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ER-mitochondria contacts control GSC surface glycan expression and sensitivity to killer lymphocytes

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Corresponding author: Denis Martinvalet, University of Geneva

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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.)

1st Editorial Decision 25 September 2016

Thank you for the submission of your manuscript (EMBOJ-2016-95429) to The EMBO Journal. Your study has been sent to three referees, and we have received reports from all of them, which I copy below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major concerns that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In particular, referee #3 points out the need for you to consolidate your findings and the proposed concept by additional experiments on mitochondrial respiration, ATP production and Ca2+ control (ref #3, pts. 2, 3). Along these lines, referee #1 asks you to explore the role of the mito-ER tether in glioma stem cell killing (ref #1, pt. 1). In addition, referee #2 states that the mechanistic details underlying the presented phenotype are in his/her view rather unclear, and asks you to investigate involvement of fission-fusion and metabolic pathways (ref #2, third paragraph). In addition, all three referees list a number of technical issues and controls that would need to be addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and we are the in principle happy to invite you to revise your manuscript experimentally to address the referees' comments.

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REFEREE REPORTS

Referee #1:

This is an outstanding report of how ER-mitochondria contacts modulate a syalylated glycan expression on the surface of glioma stem cells. Bassoy et al convincingly show that a reduction in Mfn2 levels corresponds to a decrease in ER-mitochondria tethering and this correlates with a reduction in syalylated glycan expression in GSC. A key experiment of forcing ER-mitochondria tethering by means of an artificial tether did not alter mitochondrial length but restored tethering and increased the exposure of syalylated glycans, ultimately modulating their killing by CTL. The authors have performed a plethora of experiments to convincingly prove that glioma stem cells susceptibility to killing by CTL can be modulated by the ER-mitochondria interface.

In summary, this technical tour de force proposes a novel function for ER-mitochondria tethering and therefore is of wide interest for the community studying intracellular contacts as well as for researchers interested in cancer stem cells.

I have only one experimental request and some minor concerns
1. I believe that it would be important to investigate if the artificial ER-mitochondria tether has an effect on GSC killing. This experiment would nicely complement the Mfn2 evidence presented here

Minor concerns
1. the introduction is too verbose and diffuse and can be focused and shortened
2. I suggest that the authors concentrate on the main finding that ER-mitochondria tethering controls syalylated glycan expression and therefore refocus the writing of the paper. As it is right now, the uneducated reader gets confused as to what defines the effect, mitochondrial length or tethering to the ER
3. abstract shall also be refocused accordingly

Referee #2:

The study entitled "ER-mitochondria contacts control GSC surface glycan expression and sensitivity to killer lymphocytes" by Bassoy et al. is an investigation in vitro on the mechanisms responsible for the different sensibility to cytotoxic lymphocytes of glioma stem-like cells (GSC) versus glioma differentiated cells (GDC). These are two cell types of the same tumor, normally distinguished in different subtypes/stages according to their severity levels. The Authors, although not taking into account this distinction, found a correlation between the resistance to lymphocytes-induced cell death and the levels of plasma membrane glycan expression on tumoral cells. They further found that the exposure of glycans to the cell surface depends on mitochondrial dynamics and on their effects on ER-mitochondria contact sites. Alteration of these contact sites by genetically modulating the mitochondria shape, impinges somehow on the levels of glycans exposure and thus, on tumoral cell sensibility to the lymphocyte-induced cell death. These data were nicely and interestingly confirmed in patients' samples.

This is a very interesting work that highlights the possibility that ER-mitochondria contact sites might somehow be involved on a direct and new lipid/polysaccharide trafficking pathway targeted to the plasma membrane. However, this aspect needs to be more deeply investigated. The work is well performed/presented and original, even though there are a few weakness points that would need to be resolved, together with the mechanism behind the described phenomena.

The Authors, for example, interpret and discuss RNA-seq data that are not shown in the manuscript (see pag 9 for example). By contrast, these data should be deeply analyzed to appreciate the differences between GL261 or U251 and mNS or NSU251. For instance, Authors may use RNA-seq methods to evaluate the expression profile of all proteins involved in mitochondrial fission-fusion pathway (confirming previous results), and of most relevant metabolic enzymes or kinases, in order to clarify which pathways are behind the phenotype that they present.

The Authors often state that human and mouse cells lines show very diverse results in the

GSC/GDC comparison (for example in Ca2+ entry, polarization experiments or when they forced contact between ER and mitochondria): how do they explain these differences?

Moreover, Authors' results differ significantly from what has been previously presented in an another work by Hoa N et al. (J Immunol. 2010); in the Discussion section, the Authors state that the deep differences among the two works are due to different technical approaches: here cells are detached from the plastic support before performing the experiments, while in Hoa et al. adherent cells are analyzed still attached to the plastic support. On my opinion, the latter seems to be a more physiological approach, at least apparently. Could the Authors better comment or justify the reliability of the approach they chose and used?

Point by point:
- Fig 1, related to the previous comment, it is always complicated comparing two cell lines that grow in different type of mediums and present different morphologies; for example, in ICC figures, it is possible to observe that GSC are rounded while the GDC show a larger area. In this case, it is possible that the two cell lines could produce different amounts of actin in the given experimental conditions. I would recommend the Authors to use an alternative marker to normalize Western blots (a nuclear structural protein like Histone 3 for example).
- The Authors considered ATP content in specific culture conditions (for instance, when mentioning "....when NSU251 were grown on pyruvate, this did not influence ATP content"). Is it possible that different medium conditions do affect, indeed, any results?
- Fig 1M and O, could the Authors provide other ICC pictures that better reflect the quantification of mitochondrial length (as in Fig 7)?
- The Authors need to confirm the activation of cell death (critical point for the manuscript message) in another way, for example by checking the activation of PARP and/or Caspase 8.
- Are the differences shown in Fig 7A significant?
- Apparently some significant citations are missing at page 4, in the context of mitochondria intracellular relocation for different cell needs such as ATP supply.

Referee #3:

Bassoy et al. have investigated the relationship between mitochondrial dynamics and endoplasmic reticulum(ER)/mitochondria(mito) contacts in the regulation of the expression of sialylated glycans on the surface of glioma stem-like cells (GSC), which in turn would regulate the cell susceptibility to cytotoxic immune cells. GSC have shorter but more dynamic mitochondria, less ER-mito contact sites and lower expression of specific glycans. Authors proposed that mitochondrial dynamics and ER-mito contacts sites are responsible for the change in the expression of these glycans and thus of the sensitivity of the cells to immune effector cells. This potentially opens new directions for cancer therapy, which could target mitochondria and mitochondria/ER tethering. However Authors' hypothesis need to be further reinforced and investigated.

Major issues to address:
1. The main finding is based on gene silencing. Because of the potential off target effects, at least 2 different siRNA constructs have to be used.
2. Authors showed that mitochondrial respiration is impaired in GSC compared to their differentiated counterparts. However they did not test the possibility that impaired mito respiration and metabolism is relevant for the surface glycan phenotype. Authors should check for the effects on mito respiration and ATP production upon silencing of Drp1 in GSC.
3. ER-mito interactions seem to be reduced in GSC. This might impact on calcium coupling between organelles. Authors performed a few experiments on calcium handling shown in Fig S5, challenging cells with thapsigargin and 100uM Ca2+. Cytosolic calcium elevation in response to these stimuli is faster and higher (at least for mNs) in GSC that is plausible if ER/mito coupling is impaired. Furthermore baseline of cytosolic Ca2+ looks to be higher for GSC, indicating a defective calcium handling. However, the Authors discarded the possibility that GSC have distinctive Ca2+ storing properties; this should be further addressed, by specifically checking ER calcium discharging and mito uptake.
4. To restore mito morphology, authors silenced Drp1 in GSC cells. However in these cells Mfn2, a candidate to ER/mito tether, is downregulated as well. If the hypothesis addressed in this manuscript

is true, expression of Mfn2 should be more strikingly restoring glycan expression associated to differentiated cells, because of its double function in mito fusion and ER/mito tethering. Authors should perform this experiment accordingly.
Minor issues are listed below:
Introduction
Contains long description of previous literature that seems to be irrelevant for the present study. Also, selection of the references seems to be unfitting in several cases. For example, control of mitochondrial motility by Ca2+ was described in:

Yi M. et al. 2004 J Cell Biol; and Brough M. et al 2005 Biochem J
Fig. 1A
The presented images do not allow one to evaluate the mitochondrial elongation state. Please provide higher resolution images.
Fig. 1C
Please provide quantitative evaluation of the electron microscopy results.
Fig1. B&E y-axis
Please replace uM with um
Fig.1G
Authors used actin as loading control. Tom20 signal is decreased in the NSU251 lane. This suggests a decrease an overall decrease in mito proteins. Authors should introduce at least one mito marker as loading control in the figure 2. Since NSU251 cells have been shown to have defective mitochondrial respiration authors should also check for levels of expression of components of the electron transport chain.
Fig.1 M-P
Mito length is restored in GSC by silencing Drp1. Is organellar dynamics also affected? Fusion assay should be performed for this condition as well.
Fig.S1 E-G
It seems to be a conflict that NSU251 cells have very little respiratory activity but these cells can maintain ATP level when they switched from glucose to pyruvate.
Fig.S2B
Error bar is missing on the plot on the right side of the figure.
Fig.S6A, B
Authors should check for expression of alternative ER/mito tether candidates: IP3R/GRP75/VDAC1 complex (Szabadkai et al., 2006), Fis1 (Iwasawa et al., 2011) and PTPIP15/VAPS (De Vos et al., 2012)

1st Revision - authors' response 06 December 2016

A point by point reply to the reviewers follows.

Referee #1:
This is an outstanding report of how ER-mitochondria contacts modulate a syalylated glycan expression on the surface of glioma stem cells. Bassoy et al convincingly show that a reduction in Mfn2 levels corresponds to a decrease in ER-mitochondria tethering and this correlates with a reduction in syalylated glycan expression in GSC. A key experiment of forcing ER-mitochondria tethering by means of an artificial tether did not alter mitochondrial length but restored tethering and increased the exposure of syalylated glycans, ultimately modulating their killing by CTL. The authors have performed a plethora of experiments to convincingly prove that glioma stem cells susceptibility to killing by CTL can be modulated by the ER-mitochondria interface.

In summary, this technical tour de force proposes a novel function for ER-mitochondria tethering and therefore is of wide interest for the community studying intracellular contacts as well as for researchers interested in cancer stem cells.
I have only one experimental request and some minor concerns

1. I believe that it would be important to investigate if the artificial ER-mitochondria tether has an effect on GSC killing. This experiment would nicely complement the Mfn2 evidence presented here

We thank this reviewer for his appreciation of our work and for his suggestion. OMM-ER artificial tether indeed protects the GSC from cytotoxic lymphocytes killing. These new results have been added to the new fig3.

Minor concerns

1. the introduction is too verbose and diffuse and can be focused and shortened
2. I suggest that the authors concentrate on the main finding that ER-mitochondria tethering controls syalylated glycan expression and therefore refocus the writing of the paper. As it is right now, the uneducated reader gets confused as to what defines the effect, mitochondrial length or tethering to the ER
3. abstract shall also be refocused accordingly

We also thank the reviewer for his suggestion. We have shortened and re-centered the introduction and abstract on our main finding.

Referee #2:

The study entitled "ER-mitochondria contacts control GSC surface glycan expression and sensitivity to killer lymphocytes" by Bassoy et al. is an investigation in vitro on the mechanisms responsible for the different sensibility to cytotoxic lymphocytes of glioma stem-like cells (GSC) versus glioma differentiated cells (GDC). These are two cell types of the same tumor, normally distinguished in different subtypes/stages according to their severity levels. The Authors, although not taking into account this distinction, found a correlation between the resistance to lymphocytes-induced cell death and the levels of plasma membrane glycan expression on tumoral cells. They further found that the exposure of glycans to the cell surface depends on mitochondrial dynamics and on their effects on ER-mitochondria contact sites. Alteration of these contact sites by genetically modulating the mitochondria shape, impinges somehow on the levels of glycans exposure and thus, on tumoral cell sensibility to the lymphocyte-induced cell death. These data were nicely and interestingly confirmed in patients' samples. This is a very interesting work that highlights the possibility that ER-mitochondria contact sites might somehow be involved on a direct and new lipid/polysaccharide trafficking pathway targeted to the plasma membrane. However, this aspect needs to be more deeply investigated. The work is well performed/presented and original, even though there are a few weakness points that would need to be resolved, together with the mechanism behind the described phenomena. The Authors, for example, interpret and discuss RNA-seq data that are not shown in the manuscript (see pag 9 for example). By contrast, these data should be deeply analyzed to appreciate the differences between GL261 or U251 and mNS or NSU251. For instance, Authors may use RNA-seq methods to evaluate the expression profile of all proteins involved in mitochondrial fission-fusion pathway (confirming previous results), and of most relevant metabolic enzymes or kinases, in order to clarify which pathways are behind the phenotype that they present.

We thank reviewer #2 for his appreciation of our work and for his comment. We have thoroughly analyzed the RNAseq data and performed different heat maps and principal component analyses to investigate whether there were pathways obviously dysregulated between mNS and GL261. However no pathway was obviously up or downregulated even if some discrete genes had their expression up or down regulated. Nevertheless, we gave a WB analysis of the expression of the key genes involved in mitochondrial shaping (Figure 1G and I). As suggested later by the reviewer we complemented this by adding the histone H3 as an additional loading control. Using the histone H3 as control did not change the densitometry data, we therefore kept the previous dataset using actin normalization.

We also looked at the mRNA expression of the metabolic enzyme involved in glycolysis, gluconeogenesis, pentose phosphate pathway, TCA cycle, fatty acid β-oxidation and fatty acid synthesis. We added these results for the reviewers' attention only. There was no obvious difference between GDC and GSC. This is agreement with our data showing that both human and mouse pairs of GDC/GSC could maintain their ATP pool both on glu or pyruvate condition (Fig S1G and H). There was also no difference in the expression pattern on the respiratory chain subunits tested (Fig S1 I and J), nor in the expression of the restricted set of metabolic enzymes we tested (Fig S1 K and L). These results are also in agreement with the fact that there was no difference in the ability of the

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GDC and GSC to produce the glycan. In fact our lectinship data clearly showed that both GL261 and mNS have similar expression of the glycans recognized by 46 different lectins. This strongly indicates that it is more a defect in their externalization.

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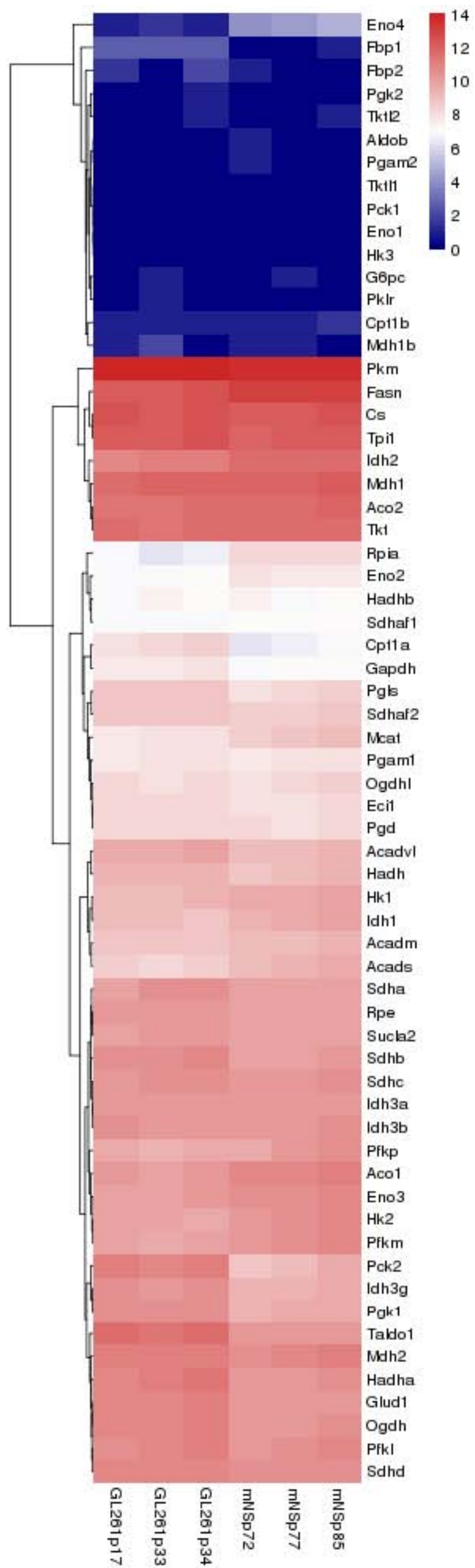

Heatmap representation of the expression profile of the metabolic enzyme involved in glycolysis, gluconeogenesis, pentose phosphate pathway, TCA cycle, fatty acid βoxidation and fatty acid synthesis

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The Authors often state that human and mouse cells lines show very diverse results in the GSC/GDC comparison (for example in Ca2+ entry, polarization experiments or when they forced contact between ER and mitochondria): how do they explain these differences?

We agree with the reviewer that both models were not identical to each other. We think that this could be due to interspecies differences and as the reviewer stated earlier because the two pairs GDC/GSC are not exactly the same stage of the tumor. We have further stressed this point in the results section. Note that this variation between the two models helped us to focus on the common determinant to explain the CTL susceptibility trait that was shared between the two models and the early passage samples.

Moreover, Authors' results differ significantly from what has been previously presented in an another work by Hoa N et al. (J Immunol. 2010); in the Discussion section, the Authors state that the deep differences among the two works are due to different technical approaches: here cells are detached from the plastic support before performing the experiments, while in Hoa et al. adherent cells are analyzed still attached to the plastic support. On my opinion, the latter seems to be a more physiological approach, at least apparently. Could the Authors better comment or justify the reliability of the approach they chose and used?

We thank the reviewer for this comment. As we stated in our manuscript we harvest the GDC (as well as the multicellular GSC spheres) using accutase in order to have the both GSC and GSC floating condition to face the CTL as it is technically easier for the killing assay. The contrary meaning having both GDC and GSC to adhere is not possible since as soon as GSC adhere they start to differentiate into GDC. Moreover comparing the killing of adherent GDC and floating GSC seem to empirically add more variables to the system that is already fairly complicated. So we chose the suspension conditions for both in.

Point by point:
-Fig 1, related to the previous comment, it is always complicated comparing two cell lines that grow in different type of mediums and present different morphologies; for example, in ICC figures, it is possible to observe that GSC are rounded while the GDC show a larger area. In this case, it is possible that the two cell lines could produce different amounts of actin in the given experimental conditions. I would recommend the Authors to use an alternative marker to normalize Western blots (a nuclear structural protein like Histone 3 for example).

We have added histone H3 loading control to fig 1G and I. This did not change the quantification so we kept the initial results.

-The Authors considered ATP content in specific culture conditions (for instance, when mentioning "....when NSU251 were grown on pyruvate, this did not influence ATP content"). Is it possible that different medium conditions do affect, indeed, any results?

Actually, it is possible. Our results showed that NSU251 barely respire in the steady state condition, as they seem to be more glycolytic. However this does not mean that they cannot respire when forced to do so like in the pyruvate condition. This is in agreement with the apparently functional respiratory chain Fig S1I and J.

-Fig 1M and O, could the Authors provide other ICC pictures that better reflect the quantification of mitochondrial length (as in Fig 7)?

We added an extended view in the support material.

-The Authors need to confirm the activation of cell death (critical point for the manuscript message) in another way, for example by checking the activation of PARP and/or Caspase 8.

We thank the reviewer for this comment and kindly remind him/her that in Fig S7D we have shown that these cells are sensitive to granzyme B and perforin killing which is the best way to test the functionality of the GDC and GSC apoptotic machinery.

-Are the differences shown in Fig 7A significant?

Yes the differences shown are very significant. We have added the p values stars.

-Apparently some significant citations are missing at page 4, in the context of mitochondria intracellular relocation for different cell needs such as ATP supply.

We apologize for missing some of the important references. As also suggested by reviewer 3 we added the Yi M. et al. 2004 J Cell Biol; and Brough M. et al 2005 Biochem J in the citation.

Referee #3:

Bassoy et al. have investigated the relationship between mitochondrial dynamics and endoplasmic reticulum(ER)/mitochondria(mito) contacts in the regulation of the expression of sialylated glycans on the surface of glioma stemlike cells (GSC), which in turn would regulate the cell susceptibility to cytotoxic immune cells. GSC have shorter but more dynamic mitochondria, less ER-mito contact sites and lower expression of specific glycans. Authors proposed that mitochondrial dynamics and ER-mito contacts sites are responsible for the change in the expression of these glycans and thus of the sensitivity of the cells to immune effector cells. This potentially opens new directions for cancer therapy, which could target mitochondria and mitochondria/ER tethering. However Authors' hypothesis need to be further reinforced and investigated. Major issues to address:

1. The main finding is based on gene silencing. Because of the potential off target effects, at least 2 different siRNA constructs have to be used.

We thank the reviewer for the point. We opted for a rescue experiment by re-expressing Drp1 or MFN2 in the knock down background. We have added these controls as additional figures FigS9 and Fig S10 respectively.

2. Authors showed that mitochondrial respiration is impaired in GSC compared to their differentiated counterparts. However they did not test the possibility that impaired mito respiration and metabolism is relevant for the surface glycan phenotype. Authors should check for the effects on mito respiration and ATP production upon silencing of Drp1 in GSC.

We thank the reviewer for this comment. As suggested by the reviewer we have tested the respiration and ATP content of the DRP1sh GSC. This new data was added in new figure Fig S2C-F. Knockdown of DRP1 did not significantly alter the respiration rate and the ATP content of the GSC. This could be explained by the fact that this knock down only increases the mitochondrial length by less than two fold, probably not enough to significantly impact on the respiration rate as one could expect higher respiration rate for longer tubular mitochondria. However this result further confirms that the difference in glycan surface glycan expression is not due to metabolic difference. We are taking this opportunity to kindly remind the reviewer that actually in the data reported two very strong piece of evidence further support the fact that we can safely exclude a metabolic defect in the differential glycan surface expression. First, the lectinship data suggest that both GDC and GSC are equally capable of producing the different branched glycans (Fig S4F). Second GDC/GDC MFN2sh and vice versa GSC/GSCDRP1sh pairs are grown in similar culture condition and the differential glycan expression depends on the status of mitochondrial morphology (Fig1u and V and Fig 8D). Together these evidence exclude metabolic differences and difference in culture condition.

3. ER-mito interactions seem to be reduced in GSC. This might impact on calcium coupling between organelles. Authors performed a few experiments on calcium handling shown in Fig S5, challenging cells with thapsigargin and 100uM Ca2+. Cytosolic calcium elevation in response to these stimuli is faster and higher (at least for mNs) in GSC that is plausible if ER/mito coupling is impaired. Furthermore baseline of cytosolic Ca2+ looks to be higher for GSC, indicating a defective calcium handling. However, the Authors discarded the possibility that GSC have distinctive Ca2+ storing properties; this should be further addressed, by specifically checking ER calcium discharging and mito uptake.

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We thank the reviewer for this comment. Indeed we have tested the ability of GSC mitochondrial calcium uptake upon ER discharged. We took advantage of the mitochondrial targeted calcium sensor 4mitD3CPV. We observed that NSU251 and mNS expressing 4mitD3CPV mitochondria uptake less calcium upon stimulation with ATP and carbachol compared to the GDC counterpart U251 and GL261. This further supports our results of less contact between ER and mitochondria in GSC. This new data was added in new fig 2F.

4. To restore mito morphology, authors silenced Drp1 in GSC cells. However in these cells Mfn2, a candidate to ER/mito tether, is downregulated as well. If the hypothesis addressed in this manuscript is true, expression of Mfn2 should be more strikingly restoring glycan expression associated to differentiated cells, because of its double function in mito fusion and ER/mito tethering. Authors should perform this experiment accordingly.

We thank the reviewer for this comment. Unfortunately we could not obtain GSC over-expressing MFN2 and therefore we were unable to perform this experiment. Troubleshooting the condition to get MFN2 expression in GSC will require much intense work. We apologize for not being able to test this hypothesis.

Minor issues are listed below:
Introduction Contains long description of previous literature that seems to be irrelevant for the present study. Also, selection of the references seems to be unfitting in several cases. For example, control of mitochondrial motility by Ca2+ was described in: Yi M. et al. 2004 J Cell Biol; and Brough M. et al 2005 Biochem J.

We have shortened the introduction. We apologize for missing the references that have now been added.

Fig. 1A The presented images do not allow one to evaluate the mitochondrial elongation state. Please provide higher resolution images.

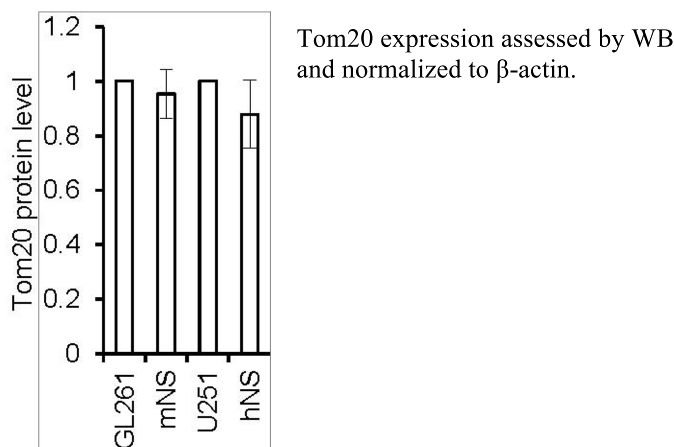
We provided higher resolution of these images as supporting information.

Fig. 1C Please provide quantitative evaluation of the electron microscopy results. Fig1. B&E y-axis Please replace uM with um

We fixed the y-axis legends

Fig.1G Authors used actin as loading control. Tom20 signal is decreased in the NSU251 lane. This suggests a decrease an overall decrease in mito proteins. Authors should introduce at least one mito marker as loading control in the figure 2. Since NSU251 cells have been shown to have defective mitochondrial respiration authors should also check for levels of expression of components of the electron transport chain.

As suggested by reviewer#2 we added histone H3 as an additional loading control which did not change the quantification.
For the reviewer intention only we provide the quantification of Tom20 for the different experiments performed.



Tom20 expression assessed by WB and normalized to β-actin.

As the reviewer can see the there is no significant difference in tom20 expression between the GDC and GSC.

Fig.1 M-P Mito length is restored in GSC by silencing Drp1. Is organellar dynamics also affected? Fusion assay should be performed for this condition as well.
We thank the reviewer for this suggestion however in the time frame of this revision we could not test this hypothesis as it would take more intense investigation to get conclusive results. We apologize for this.

Fig.S1 E-G It seems to be a conflict that NSU251 cells have very little respiratory activity but these cells can maintain ATP level when they switched from glucose to pyruvate.

As explained to reviewer #2 the fact that NSU251 do not respire at steady state does not mean they cannot respire when forced to, like in pyruvate condition. This explained why they are still able to maintain their ATP content under pyruvate. Moreover this agrees with an apparently intact respiratory chain as shown in Fig S1I and J.

Fig.S2B Error bar is missing on the plot on the right side of the figure. Fig.S6A, B Authors should check for expression of alternative ER/mito tether candidates: IP3R/GRP75/VDAC1 complex (Szabadkai et al., 2006), Fis1 (Iwasawa et al., 2011) and PTPIP15/VAPS (De Vos et al., 2012)

We thank the reviewer for this suggestion. We have added IP2R, GRP75, Fis1 PTPIP15 in new FigS6E and F.

2nd Editorial Decision 04 January 2017

Thank you for submitting your revised manuscript for consideration by the EMBO Journal, and your patience with our response at this time of the year. Your revised study has now been re-evaluated by the three referees whose comments are enclosed. As you will see, the third referee remains overall more critical on the study than the two others, however we decided - in light of the strong support of the latter - to give you the opportunity to revise your manuscript to address the referees' points.

Both referee #1 and referee #2 find that their concerns have been sufficiently addressed and are in favour of publication, pending satisfactory minor revision regarding language. Please note that while referee #2's concern on more mechanistic details regarding regulation of glycan surface expression is per se well taken, we editorially judge more experiments not needed at this point for the current manuscript. Referee #3 states, that your claims on direct causality between cell death and glycan surface are not sufficiently well supported by the current data (ref #3, pt. 1). This referee also points out that there is a need for you to revise your data on Grp75 and Park7 levels, which in his/her view are not conclusive (ref #3, pt. 2), as well as to revise statistical representation of your new findings (ref #3, pt. 3). Please note, however, that taking into account the positive comments of referee #1 and referee #4, we have decided that pending a satisfactory revision, we would go ahead with acceptance of this manuscript as soon as possible. Thus, I ask you to revise your manuscript regarding the points raised by referee #3 and evaluate, whether you would be able to add complementary data, or, alternatively, relativise your statements and introduce caveats where appropriate. I also ask you to revise the language throughout the manuscript, as alerted to by referee #2.

Please contact me if you have any questions, need further input on the referee comments or if you anticipate any problems.

Please see below for some formal formatting issues, which need to be adjusted at re-submission. In addition we request a author checklist with information on

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your final revision.

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REFEREE REPORTS

Referee #1:

I was already positive after seeing the first version of this manuscript and I am even more now. The authors did a great job in addressing the comments by me and by the other reviewers. In particular I find the experiments with the artificial tether compelling. This paper will be a milestone opening new avenues on how mitochondria-ER contact sites control surface glycan expression.

Referee #2:

The Authors addressed most of the major points pointed out by the reviewers and rather ameliorated the manuscript. Anyway, the identification of the mechanism that regulates the surface expression of glycans through modulation of ER-mitochondria contact-sites is still missing. I am still convinced that the elucidation of the specific mechanism would deeply ameliorate the work. A final revision of the language should be required.

Referee #3:

After revision, the manuscript by Bassoy et al more convincingly supports a role for ER-mitochondria contacts in the control GSC surface glycan expression and sensitivity to killer lymphocytes. The new experiments on mitochondrial matrix [Ca2+] in stem and differentiated cells also add a new mechanistic clue to the phenotype.

The Authors effort to perform additional studies is appreciated but some of the new experiments don't measure the relevant parameter or aren't presented appropriately:

1. The Authors opted to validate a role for Drp1- and Mfn2-mediated shaping of the mitochondria in the surface glycan expression by performing rescue studies in siRNA-treated conditions. However, only the siRNA experiments shown in Fig1U&V include measurements of the surface glycans whereas the rescue shown in supplementary Fig9&10 only shows cell death. Therefore, the pathway through the surface glycans remains to be established.

2. Figure S6E and F: Grp75 signal is increased in glioma stem cells. Levels of Park7 and PISD look also different. Authors should provide quantification and describe the results in the text and discuss their potential relevance for the proposed pathway. For Grp75, a possible function could be to restore the ER/mito distance.

3. The mitochondrial [Ca2+] data are based on 2 experiments and so the statistical reliability of the stated difference is unclear. As a minimum, the results of obtained in the individual experiments have to be shown with distinctive symbols so the Reader can see whether the pattern indicated by the average was reproducible in each experiment.

2nd Revision - authors' response 22 January 2017

A point-by-point reply to the reviewers follows.

Referee #1:

I was already positive after seeing the first version of this manuscript and I am even more now. The authors did a great job in addressing the comments by me and by the other reviewers. In particular I find the experiments with the artificial tether compelling. This paper will be a milestone opening new avenues on how mitochondria-ER contact sites control surface glycan expression.

We thank the reviewer for his appreciation of the improvement made in this manuscript.

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Referee #2:

The Authors addressed most of the major points pointed out by the reviewers and rather ameliorated the manuscript. Anyway, the identification of the mechanism that regulates the surface expression of glycans through modulation of ER-mitochondria contact-sites is still missing. I am still convinced that the elucidation of the specific mechanism would deeply ameliorate the work. A final revision of the language should be required.

We thank this reviewer for his comments. We have stressed that further investigation is required for the full understanding of the process investigated in this work. We have also thoroughly revised the language of the manuscript.

Referee #3:

After revision, the manuscript by Bassoy et al more convincingly supports a role for ER-mitochondria contacts in the control GSC surface glycan expression and sensitivity to killer lymphocytes. The new experiments on mitochondrial matrix [Ca2+] in stem and differentiated cells also add a new mechanistic clue to the phenotype. The Authors effort to perform additional studies is appreciated but some of the new experiments don't measure the relevant parameter or aren't presented appropriately:

1. The Authors opted to validate a role for Drp1-and Mfn2-mediated shaping of the mitochondria in the surface glycan expression by performing rescue studies in siRNA-treated conditions. However, only the siRNA experiments shown in Fig1U&V include measurements of the surface glycans whereas the rescue shown in supplementary Fig9&10 only shows cell death. Therefore, the pathway through the surface glycans remains to be established.

We thank the reviewer for this comment. We wish to clarify the fact that, the rescue experiment is intended to show the specificity of the knockdown for the phenotype observed. We showed that sh DRP1 increased the mitochondrial length, which resulted in the protection from cytotoxic killer cells most likely by the alteration of the surface glycan expression. Our rescues experiments showed that re-expression of DRP1 in the DRP1sh background restored the initial mitochondria length and restored the initial sensitivity to killer lymphocytes indicating that the effect observed was not due to some off target effect. This is just a control experiments. With more time and space we could have also check all the other parameters such glycan surface expression, conjugate formation and cytotoxic granule polarization frequency and time.

2. Figure S6E and F: Grp75 signal is increased in glioma stem cells. Levels of Park7 and PISD look also different. Authors should provide quantification and describe the results in the text and discuss their potential relevance for the proposed pathway. For Grp75, a possible function could be to restore the ER/mito distance.

We thank the reviewer for this observation, the expression of Grp75 and Perk7 is mildly increased. This mild increase could be a compensation mechanism to restore some ER-mitochondria contacts function such as mitochondria capacity to uptake calcium in the GSC. We have had a sentence in this sense in the discussion section of the manuscript.

3. The mitochondrial [Ca2+] data are based on 2 experiments and so the statistical reliability of the stated difference is unclear. As a minimum, the results of obtained in the individual experiments have to be shown with distinctive symbols so the Reader can see whether the pattern indicated by the average was reproducible in each experiment.

We thank the reviewer for these comments. Of course the more repeats the stronger the data. We have had on sentence to stress this point in the result section.

3rd Editorial Decision 26 January 2017

Thank you for sending us the revised version of your manuscript.

I am pleased to inform you we judge most of the remaining referee's concerns were satisfactorily addressed. Thus we would in principle like to proceed with accepting your manuscript for publication in the EMBO Journal as soon as possible.

However, we would still need you to take care of some minor formatting changes and textual adjustments, which need to be considered:

>> Figure 4D, G., 5E, H, 6A, D, G, J - labeling needs to be enlarged to be readable.

>> Scale bars should be removed for all but one image in series in Fig.4D,G Fig.5E,H; Fig6A,D,G,J

>> please move qPCR primer information to the appendix and provide an updated appendix text file.

>> Appendix Fig S5 is missing scale bars. Please provide a revised .pdf file for this figure.

Please approach us at any point should you have additional questions related to these points.

Also please provide a graphical synopsis figure as well as highlight bullet points summarizing the main conclusions of the work.

Looking forward to the final revised version of your manuscript.

3rd Revision - authors' response 27 January 2017

Authors made requested editorial changes.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Denis Martinvalet

Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-95429R1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Question	Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We used standard cell based cytotxic assays with at least tree independent repeats.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA no animal studies.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA no animal studies.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA no animal studies.
For animal studies, include a statement about randomization even if no randomization was used.	NA no animal studies.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA no animal studies.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA no animal studies.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Statistical significance has been calculated by a two-tailed or one-tailed Student t-test between samples and P values are indicated in the legends.
Is there an estimate of variation within each group of data?	no
Is the variance similar between the groups that are being statistically compared?	not tested

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Rabbit polyclonal anti-MFN1 clone H65, sc-50330; Goat polyclonal anti-Eif2ak3/PERK, clone C16, sc-9481;Mouse monoclonal anti-DJ-1/Park7 clone A9), sc-55573; Rabbit polyclonal anti-HSP60 H-300, sc-13966; Rabbit polyclonal anti-PISD E15, sc-86197; Rabbit polyclonal anti-Tom40, H300, sc-11414 (Santa. Cruz Biotecnology); Mouse monoclonal anti-Tom20 clone 4F3 MCA4300Z (AbD Serotec); Mouse monoclonal anti-MFN2 clone 4H8, H00009927-M03 (Abnova); Mouse monoclonal anti-OPA1 clone 18/OPA1, 612607; Mouse monoclonal anti-DLP1 clone 8/DLP1, 611113 (BD Biosciences); Mouse monoclonal anti-β-actin (Sigma); Rabbit poly clonal anti BAP31, GTX111939 (Genetex); anti-mouse CD86 clone GL1; anti-mouse CD80 (B7-1); anti-mouse MHC Class-I; anti-mouse CD54 clone 3E2, 553252; anti-human; CD86 clone IT2.2, 555663; anti-human CD80 clone L307.4, 557223; anti-human HLA-ABC, 562006 and MICA/B from BD biosciences.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell line have been screened for mycoplasma and tested as negative. U251 cells were from the European Collection of Cell Cultures (ECACC). U251 and GE904 pairs were characterized by HLA typing at the DRB1 locus at 4 digit-resolution to confirm the same origin of the GSC/GDC pairs.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	
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G- Dual use research of concern

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