three systems, but unfortunately the effects on mitophagy are only studied in yeast. This is a little concerning, because some effects seem are different in the different organisms. Yeast Arf mutants have globular mitochondria, worms have hyper-connected mitochondria and mammals have swollen mitochondria with tubular extensions. I am not sure what this means or whether these are general phenomena. Many questions also remain regarding the mechanism, but I suppose that could be saved for a later paper.

Here are some suggestions for improvements in the text:

In abstract: I would not say due to a deficiency in lipid environment, because there is no evidence for or against this. Too speculative.

Intro, p4: "mutation caused clustering/aggregation of Fzo1 at the restrictive temperature"
Should say: "mutation caused clustering/aggregation through the actions of Fzo1 at the restrictive temperature"

Intro p5: I would not say a functional contribution in lipid and/or Ca transfer, since there is no evidence for or against this.

Results p8. Knock down of COPB-1; how do you know it worked?

P9: what is the redundancy with arf3 based on?

P11: title: "arf1 is present on purified mitochondria" replace with "Arf1 cofractionates with ER and mitochondria"

P11: title bit of an overstatement: "GBF-1 and ARF-1 do not directly regulate mitochondrial fission dynamics" replace with "GBF-1 and ARF-1 deficiencies do not suppress fission or fusion defects"

P12. It is tough to enhance the effects of drp1 or dnmm1 deletions, since those are very strong already. maybe better say: "As expected, ..."

P14: what analysis revealed that the AAA-ATPase Cdc48 interacted specifically with Arf1 bound to GTP ? data not shown?

1st Revision - authors' response 29 July 2014

Referee #1:

The manuscript has been extensively revised. The Sar1 part has been removed, the negative results put in the supplementary data and the CDC48 part has been developed.
The added results give a welcome mechanistic flavor to the mitochondria phenotype in arf/gbf mutant.

In short, independently of Arf function in the secretory pathway/COPI, in Arf mutant, the mitofusin Fzo is mislocalised on clusters along mitochondria perhaps due to the impaired function of ER/mitochondria contact sites (even though those sites still exist). This is and this has two consequences: impaired mitochondria dynamics and function and initiation of mitophagy.
There is no doubt that the results and conclusions are new and very interesting. Because there is so much new data, the reviewer's comments are again long, but after fixing the writing and point 6.3, I definitely advise for publication to the EMBO Journal.

1) Unlike the first version, the manuscript is not written as neatly. In particular some transitions are abrupt and parts miss conclusions.
Furthermore, although I have found the back-and-forth between C.elegans and yeast powerful in the original version, it is more confusing this time. This is not helped by the fact that the authors do not always specify which systems they are dealing with, and the reader is left to guess with RNAi equating C.elegans and real mutations equating yeast. This should be fixed. I also strongly advised the authors to improve the writing by reading their manuscript calmly and carefully and provide a model.

We appreciate the reviewer's point. The manuscript underwent extensive restructuring and re-writing, which improved the readability. *C. elegans* data and yeast data have been largely separated. We also include a model at the end of the manuscript.

About the scientific message:
2) The first part is about the C.elegans gbf and arf mutations leading to hyperconnected mitochondria phenotype both in term morphology (hyperconnected) and function.

3) The second part is to recapitulate this phenotype in yeast. Arf and gfp yeats mutant have disrupted mitochondria and the same disruption, showing a conserved role.
However, (and I have asked this question in my original review without getting an adequate answer), I am not sure how the yeast phenotype in Arf and gea mutant (fragmented, round mitochondria) relates to c.elegans of hyperconnected mitochondria.
There is clearly a phenotype in both model organisms but there seem to be almost the opposite. THIS needs to be discussed. In light of the new data on the mitofusin Fzo (that is less abundant in arf mutant), it seems logical that the phenotype should be toward more fragmented (as in yeast). Yet, in C.elegans, the mitochondria appear hyperconnected.
Could the difference between the two model organisms be at least discussed? Could the mislocalised fzo in one case leads to its activation and in other, its inhibition (see point 5)?

We included a more extensive discussion of the phenotypic differences.

4) Arf is found in mitochondria with the caveat that it could come from the ER and be located to ER/mitochondria contact sites.

We agree with the reviewer that we cannot exclude that Arf1 is specifically enriched on the ER site of ER-mitochondrial contact sites and this possibility is now also mentioned in the text.

5) Arf mutant phenotype is not enhanced by loss or overexpression of Drp1 or Fzo. However, Fzo level is decreased in Arf mutant.

The levels are reduced but Fzo1 is still there. We also envisage that the arf1-11 mutation affects the mitochondrial lipid composition and therefore clustering of Fzo1. We tried to make this possibility clearer in the manuscript. We also hope that the model will help.

6) Arf is found to biochemically interact with CDC48, and O/e of CDC48 rescues Arf loss of function in yeast.
6.1: I found this result difficult to understand at least in the way it is presented. The reasoning is CDC48 would lead to a greater Fzo degradation, so it should enhance the Arf mutant phenotype (since Fzo level are also found to be reduced in this background). How can almost total loss of Fzo rescue the fragmented mitochondrial phenotype?
In support of this, CDC48 overexpressing yeast have fragmented mitochondria (Fig.6A).
What happens to CDC48 level in Arf mutant yeast and C.elegans? I guess nothing since combined mutations of Arf and CDC48 do not affect the mitochondria phenotype.

We had provided the levels of Cdc48 in arf1-11 mutant cells in Fig. 7A. The levels in the WT situation seemed to be lower at 37°C than at 23°C and in the mutant situation.

What Arf1 and 2 do is to mislocalise Fzo to specific spots as shown in Figure 7. This is irrespective of CDC48 (even though this aspect has not been addressed in a combined arf/CDC48 double mutant yeast). These spots are the origin of the problem. Again, the mitochondria phenotype is not due to the decreased Fzo level but to its mislocalisation
The subtitle on page 15 should not be "Fzo levels are reduced in arf mutant cells" but "Fzo is mislocalised in arf mutant cells in deleterious clusters along mitochondria" followed by "CDC48 in MAD removes these deleterious clusters".

Thank you very much for this suggestion. We changed the titles of the sections accordingly

This is really not clearly written, and the conclusion comes too late (a page later) at the end of the section on UPR (that is not involved) and MAD.
It is imperative that the authors should provide more mechanistic explanations on how they think Arf/Gbf works with regard with CDC48 and Fzo. A model would be welcome. Also, an explanation on how these Fzo clusters could lead to hyperconnected mito in C elegans and little round mito in yeast should be provided.

We improved the writing.

6.2: Since Fzo mislocalisation is critical for the phenotype, is this also true in C.elegans? Again, what is the speculation of Fzo clustering on its function? Do the clusters co-localise with ERMES in yeast?

We cannot do the experiment in *C. elegans* because to our knowledge nobody has constructed a FZO-1::GFP fusion. Anti-FZO-1 antibodies are available for the Conradt lab, but we did not manage to keep the mitochondrial network intact in body wall muscle cells upon fixation for whole-mount stainings. Yet, we checked for the co-localization of Mdm34 with Fzo1 in yeast. We could not find a significant co-localization between Fzo1 clusters and Mdm34 (the ERMES). Since these are negative results, we did not include them in the manuscript.

6.3: I am also a little puzzled by the statement that CDC48 acts in MAD and UPR. I was also under the impression that it also acts in ERAD (at least from Jeff Brodsky' s work and others).
Surprisingly, this is mentioned in the discussion but nowhere in the result section.

Done!

6.4: Vms1 is not introduced. What is this? This paragraph is not concluded.

We now introduce Vms1 properly and added a conclusion.

7) The authors then show that ER/mitochondria contacts (tethering) are not seemingly impaired using morphological evidences but also overexpressing the CHIMERA construct that have not effect on the arf mutant phenotype.

8) Then comes the MIRO/VDAC-1 part. Again, I fail to understand how the authors move from saying the ERMES is normally localized to actually investigate the function of ERMES components. A sentence such as " although ER to mitochondria connections are seemingly non-affected, we addressed their functionality as it is possible that they are affected in line with defects in mitochondrial function".
The data are convincing but again, what is the relationship between Arf and Miro? How can miro function be affected by Arf?
How does this relate to Fzo mislocalisation?
Again, an integrative model would be welcome.

We hope the model will make it clearer. We also modified the writing.

9) the mitophagy part is short but convincing.

Thank you!

10) Can the title be more specific, perhaps more mechanistic mentioning Fzo mislocalisation and CDC48/MAD and the function of ER/Mito sites.

We suggested a different title, but the editor felt the initial one was better. So we stayed the with initial title

11) The figures were either way too small or way too big. Difficult to print and/or to look at.

We apologize to the reviewers but this was probably due to the reduction of the file size during small pdf generation. In the final manuscript this should not be a problem.

Referee #2:

The manuscript by Ackema et al. is improved by the renewed focus on Arf1 and its GEF. The interplay with cdc48 in the regulation of fzo1 helps some, while cofractionation with the MAM and the effects on mitophagy in yeast make this paper very interesting. A lot of the observations are repeated in three systems, but unfortunately the effects on mitophagy are only studied in yeast. This is a little concerning, because some effects seem are different in the different organisms. Yeast Arf mutants have globular mitochondria, worms have hyper-connected mitochondria and mammals have swollen mitochondria with tubular extensions. I am not sure what this means or whether these are general phenomena. Many questions also remain regarding the mechanism, but I suppose that could be saved for a later paper.

Here are some suggestions for improvements in the text:

In abstract: I would not say due to a deficiency in lipid environment, because there is no evidence for or against this. Too speculative.

We agree with the reviewer and changed the abstract accordingly

Intro, p4: "mutation caused clustering/aggregation of Fzo1 at the restrictive temperature"
Should say: "mutation caused clustering/aggregation through the actions of Fzo1 at the restrictive temperature"

Done

Intro p5: I would not say a functional contribution in lipid and/or Ca transfer, since there is no evidence for or against this.

Done

Results p8. Knock down of COPB-1; how do you know it worked?

We perfomed qPCR (Former Fig. S7; now Fig. S2). In addition, the knockdown causes embryonic lethality.

P9: what is the redundancy with arf3 based on?

In yeast to mammals, different Arf proteins can act redundantly. In yeast Arf1 and Arf2 act redundantly, and in mammals Arf1 and Arf4 and Arf5 have overlapping functions. We added an explanation and the appropriate references.

P11: title: "arf1 is present on purified mitochondria" replace with "Arf1 cofractionates with ER and mitochondria"

Thank you for the suggestion. We changed the title accordingly.

P11: title bit of an overstatement: "GBF-1 and ARF-1 do not directly regulate mitochondrial fission dynamics" replace with "GBF-1 and ARF-1 deficiencies do not suppress fission or fusion defects"

Again we changed the title accordingly.

P12. It is tough to enhance the effects of drp1 or dnmm1 deletions, since those are very strong already. maybe better say: "As expected, ..."

We rephrased this part.

P14: what analysis revealed that the AAA-ATPase Cdc48 interacted specifically with Arf1 bound to GTP ? data not shown?

The differential Arf1 affinity chromatography selects for proteins that specifically interact with either Arf1-GTP or Arf1-GDP. We describe this better in the main text now.

3rd Editorial Decision 01 August 2014

Thank you for submitting your revised manuscript to us. I am happy with the way it is structured now. Could you please include the MS data for Cdc48-Arf1-GTP interaction in your final version of the manuscript as well as do the small textural amendments that we have discussed?

4th Editorial Decision 07 August 2014

I appreciate the introduced changes in the revised version of your manuscript and I am pleased to accept the manuscript for publication here.
Please see below for important information on how to proceed.

Thank you for contributing to the EMBO Journal.

