EMBO reports

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Endoplasmic reticulum chaperone Gp96 controls actomyosin dynamics and protects against pore-forming toxins

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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 07 July 2016

Thank you for the submission of your manuscript to EMBO reports. I apologize for the delay in getting back to you; we have only now received the final referee report, and all reports are copied below. I am sorry to say that the evaluation of your manuscript is not a positive one.

As you will see, while referee 2 and 3 acknowledge the potential interest of your findings, referee 1 is clearly not convinced about the data and the conclusions drawn from it. And although referee 2 is slightly more positive about the imaging data and suggests quantification to support the conclusions drawn, also this referee points out that the localization, redistribution or enrichment of proteins in many instances is unclear and not readily apparent from the images shown. Both, referee 1 and 2 indicated in the summary evaluation sheet returned with their reports that the technical quality is low/unacceptable. Referee 3 suggests further in vivo experiments to demonstrate that blebbing is not an artifact of cell culture.

Given these concerns, the amount of work required to address them, the uncertain outcome of these experiments, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from a majority of referees, I am sorry to say that we cannot offer to publish your manuscript.

I am sorry to disappoint you on this occasion, and hope that the referee comments will be helpful in your continued work in this area.

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REFEREE REPORTS

Referee #1:

The authors have identified the endoplasmic reticulum chaperone Gp96 as a novel regulator of plasma membrane blebbing following the attack by pore-forming toxins (listeriolysin, aerolysin, -hemolysin, streptolysin O). They show that Gp96 interacts with cortical non-muscle myosin (NMHCIIA) networks. Downregulation of Gp96 leads to an increase in membrane permeability and blebbing, and gives rise to epithelial-mesenchymal transformation.
The authors have investigated Gp96 and NMHCIIA interacting partners, their role during cell migration and in zebrafish larvae. They conclude that Gp96 is required for cellular protection against injury by pore-forming toxins and bacterial invasion by Listeria monocytogenes.

Critique: This study contains a wealth of experimental data. As confirmed before and now by Mesquita et al., there is no doubt about the formation of subcortical acto-myosin networks or ER-cisternae vacuolation after toxin-induced membrane injury, yet the question remains: are those structural manifestations related and do they act conjointly in the defense against plasma membrane damage?

This reviewer is not convinced by the confocal data. Neither the antibody labelling for Gp96/NMHCIIA (Fig 1D,E) nor the mCherryGp96/KDELGFP videos (Fig 2B, movies, 2,3,4) show more than a spurious colocalization and are more characterized by a general lack of focus.
In Fig. 3G and movies 5,6,7 which show blebbing cells, the confocal image is blurred - in contrast to the DIC sequence which is in focus. Since DIC is not a confocal technique, it is likely that the myosin translocation and the membrane movement are not in the same focal plane. HeLa cells have a particularly thin and extended cytoplasm, in this cell type, blebs therefore develop above the focal plane of the acto-myosin cytoskeleton.

Plasma membrane injury and consequently the influx of Ca2+ lead to the reorganisation of many membrane components and to the redistribution of cytoplasmic molecules. The observed colocalization of NMHCIIA and LAMP1, actin and calpain-2, NMHCIIA and ERK or NMHCIIA and -tubulin (Fig 4A) are by no means convincing since the images are of poor quality. Their specific "recruitment" is not corroborated by experimental data.

Referee #2:

Summary: Here the authors investigate the molecular mechanisms that protect cells against plasma membrane damage by bacterial pore forming toxins. Previous studies suggested that infection with Listeria monocytogenes, which produces the pore forming toxin LLO, causes redistribution of the endoplasmic reticulum (ER) chaperone Gp96 to the plasma membrane. Based on this, and the known involvement of ER pathways in the response to pore forming toxins, the authors investigated the role of Gp96 in the cellular response to LLO. They found that LLO triggers the interaction of Gp96 with non-muscle myosin heavy chain IIA (NMHCIIA). Moreover, LLO triggers the formation of cortical bundles containing NMHCIIA in association with plasma membrane blebs and Gp96-positive structures. Gp96 expression is important for NMHCIIA bundle formation and for reducing blebbing and increasing bleb retraction. Gp96 is also important in uninfected cells for regulating stress fiber formation and cell migration. Both Gp96 and NMHCIIA are important for preserving plasma membrane integrity upon LLO expression or Listeria monocytogenes infection. Finally, the authors use zebrafish as a model system to show that Listeria monocytogenes infection also promotes the interaction of Gp96 with NMHCIIA, and that Gp96 plays a role in enhancing survival following bacterial infection.

Major points:
Overall this is a nice study that sheds light on the mechanisms that protect cells from pore forming

toxins, and reveals connections between the ER chaperone Gp96 and NMHCIIA that are important in protecting against membrane damage in infected cells and animals. The paper is a good candidate for publication if the authors can address the specific points below.

Specific points:
1. Figures 1 and 2: The authors should attempt to quantify the association of Gp96 vacuoles with the cell periphery, membrane blebs, and NMHCIIA bundles in LLO treated cells. It seems that Gp96 vacuoles are associated with these structures in LLO-treated but not untreated cells, but quantification of this result would lend support to these conclusions. Also, in the Introduction, the authors mention that Gp96 redistributes to the plasma membrane in LLO-treated cells, however that is no apparent from the images. This should be explained.

2. Figure 2A and Video 1: The color scheme for showing NMHCIIA is confusing as it makes it look like more than one protein is being visualized. A single color might be sufficient. Moreover, in the video, it seems that NMHCIIA is highly overexpressed, so that the bundles in the blebs are not readily apparent. It might be worth to image cells expressing lower amounts of NMHCIIA so that bundles can be more readily seen over the background fluorescence.

3. Figure 4A: The enrichment of most of these markers with NMHCIIA bundles in cellular blebs is not really clear. Most of the markers appear to be everywhere in the cell, and many are not particularly enriched in near the NMHCIIA structures. Perhaps quantification of these images would provide better support for the conclusions.

4. Figure 7D, E: It would be helpful to also show in Figure 7 the results for the delta-hly mutant alone, in the absence of the Gp96 morpholino (this is currently shown in the supplementary material and could be moved to the main figure).

5. The connections between Gp96, NMHCIIA and blebbing in the protection against membrane damage are somewhat confusing. In particular, blebbing is proposed to be a protective mechanism against pore forming toxins. However, Gp96 and NMHCIIA silencing, which both protect against toxins, have opposing effects on blebbing. Gp96 silencing enhances blebbing and NMHCIIA silencing inhibits blebbing. This suggests that the connections between Gp96, NMHCIIA and blebbing are more complicated than the authors acknowledge. This should be discussed in the paper.

Referee #3:

This is an excellent piece of work that thoroughly examines the molecular association of GP96 with NMHCIIA during L. monocytogenes infections and identifies LLO as the trigger for their enhanced association. Although most experiments are conducted in HeLa cells (likely due to the ease of the studies with that cell line) crucial experiments are validated in CaCo-2 cells and with another toxin that shows similar effects. The authors attempt to go to the in vivo system to validate their findings.

Major comments:
Fig S2F. The Macrophage LLO treated data is weak as there appears to be essentially no Gp96 in the NMHCIIA zone and the increased concentrations of NMHCIIA may be because the cells appear to round-up more than the untreated cells (there are definitely fewer projections in the LLO treated cells). I actually think that the macrophage data is not really needed for the paper and if the authors wanted to remove it I wouldn't have a real problem with that.

The identification of comet tails in vivo is not crucial for the study, but the authors mention they are present in Fig 7 and I can't see any. Yes, there are actin concentrations, but clear comet tails are lacking.

I don't think that the identification of the KDEL puncta go far enough to demonstrate the blebbing events described throughout the manuscript. The ultimate confirmation of the blebbing events not being an artifact of cell culture would be to see NMHCIIA-based blebs in vivo. Also, the hly in vivo survival work (Fig 7C) is not that surprising as there are much less bacteria in the animals and those bacteria would presumable not be able to replicate or spread very well as they would theoretically be contained within a vacuole. If the point was to just show that zebrafish could be

used for in vivo analysis that might be best put into another manuscript.

Minor comments:
Pg 3. "NMII activity is required to PFT-induced PM blebbing" should read "NMII activity is required for PFT-induced PM blebbing"
Fig S4. All of the lanes do not appear to be necessary as some were not knocked-down. Just include the cell lines that were pursued.

1st Author Response - appeal 18 July 2016

Thank you for the interest you demonstrated towards our manuscript submitted to EMBO reports (Manuscript ID # EMBOR-2016-42833V1).

We are grateful to all referees for carefully revising our manuscript and providing suggestions that will strengthen our work. However we are disappointed and surprised to not have the opportunity to resubmit. We believe that, should we be given the opportunity to address the issues raised, we can submit a significantly improved manuscript showing that the ER chaperone Gp96 is a key regulator of cytoskeletal rearrangements taking place at sites of plasma membrane blebbing thus contributing to recovery plasma membrane integrity upon bacterial pore-forming toxins attack.

Most of the criticism originated from Referee#1. This referee pointed to the poor quality of our microscopy images. We consider that such problems can be rapidly overcome (details below). Referee#2 and Referee#3 appeared very positive ("overall this is a nice study...the paper is a good candidate for publication" and "This is an excellent piece of work..." respectively). Given that we have already gathered a significant amount of data required to respond to the issues raised by these two referees (details below), we are confident we can revise our manuscript in 2 months and therefore meet the criteria for publication in EMBO Reports.

Referee#1

This referee is not convinced by data on:

∑ Figs 1D and E: We will provide images where the colocalization of Gp96 and NMHCIIA is more evident, and importantly show quantifications (Pearson's coefficient). We already have these data.

∑ mCherryNMHCIIA/KDELGFP (Fig 2B, movies 2, 3, 4): The referee mentions mCherryGp96 but we believe he/she refers to mCherryNMHCIIA. This movie shows that Endoplasmic Reticulum (ER) vacuolation, plasma membrane (PM) blebbing and NMHCIIA rearrangements occur simultaneously, and support other data in our manuscript that demonstrate the recruitment of ER to cortical NMHCIIA bundles. We agree that the quality of the imaging (in particular the mCherry NMHCIIA channel) can be improved. We are currently establishing better quality movies.

∑ Fig 3G and movies 5, 6, 7: We believe that this referee missed the related technical description. The figure/movies do not show individual confocal sections, but instead they show projections of the total thickness of the cells "data sets with 0.5 m steps (in Exp. Proc.) and 'Fluorescence image corresponds to Z-stack' in Fig legend. Thus NMHCIIA-bundles clearly accumulate at PM blebbing areas. In a revised version we will revise the technical description.

∑ Fig 4A: Together with the Gp96 IP upon Wnt5A treatment which demonstrates NMHCIIA-Gp96 association, this figure aims to point further similarities between LLO-induced cortical structures and uropod-like structures. The examples provided show the presence of uropod components in LLO-induced cortical structures, and we propose to provide new images accompanied by quantifications as suggested by Referee #2.

Referee#2

∑ Figs 1 and 2: We will provide the quantifications proposed by the referee in support to the images and conclusions.
∑ Concerning Gp96 redistribution to the cell surface we will provide FACS quantifications and/or biotinylation assays that we already have.
∑ Fig 2A and video 1: We will provide additional videos showing cells expressing lower levels of GFPNMHCIIA. Color scheme will be removed.
∑ Fig 4A: Quantifications will be provided as suggested.
∑ Fig 7D and E: Data for the delta-hly mutant will be moved from the supplementary material to the main Figure.
∑ Concerning the connection between Gp96, NMHCIIA and blebbing in the protection against plasma membrane damage, we believe that our data argues against the dogma that blebbing is always protector of plasma membrane integrity and show that, to be protector, blebbing must be tightly controlled. We demonstrate that PFT-induced blebbing requires a dynamic regulation of the cytoskeletal and ER networks, which culminates with the concentration of ER, NMHCIIA and other uropod components at blebbing sites that facilitate PM stability and retraction. In Gp96-depleted cells, blebbing is uncontrolled, the PM fails to contract and repair does not occur. Consecutively, in NMHCIIA-depleted cells the entire cellular contractility and trafficking mechanisms are compromised the cell does not bleb but also fails to establish any repair mechanism. Importantly we also establish a parallel between retraction of the PM during blebbing and polarized migration. These considerations supported by recent publications in the field will be further integrated in the discussion, as suggested.

Referee#3

∑ Fig S2F: We agree with the referee that macrophage data is not central to our manuscript message. We will remove these data as suggested.
∑ Fig 7: We agree that data on actin comet tails is not crucial for this study. As the referee suggests we will remove actin comet tails from the text.
∑ NMHCIIA-based blebs in vivo: PM blebbing and bleb-like structures have been established and/or visualized in vivo during different processes including efferocytosis, development, angiogenesis, migration and lung metastasis (Gebala et al., 2016; Headley et al., 2016; Tozluoglu et al., 2013). In support to the existence of blebbing in response to in vivo Listeria infection, we already have some images, from fixed samples of zebrafish larvae infected with Listeria at the tail muscle, showing bleb-like structures labeled for Actin and KDEL. Unfortunately we couldn't use anti-NMHCIIA antibodies in immunofluorescence staining of Listeria -infected zebrafish. Nevertheless, if absolutely necessary, we may explore other in vivo models in which we could directly visualize NMHCIIA-driven blebs.

In summary, taking into account the amount of work done, the positive evaluations from both Referees #2 and #3, and our capacity to address the issues raised by the 3 referees in a timely manner (eg 2 months), we sincerely hope that you will reconsider your decision and give us the opportunity to resubmit a significantly revised version of our manuscript. Our data provide new and exciting discoveries highlighting the interplay between ER and cytoskeleton components in processes of membrane remodeling and repair that occur not only upon pore-forming toxin attack, but also during cell migration and development. The data reported in our manuscript will thus be of great interest to both cell biologists and infection biologists.

Thank you for your consideration.

Associated references:
Gebala, V., Collins, R., Geudens, I., Phng, L.K., and Gerhardt, H. (2016). Blood flow drives lumen formation by inverse membrane blebbing during angiogenesis in vivo. Nat Cell Biol 18, 443-450.
Headley, M.B., Bins, A., Nip, A., Roberts, E.W., Looney, M.R., Gerard, A., and Krummel, M.F. (2016). Visualization of immediate immune responses to pioneer metastatic cells in the lung. Nature 531, 513-517.
Tozluoglu, M., Tournier, A.L., Jenkins, R.P., Hooper, S., Bates, P.A., and Sahai, E. (2013). Matrix geometry determines optimal cancer cell migration strategy and modulates response to interventions. Nat Cell Biol 15, 751-762.

2nd Editorial Decision 19 July 2016

Thank you again for your e-mail asking us to reconsider our decision and invite revision of your manuscript. I have now read your letter carefully and re-read the reviewer's reports.

I note in your letter that you would be able to address the issues that reviewer 1 - but also reviewer 2 - pinpoints. You outline that you can provide new confocal data of better quality and provide quantification of the observed effects.

I am sorry to say that I cannot reverse the decision, but given the potential interest of your findings, I would not oppose to consider a re-submission of your paper. Please address all referee concerns in a point-by-point response upon re-submission.

I must however stress that the new manuscript would be treated as a new submission and an editorial re-evaluation would be made at the time of submission that would take into account any novel literature on the topic, and would assess the changes that were made to address the reviewer's criticism. In case the submission would be assessed positively at the editorial stage, we will try to contact the same referees so as to provide you with a fast review and decision.

Wishing you success with the work ahead.

2nd Revision - authors' response 23 September 2016

Referee #1:

Critique: This study contains a wealth of experimental data. As confirmed before and now by Mesquita et al., there is no doubt about the formation of subcortical acto-myosin networks or ER-cisternae vacuolation after toxin-induced membrane injury, yet the question remains: are those structural manifestations related and do they act conjointly in the defense against plasma membrane damage?

Author's response: Although we agree that mechanisms of recovery from plasma membrane damage in response to toxins are not yet fully understood, it is our opinion that our study contributes to advances in this field. We show here that without NMHCIIA and Gp96 host cells are no longer able to coordinate cytoskeletal rearrangements and to assemble cortical actomyosin bundles that coordinate PM blebbing and protect cells against PFTs. These events correlate with redistribution of the ER and Gp96 to the cell periphery and cortical bundles formation. We believe our data shed new light regarding the control of cytoskeletal dynamics upon membrane injury. In particular, we demonstrate a new function of an ER chaperone and provide exciting discoveries that highlight the crosstalk between ER and cytoskeleton components in processes of membrane remodeling and repair that occur not only upon pore-forming toxin attack, but also during cell migration and development.

This reviewer is not convinced by the confocal data. Neither the antibody labelling for Gp96/NMHCIIA (Fig 1D,E) nor the mCherryGp96/KDELGFP videos (Fig 2B, movies, 2,3,4) show more than a spurious colocalization and are more characterized by a general lack of focus.

Author's response: The new version of our manuscript includes new images where co-localization of Gp96 and NMHCIIA is more evident (Figures 1D and F). Importantly, we also provide quantifications for Gp96 and NMHCIIA co-localization in response to LLO (Figure 1E). These quantifications, which fully support our conclusions, were performed on confocal microscopy images in which we define different individual regions of interest (ROI). We considered the entire cell, the cortical bundles and equivalent size cellular regions outside cortical bundles (Control ROI), and obtained Pearson's correlation coefficient using Coloc 2 for FIJI-ImageJ. At least 30 individual cells and 50 bundles or Ctrl ROIs were quantified in 6 independent experiments. Concerning mCherryNMHCIIA/KDELGFP video, we agreed that the quality of the imaging (in particular the mCherryNMHCIIA channel) needed to be improved. We provide a new and better

quality video (Supp Movie 2) and the corresponding sequential frames (Figure 2B), in which we used GFPNMHCIIA and mCherryKDEL. It is our opinion that the new video clearly shows that ER vacuolation, plasma membrane blebbing and NMHCIIA rearrangements occur simultaneously, and support other data in our manuscript that demonstrate the presence of ER structures/vacuoles at cortical NMHCIIA bundles and PM blebs.

In Fig. 3G and movies 5,6,7 which show blebbing cells, the confocal image is blurred -in contrast to the DIC sequence, which is in focus. Since DIC is not a confocal technique, it is likely that the myosin translocation and the membrane movement are not in the same focal plane. HeLa cells have a particularly thin and extended cytoplasm, in this cell type, blebs therefore develop above the focal plane of the acto-myosin cytoskeleton.

Author's response: We believe that the referee missed the related technical description for Figure 3G and corresponding videos. The figure/movies do not show individual confocal sections, but instead they show projections of the total thickness of the cells. Thus the NMHCIIA distribution rearranges from its cytosolic and filamentous distribution into cortical accumulations/clusters at sites of membrane remodeling which finally culminate into distinct bundles at the base of PM blebbing areas. Such redistribution was not detected in Gp96-depleted cells or upon blebbistatin treatment. Legends of supplementary movies were revised to include clear technical details.

Plasma membrane injury and consequently the influx of Ca^{2+} lead to the reorganisation of many membrane components and to the redistribution of cytoplasmic molecules. The observed colocalization of NMHCIIA and LAMP1, actin and calpain-2, NMHCIIA and ERK or NMHCIIA and α-tubulin (Fig 4A) are by no means convincing since the images are of poor quality. Their specific "recruitment" is not corroborated by experimental data.

Author's response: In the new version of our manuscript, we provide new and better quality images for Figure 4A. In addition, we also provide image quantifications (shown in Figure 4Aii) that fully support our conclusions. For quantifications we used confocal microscopy images in which we define different individual regions of interest (ROI). We considered the entire cell, the cortical bundles and equivalent size cellular regions outside cortical bundles (Control ROI), and obtained Pearson's correlation coefficient using Coloc 2 for FIJI-ImageJ. At least 30 individual cells and 50 bundles or Ctrl ROIs were quantified in 3 independent experiments.

Referee #2:

Major points:
Overall this is a nice study that sheds light on the mechanisms that protect cells from pore forming toxins, and reveals connections between the ER chaperone Gp96 and NMHCIIA that are important in protecting against membrane damage in infected cells and animals. The paper is a good candidate for publication if the authors can address the specific points below.

Specific points:
1. Figures 1 and 2: The authors should attempt to quantify the association of Gp96 vacuoles with the cell periphery, membrane blebs, and NMHCIIA bundles in LLO treated cells. It seems that Gp96 vacuoles are associated with these structures in LLO-treated but not untreated cells, but quantification of this result would lend support to these conclusions. Also, in the Introduction, the authors mention that Gp96 redistributes to the plasma membrane in LLO-treated cells, however that is no apparent from the images. This should be explained.

Author's response: We provide in the new version of our manuscript data showing quantifications of NMHCIIA-Gp96 association at the cell cortex within cortical bundles (Figure 1E). The details regarding the quantifications are explained above, in our response to Referee #1. We also provide FACS analysis data showing that the percentage of cells displaying surface-exposed Gp96 significantly increases upon incubation with LLO thus confirming the presence of surface exposed Gp96. The amount of surface-exposed Gp96 is rather low as compared to the amount of intracellular ER-associated Gp96. Nevertheless, we were able to show distinct Gp96-ER structures/vacuoles very close to the cell periphery within cortical bundles. By microscopy we did not observed that Gp96 displays a homogenous PM contour yet it is possible that such weak Gp96 signal from the membrane could be difficult to detect by immunofluorescence in permeabilised cells.

2. Figure 2A and Video 1: The color scheme for showing NMHCIIA is confusing as it makes it look like more than one protein is being visualized. A single color might be sufficient. Moreover, in the video, it seems that NMHCIIA is highly overexpressed, so that the bundles in the blebs are not readily apparent. It might be worth to image cells expressing lower amounts of NMHCIIA so that bundles can be more readily seen over the background fluorescence.

Author's response: The color scheme was removed from Figure 2A as well as from Figure 3G, only single color images are shown in the new version of our manuscript. In addition, in agreement with referee's request, we provide a new video 1 and corresponding sequential frames shown in Figure 2A. The new video shows a cell expressing lower levels of NMHCIIA.

3. Figure 4A: The enrichment of most of these markers with NMHCIIA bundles in cellular blebs is not really clear. Most of the markers appear to be everywhere in the cell, and many are not particularly enriched in near the NMHCIIA structures. Perhaps quantification of these images would provide better support for the conclusions.

Author's response: In the new version of our manuscript, we provide new and better quality images for Figure 4A. In addition, we also provide image quantifications (shown in Figure 4Aii) that fully support our conclusions. The details regarding the quantifications are explained above, in our response to Referee #1.

4. Figure 7D, E: It would be helpful to also show in Figure 7 the results for the delta-hly mutant alone, in the absence of the Gp96 morpholino (this is currently shown in the supplementary material and could be moved to the main figure).

Author's response: As suggested by the referee, the data previously included in supplementary material Figure S8B were moved to the main figures and shown in Figure 7F.

5. The connections between Gp96, NMHCIIA and blebbing in the protection against membrane damage are somewhat confusing. In particular, blebbing is proposed to be a protective mechanism against pore forming toxins. However, Gp96 and NMHCIIA silencing, which both protect against toxins, have opposing effects on blebbing. Gp96 silencing enhances blebbing and NMHCIIA silencing inhibits blebbing. This suggests that the connections between Gp96, NMHCIIA and blebbing are more complicated than the authors acknowledge. This should be discussed in the paper.

Author's response: We demonstrate that PFT-induced blebbing requires a dynamic regulation of the cytoskeletal and ER networks, which culminates with the concentration of ER, NMHCIIA and other uropod components at blebbing sites that facilitate PM stability and retraction. In Gp96-depleted cells, blebbing is uncontrolled, the PM fails to contract and repair does not occur. In absence of NMII activity both cellular contractility and trafficking mechanisms are compromised, impairing blebbing and compromising any repair mechanism. Importantly, we also establish a parallel between retraction of the PM during blebbing and polarized migration. We propose that to be protector, blebbing must be tightly controlled. These considerations were integrated in the second paragraph of discussion.

Referee #3:

Major comments:
Fig S2F. The Macrophage LLO treated data is weak as there appears to be essentially no Gp96 in the NMHCIIA zone and the increased concentrations of NMHCIIA may be because the cells appear to round-up more than the untreated cells (there are definitely fewer projections in the LLO treated cells). I actually think that the macrophage data is not really needed for the paper and if the authors wanted to remove it I wouldn't have a real problem with that.

Author's response: Following referee's suggestion, the images concerning LLO-treated macrophage-like cell line, previously shown in Figure S2F, were removed from the new version of our manuscript.

The identification of comet tails in vivo is not crucial for the study, but the authors mention they are present in Fig 7 and I can't see any. Yes, there are actin concentrations, but clear comet tails are lacking.

Author's response: We agree that the identification of comet tails in vivo is not crucial for the study, however comet tails are an hallmark of Listeria monocytogenes infection thus validating the infection model in use in our study. We also agree that the comet tails were extremely difficult to detect in the figures we provided. The new version of our manuscript includes (in supplementary material Figure S8D) enlarged images clearly showing actin comet tails in tissues of zebrafish-infected larvae.

I don't think that the identification of the KDEL puncta go far enough to demonstrate the blebbing events described throughout the manuscript. The ultimate confirmation of the blebbing events not being an artifact of cell culture would be to see NMHCIIA-based blebs in vivo. Also, the Δhly in vivo survival work (Fig 7C) is not that surprising as there are much less bacteria in the animals and those bacteria would presumable not be able to replicate or spread very well as they would theoretically be contained within a vacuole. If the point was to just show that zebrafish could be used for in vivo analysis that might be best put into another manuscript.

Author's response: PM blebbing and bleb-like structures have been established and/or visualized in vivo during different processes including efferocytosis, development, angiogenesis, migration and lung metastasis (Gebala et al., 2016; Headley et al., 2016; Tozluoglu et al., 2013). In support to the existence of blebbing in response to in vivo Listeria infection, we gathered some initial images, from fixed samples of zebrafish larvae infected with Listeria at the tail muscle, showing bleb-like structures labeled for Actin and KDEL (images shown below, annex 1). Unfortunately, due to technical issues we couldn't assess the NMHCIIA labeling in such structures. This topic deserves further in-depth analysis. We agree with the referee that numbers of Δhly bacteria in zebrafish larvae are decreased as compared to wt bacteria. For that reason we performed Δhly infections with 10-fold more bacteria.

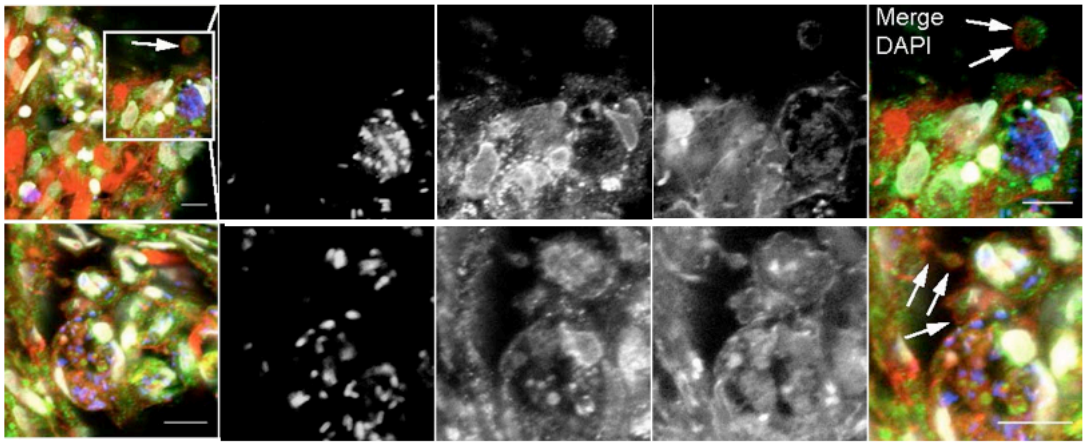
Minor comments:
Pg 3. "NMII activity is required to PFT-induced PM blebbing" should read "NMII activity is required for PFT-induced PM blebbing"

Author's response: This was corrected in the new version of our manuscript.

Fig S4. All of the lanes do not appear to be necessary as some were not knocked-down. Just include the cell lines that were pursued.

Author's response: Indeed the lanes corresponding to cells that were not knocked-down and not used are not crucial. Nevertheless, we would prefer to keep all the lanes as they really show different efficiencies of protein depletion.

Associated references:
Gebala, V., Collins, R., Geudens, I., Phng, L.K., and Gerhardt, H. (2016). Blood flow drives lumen formation by inverse membrane blebbing during angiogenesis in vivo. Nat Cell Biol *18*, 443-450.
Headley, M.B., Bins, A., Nip, A., Roberts, E.W., Looney, M.R., Gerard, A., and Krummel, M.F. (2016). Visualization of immediate immune responses to pioneer metastatic cells in the lung. Nature *531*, 513-517.
Tozluoglu, M., Tournier, A.L., Jenkins, R.P., Hooper, S., Bates, P.A., and Sahai, E. (2013). Matrix geometry determines optimal cancer cell migration strategy and modulates response to interventions. Nat Cell Biol *15*, 751
762.



Annex Figure: Confocal microscopy images of wt zebrafish larvae infected (low dose) in the tail muscle, with the GFP-expressing wt Lm for 24 h, stained with phalloidin (actin, red) and DAPI (white) and immunolabelled for KDEL-proteins (green). Arrows indicate actin/KDEL positive bleb-like structures (arrows).

3rd Editorial Decision 14 October 2016

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see all three referees are now positive about the study and support publication in EMBO reports. The additional lanes in Figure S4D are certainly not essential data, as referee 3 points out, but it is not necessary to remove them from the figure.

From the editorial side, there are a few things that we need before we can proceed with the official acceptance of your study.

- Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Also movies are part of the Expanded View content (Movie EV1 etc.)

- Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

- All Materials and Methods have to be part of the main manuscript. Please move the M&M currently in the Supplementary Information to the main manuscript and relabel this section "Materials and Methods".

- Regarding data quantification, can you please specify the test used to calculate p-values in the respective figure legends? This information is currently incomplete and must be provided in the figure legends. Please also increase the line thickness of the scale bars in all microscopy images to ensure their visibility in the print version of the manuscript.

- Please update the reference style to match the EMBO reports style. The respective EndNote file can be downloaded from our "Author Guidelines."

REFEREE REPORTS

Referee #1:
The authors have improved the quality of the micrographs. This reviewer has no objections to publication

Referee #2:
The authors have addressed my concerns and the manuscript is now suitable for publication.

Referee #3:
I feel that the authors have adequately addressed my concerns despite the fact that I do not agree that all lanes of their KD cells in Fig S4 should be included for publication. This is primarily as most were not used for further study. However, despite that point of contention I do not think that their inclusion of all of those lanes should preclude the paper being published. I will leave that decision to the editor of the journal.

3rd Revision - authors' response 07 November 2016

Authors made requested editorial changes and resubmitted their manuscript.

4th Editorial Decision 16 November 2016

Thank you for the submission of your revised manuscript to our offices. Browsing through the manuscript I have noticed several issues that still need to be resolved before we can officially accept the study.

- I have noticed that the EV movie files mentioned in the text and Expanded View Figure Legends have not been uploaded. Could you please submit the movie files?

- Moreover, Fig. EV4a misses a scale bar and I have noticed that the stills showing timepoints 75 and 90 minutes in Fig. EV5C look exactly the same. Could you please compare these stills to the movie and confirm that this is correct or if you unintentionally used the same timepoint twice?

- Please provide a conflict of interest statement in the mansucript.

- Thank you for the submission of the synopsis. You sent the synopsis image embedded into the Word document. Could you please supply it as individual .tif or .jpeg file?

- Finally, the title should not exceed 100 characters including spaces and the abstract should be in present tense. Please find my suggestions pasted below the abstract but feel free to modify it.

4th Revision - authors' response 16 November 2016

Authors made remaining changes and resubmitted their manuscript.

5th Editorial Decision 18 November 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name:	Sandra Sousa
Journal Submitted to:	EMBO Reports
Manuscript Number:	EMBOR-2016-42833

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

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?	Sample sizes and statistical analysis used are specified in figure legends and/or Material and methods (pg 14 immunofluorescence quantifications; pg 15 wound closure assay and flow cytometry of LLO-treated and infected cells; pg 16 determination of bacterial burden in vivo and statistics)
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	All experiments were done using immortalised cell lines (pg 11, Materials and Methods - Cell lines and Zebrafish)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All quantifications are specified in figure legends and/or Material and methods (pg 14 immunofluorescence quantifications; pg 15 wound closure assay and flow cytometry of LLO-treated and infected cells; pg 16 determination of bacterial burden in vivo and statistics)
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
For animal studies, include a statement about randomization even if no randomization was used.	NA
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.	When appropriate experiments were conducted by different laboratory members and microscopic quantifications undertaken in a blind-scoring manner
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes (see figure legends and materials and methods)
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Such distributions were not tested
Is there an estimate of variation within each group of data?	Statistical analysis are detailed in figure legends and/or materials and methods
Is the variance similar between the groups that are being statistically compared?	Statistical analysis are detailed in figure legends and/or materials and methods

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).	The complete detailed information regarding all reagents and antibodies is specified in the material and methods, pg 10
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The complete detailed information regarding all cell lines used is specified in the materials and methods, pg 11. All cell lines were routinely analysed for possible mycoplasma contaminations

* 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	NA
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).	NA
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).	NA
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	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NO