THE EMBO JOURNAL

Manuscript EMBO-2016-95028

Mycobacterium tuberculosis protease MarP activates a peptidoglycan hydrolase during acid stress

Helene Botella, Julien Vaubourgeix, Myung Hee Lee, Naomi Song, Weizhen Xu, Hideki Makinoshima, Michael S Glickman, Sabine Ehrt

Corresponding author: Sabine Ehrt, Weill Cornell Medical College

Review timeline:

Submission date:	15 June 2016
Editorial Decision:	26 July 2016
Revision received:	24 October 2016
Editorial Decision:	21 November 2016
Revision received:	28 November 2016
Accepted:	02 December 2016

Editor: Hartmut Vodermaier

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 26 July 2016

Thank you for submitting your manuscript on MarP cleavage of RipA in the Mtb acidic stress response to our journal. After some delay due to limited reviewer availability at this time of the year, we have now received reports from three expert referees, copied below for your information. As you will see, all referees consider your findings of interest and potential importance, but there are are also several substantive concerns that would need to be satisfactorily addressed before publication may be warranted. The most salient of these points is raised by referee 3 and concerns the unexplained specificity for acidic conditions despite RipA cleavage at neutral pH, and the untested interaction of MarP-RipA in non-stressed cells.

Should you be able to decisively extend the study and conclusively clarify the principle concern of referee 3, we would be interested in considering a revised manuscript further for EMBO Journal publication. Since our editorial policies allow only a single round of major revision, please however make sure to also diligently address and respond to the various other points specified by all three reviewers, including those about microscopy, reproducibility/repeats and internal consistencies. Should you have any questions/feedback regarding the referee reports or the revision work, please do not hesitate to contact me ahead of resubmission.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may

be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

This paper by Botella et all identifies a specific molecular function for the MarP protease, which had previously been shown to be important for TB to survive acid stress. The authors show that MarP cleaves the PG endopeptidase RipA, and that this cleavage is important for mycobacterial cells to survive in acid stress. This work is well conceived and executed and makes an important contribution to our understanding of how Mtb survives during stress. I like the cell wall staining experiments and I especially appreciate that the authors reconstituted the system in vitro. Well done! Most of my comments are on writing, annotation and data presentation. My only major concern is reproducibility of the microscopy data. I understand that you did some statistical analyses, but it seems like the microscopy experiments were only done once. Reproducibility is more important than statistics. If the experiments were done on replicate cultures, then please indicate that and show error bars where appropriate. If these experiments were one done on a single culture, then they need to be repeated.

Major comments:

Figure 1A&B - I really like this data, but I don't like the presentation. There are no numbers on the X axis and I don't even know what number the tick mark represents. I think it would be easier to interpret if the bars for each strain were intercalated and colored differently, with a separate small graph for each pH. You could just show pH 7.2 and pH5 for TB and pH 7.2 and pH 4.5 for smeg in the body of the paper, and leave the rest in the supplement - all those extra pH points don't really add anything to the story. Also, is this data and that in 1D reproducible? Please include in the figure legend information about how many replicate cultures for each strain were combined for A&B, and please include error bars in D.

Figure 2C - This presentation of the data could be improved. I think it would help to remove the pH 5 data - it's not clear to me that this adds anything. As far as I can tell, the main point here is that the septa that are formed during the initial staining are mostly resolved by 16 hours in the WT in acid, but fewer of them are resolved in the mutant. The NADA alone data just obscures this point, because it stretches the axis so that you really have to squint to see the difference between the NADA+HADA data at the bottom of the graph. Can you remove (or put in supp) the NADA alone data so that the differences you care about are emphasized? OR, perhaps split the Y axis and make the bottom of the graph where all the interesting data is 75% of the Y axis. Also, importantly, is this reproducible? Has this experiment been conducted on replicate cultures? Please present the data with error bars representing the spread of percentages between independent replicates.

Minor comments:

Pg 2 , end of abstract - don't you think PG hydrolysis is essential under all conditions? Just maybe the degree and factors involved changes in acid stress? Maybe reword slightly.

Pg 4 - I think you should cite the Kuru 2012 paper in addition to the nature protocols paper.

The genetic notation ΔmarP¬TB is confusing and non-standard. I believe it should be annotated Mtb ΔmarP. Then, use the subscript to indicate which species a complemented gene comes from, so the smeg complement strain should be: Msm ΔmarP::marPTB. Also, please don't use the subscript to mean the species in one place, and to indicate a mutation in another - I thought we were talking about MarP from staph aureus for a minute. The correct annotation is:
marP S343A

Figure 4B - in the fifth lane, according to your table at the top you added both MarP and MarP

S343S to this reaction - I think this is just a typo, please correct.

Page 8 and 9 - The correct annotation for amino acid substitutions is L234G. Please correct throughout.

Page 9 first line - you repeat L to G.
Third line - "with either mutant"

Figure 4 C - You say there is not a 25 kD band upon RipA cleavage, but it sure looks like there is one on the gel. I understand that MarP is in there too, but it's hard to tell what's going on, as there seems to be a few extra bands compared to fig 4B. Can you please label the MarP bands and explain what that 25 kD band is? It looks to me like these mutations greatly slow processing, but don't stop it entirely. I think that's totally fine, just please reconcile the language in the text with the figures.

The correct annotation for superfolderGFP is sfGFP.

Figure 5 legend - the past tense of pull down is "pulled down".

Page 10 first paragraph - correct spelling to "fourth panel"

Figure 6 - why are the colors false-colored in red if you NADA stained them? Might as well color them in green so it looks different from the membrane staining in the other figs. Again, I think the data in Fig 6B could be presented in a better way - same as my suggestions for fig 1.

Pg 16 first paragraph - please include a citation for the tweety integration vectors.

Referee #2:

This manuscript describes the discovery that RipA, a cell wall endopeptidase in mycobacteria, is substrate of MarP, a protease important for the survival of M. tuberculosis in the mouse model and under acidic conditions. The authors show that under acidic stress conditions, a MarP mutant exhibits a septation defect that mirrors that seen in a RipAB depletion mutant under normal growth conditions. Furthermore, they show that this septation defect is also seen in a RipA deletion mutant experiencing acidic stress. These observations tie together two aspects of mycobacterial physiology that heretofore have not been linked: cell division and survival under acidic conditions. Overall, the experiments are straightforward and the authors have taken particular pains to quantify septation defects in the experiments described in the manuscript. The main conclusions are supported by the data presented in the work, although there I have a few questions and concerns that I would like the authors to clarify and address.

1. The authors studied marP mutants of both M. smegmatis and M. tuberculosis, and complemented strains of both. However, the M. smegmatis mutant was complemented with the wild type marP of M. tuberculosis and not that of M. smegmatis. I am a bit concerned by this, since in several of the experiments, the ΔmarP/marPTB strain is does not display a fully wild-type phenotype. It's been previously shown that RipA processing may be species-specific, and the M. tuberculosis ripA gene does not complement a M. smegmatis ΔripA mutant, presumably because of inefficient processing of the M. tb. RipA protein by the M. smegmatis MarP protease. I wondered why the M. smegmatis marP gene wasn't used for complementation. I think it would be a good idea for the authors to address the potential species-specific processing of RipA as an explanation for the differences between the wild type M. smegmatis and them. Msm ΔmarP/marPTB. This is particularly important since the form of RipA that is IP'd with MarP in M. smegmatis is the unprocessed form (See Fig. 5).

2. The microscopy data appears to be quantified appropriately with statistical analysis, but the images shown in Figs 2B and S2C are a little confusing to me. I had a difficult time figuring out what I was looking for. I also thought that the red NADA staining pattern for M. smegmatis looked very different than that of M. tuberculosis. The former had uniform staining, while the latter had very strong polar staining.

3. The differences in HADA/NADA septal staining were interpreted as a delay in septa formation. It

seems this stems from the idea that the HADA/NADA D-alanine analogs are incorporated into nascent PG precursors, but it's my understanding that D-amino acids can be incorporated into PG peptides by a variety of mechanisms-mostly depending upon exchange reactions performed by various PG transpeptidases acting on extracytoplasmic PG peptides. Alterations in peptide degradation by modulating RipA activity could potentially affect introduction of the D-alanine analogs into peptides at the septum, which would look like an increase in the number of septa, as opposed to an increase in the ability of septa to be labeled with these reagents. Could the authors explain the labeling mechanism in a bit more detail?

3. In the discussion, the authors describe the MarP acidic response as something that has evolved in M. tuberculosis for adaptation to its intracellular lifestyle. However, since the MarP acidic response also exists in M. smegmatis, it would seem that it is an older, general mycobacterial adaptation to environmental stress that has been maintained by M. tuberculosis for intracellular survival.

4. On page 13 of the discussion pertaining to published studies on the activity of RipA on various PG substrates, the authors mention that the peptide structure of the PG of B. subtilis is the same as that of M. tuberculosis. The peptides are not identical. The D-glutamate residues in the PG of mycobacteria are amidated, and are formally described as D-iso-glutamine. I can't find any reference that states that this same modification occurs on B. subtilis PG peptides.

5. There is an error in the model in Figure 7. The scissors depicting RipA are shown cutting the PG peptide between the meso-DAP at position 3 and the D-alanine at position 4. However, RipA cuts the peptide between the D-iso-glutamine at position 2 and the meso-DAP. The figure should be modified to show this.

Referee #3:

In prior work, the Ehrt group identified the protease MarP as being required for acid tolerance of M. tuberculosis and for maintaining the intrabacterial pH of the bacteria. Here through a candidate analysis, they identify the peptidoglycan hydrolase RipA as a substrate of MarP. They convincingly show that MarP cleaves RipA and that these proteins interact. In vitro, they show in Figure 4 B that MarP can cleave RipA at a pH of 7.4 (or so it appeared from reading the methods section). Then, in their in vivo binding assay, they show binding of the two proteins in bacteria grown at pH 4.5. Do MarP and RipA interact in non-acid-stressed cells? (Fig 5) The authors state in the abstract "Here we used biochemical methods coupled with supravital chemical probes that facilitate imaging of nascent peptidoglycan to demonstrate that - during acid stress - MarP cleaves the peptidoglycan hydrolase RipA, a process required for RipA's activation". Similarly, the heading for the results section for Figure 5 is "RipATB and MarPTB interact in acid stressed cells." Are they implying that this interaction specifically happens during acid stress? Or are one or both proteins only expressed during acid stress? (I could not find specific information on this either here or in they're past work.) If so this should be shown. Since cleavage appears to be occurring at pH7.4, how do their findings explain the specific or at least exaggerated failure to make and break septa?

In summary, this very nice work falls short of getting at the question of why either mutant accumulates septa secondary to failure of division in an acidic environment. In other words, why do these cells fail to divide in acid? Also, we already know from their prior paper that most of the MarP mutant cells die at acidic pH, could that be related to failed cell division attempts?

Minor points:

- The way bar graphs in Fig 2C and Fig 2D are presented is confusing. It would be simpler if only three colors were used for each outcome (as was done in Fig 2E). As the data are already separated by genotype, it is unnecessary and confusing to further differentiate by color.

- Fig 2D and 2E need n values.

- What is the basis in the introduction for implicating MarP in latent infection only? Have they tested the MarP mutant's ability to grow in naïve macrophages? What about the RipA mutant?

1st Revision - authors' response 24 October 2016

Point-by-point response to the reviewers' comments:

We thank the reviewers for their positive responses, constructive critiques and helpful suggestions. We have addressed the specific points raised in their reviews and believe the manuscript has been improved. Below, please find the reviewers' comments, followed by our responses.

Referee #1:

This paper by Botella et all identifies a specific molecular function for the MarP protease, which had previously been shown to be important for TB to survive acid stress. The authors show that MarP cleaves the PG endopeptidase RipA, and that this cleavage is important for mycobacterial cells to survive in acid stress. This work is well conceived and executed and makes an important contribution to our understanding of how Mtb survives during stress. I like the cell wall staining experiments and I especially appreciate that the authors reconstituted the system in vitro. Well done!

Thank you for your appreciation and enthusiasm.

Most of my comments are on writing, annotation and data presentation. My only major concern is reproducibility of the microscopy data. I understand that you did some statistical analyses, but it seems like the microscopy experiments were only done once. Reproducibility is more important than statistics. If the experiments were done on replicate cultures, then please indicate that and show error bars where appropriate. If these experiments were one done on a single culture, then they need to be repeated.

All experiments presented in this manuscript were repeated at least 3 times. In this revised version, we have indicated the number of repeats in the figure legends.

Major comments:

Figure 1A&B - I really like this data, but I don't like the presentation. There are no numbers on the X axis and I don't even know what number the tick mark represents. I think it would be easier to interpret if the bars for each strain were intercalated and colored differently, with a separate small graph for each pH. You could just show pH 7.2 and pH5 for TB and pH 7.2 and pH 4.5 for smeg in the body of the paper, and leave the rest in the supplement - all those extra pH points don't really add anything to the story. Also, is this data and that in 1D reproducible? Please include in the figure legend information about how many replicate cultures for each strain were combined for A&B, and please include error bars in D.

Thank you for your suggestion to improve the clarity of the results presented in Figure 1. In its revised version, following your suggestion, only the data obtained at pH 7.2 and pH 5 for Mtb, and pH 7.2 and pH 4.5 for Msm are reported to improve clarity.

In panels A and B, we compiled data from 3 independent experiments. We added this information and the number of cells for which length was measured to the figure legend. Box plots indicate the median and the 25th and 75th percentiles; the whiskers are drawn down and up to the 10th and 90th percentiles, respectively. We opted for the same representation in Figure 6.

For panel D of Figure 1 and panel C of Figure 6, we also combined the results of 3 independent experiments. The error bars for the total number of bacteria with multiple septa (> 1) have been added.

Figure 2C - This presentation of the data could be improved. I think it would help to remove the pH 5 data - it's not clear to me that this adds anything. As far as I can tell, the main point here is that the septa that are formed during the initial staining are mostly resolved by 16 hours in the WT in acid, but fewer of them are resolved in the mutant. The NADA alone data just obscures this point, because it stretches the axis so that you really have to squint to see the difference between the NADA+HADA data at the bottom of the graph. Can you remove (or put in supp) the NADA alone data so that the differences you care about are emphasized? OR, perhaps split the Y axis and make the bottom of the graph where all the interesting data is 75% of the Y axis.

Thank you again for your suggestion. To improve clarity, the results obtained at pH 5 have been omitted in the revised version of Figure 2. In panel C, we split the Y-axis to better visualize all data.

Also, importantly, is this reproducible? Has this experiment been conducted on replicate cultures? Please present the data with error bars representing the spread of percentages between independent replicates.

Panels C, D and E of figure 2 report the results of one experiment representative of three independent experiments. The difference between WT and mutant strains was reproducible in all replicates. The statistical analysis is provided in Appendix Table S2. The same statistical analysis was computed for the repeat experiments and is provided below.

In the tables reported below: values in red font have a p-value $<10^{-4}$, values in orange font have a p-value between 10^{-4} and 0.03, actual values are indicated in brackets, and values in black font are not statistically different. ND: not determined.

M. smegmatis – Experiment 2				
Percentage of bacteria with at least 1 septum (HADA+ NADA) + (HADA)				
		KO vs WT	**Comp vs WT**	**Comp vs KO**
4h	**pH 7**	-0.004%	-0.2%	-0.1%
	pH 4.5	6.2% (0.0038)	-4.6% (0.0088)	-10.7%
16h	**pH 7**	0.07%	0.01%	-0.08%
	pH 4.5	6.4% (0.0001)	0.5%	-5.9% (0.0003)
Interaction strain and pH				
	KO vs WT	**Comp vs WT**	**Compl vs KO**	
	pH 4.5 vs pH 7	**pH 4.5 vs pH 7**	**pH 4.5 vs pH 7**	
4h	6.2% (0.0036)	-4.4% (0.0119)	-10.2%	
16h	6.3% (0.0001)	0.5%	-5.8% (0.0003)	
Percentage of bacteria with at least 2 septa				
		KO vs WT	**Comp vs WT**	**Comp vs KO**
4h	**pH 7**	ND	ND	ND
	pH 4.5	0.01%	0.02%	0.003%
16h	**pH 7**	ND	ND	ND
	pH 4.5	4.8%	-0.5%	-5.3%

M. smegmatis – Experiment 3				
Percentage of bacteria with at least 1 septum (HADA+ NADA) + (HADA)				
		KO vs WT	**Comp vs WT**	**Comp vs KO**
4h	**pH 7**	0.04%	-0.005%	-0.04%
	pH 4.5	9.6%	-1.6%	-11.2%
16h	**pH 7**	0.03%	0.007%	0.03%
	pH 4.5	9.8%	2.4%	-7.4% (0.0008)
Interaction strain and pH				
	KO vs WT	**Comp vs WT**	**Compl vs KO**	
	pH 4.5 vs pH 7	**pH 4.5 vs pH 7**	**pH 4.5 vs pH 7**	
4h	9.5%	-1.6%	-11.1%	
16h	9.7%	2.4%	-5.8% (0.0008)	
Percentage of bacteria with at least 2 septa				
		KO vs WT	**Comp vs WT**	**Comp vs**
4h	**pH 7**	ND	ND	ND
	pH 4.5	0.4%	-0.07%	-0.5%
16h	**pH 7**	ND	ND	ND
	pH 4.5	6.5%	-1.5%	-8%

M. tuberculosis – Experiment 2				
Percentage of bacteria with at least 1 septum (HADA+ NADA) + (HADA)				
		KO vs WT	Comp vs WT	Comp vs KO
16h	**pH 7**	2.9%	0.5%	-2.4% (0.005)
	pH 5	8.7%	-1.6%	-7.1% (0.0009)
Interaction strain and pH				
	KO vs WT	**Comp vs WT**	**Compl vs KO**	
	pH 5 vs pH 7	**pH 5 vs pH 7**	**pH 5 vs pH 7**	
16h	5.8% (0.0029)	1.1%	-4.7% (0.0105)	

M. tuberculosis – Experiment 3				
Percentage of bacteria with at least 1 septum (HADA+ NADA) + (HADA)				
		KO vs WT	Comp vs WT	Comp vs KO
16h	**pH 7**	1.5%	0.2%	-1.3%
	pH 5	6.7%	1.8%	-4.9% (0.0037)
Interaction strain and pH				
	KO vs WT	**Comp vs WT**	**Compl vs KO**	
	pH 5 vs pH 7	**pH 5 vs pH 7**	**pH 5 vs pH 7**	
16h	5.2% (0.0018)	1.6%	-3.5% (0.06)	

Minor comments:

Pg 2, end of abstract - don't you think PG hydrolysis is essential under all conditions? Just maybe the degree and factors involved changes in acid stress? Maybe reword slightly.

We agree that PG hydrolysis is essential in all conditions and we apologize if the last sentence of the abstract was misleading. We edited it as follows: "Our results suggest that sustaining peptidoglycan hydrolysis, a process required for cell elongation, separation of progeny cells, and cell wall homeostasis in growing cells, may also be essential for Mtb's survival in acidic conditions."

Pg 4 - I think you should cite the Kuru 2012 paper in addition to the nature protocols paper.

Thank you for catching this omission. The citation has been added.

The genetic notation ΔmarP¬TB is confusing and non-standard. I believe it should be annotated Mtb ΔmarP. Then, use the subscript to indicate which species a complemented gene comes from, so the smeg complement strain should be: Msm ΔmarP::marPTB. Also, please don't use the subscript to mean the species in one place, and to indicate a mutation in another - I thought we were talking about MarP from staph aureus for a minute. The correct annotation is: marP S343A.

Page 8 and 9 - The correct annotation for amino acid substitutions is L234G. Please correct throughout.

The correct annotation for superfolderGFP is sfGFP.

We have corrected this annotation throughout the manuscript.

Figure 4B - in the fifth lane, according to your table at the top you added both MarP and MarP S343S to this reaction - I think this is just a typo, please correct.

Thank you for noticing. We have corrected the figure.

Page 9 first line - you repeat L to G.

The second L to G was to indicate the double mutation LGVG. To avoid confusion, we modified this as follows: "We further introduced the substitutions L234G (RipA-LG) and L234G-V235G (RipA-LGVG) in the amino acid sequence of $RipA_{TB}$."

Third line - "with either mutant"

This has been corrected.

Figure 4 C - You say there is not a 25 kD band upon RipA cleavage, but it sure looks like there is one on the gel. I understand that MarP is in there too, but it's hard to tell what's going on, as there seems to be a few extra bands compared to fig 4B. Can you please label the MarP bands and explain what that 25 kD band is? It looks to me like these mutations greatly slow processing, but don't stop it entirely. I think that's totally fine, just please reconcile the language in the text with the figures.

We agree with the reviewer's comment. We modified the text as follows:

"$MarP_{TB}$ did not fully process these mutant proteins, as the ~ 25 kDa fragment of RipA was less abundant after prolonged incubation with either mutant compared to $RipA_{TB}$. However, the ~ 35 kDa fragment appeared to be more stable when we used the two mutant proteins as substrates of $MarP_{TB}$ (Fig 4C, red arrows).

Figure 5 legend - the past tense of pull down is "pulled down".

Page 10 first paragraph - correct spelling to "fourth panel"

These mistakes have been corrected.

Figure 6 - why are the colors false-colored in red if you NADA stained them? Might as well color them in green so it looks different from the membrane staining in the other figs. Again, I think the data in Fig 6B could be presented in a better way - same as my suggestions for fig 1.
To visualize septa following incorporation of both D-alanine analogs, we false-colored NADA and HADA labeled septa in red and green, respectively. To avoid confusion with FM-5-95 staining, we added an inset indicating FM 5-95 when it applied (Figures 3C & 4E).

Pg 16 first paragraph - please include a citation for the tweety integration vectors.
The citation has been added (Pham, Jacobs Sera et al., Microbiology, 2007;153(Pt 8):2711-23.).

Referee #2:

This manuscript describes the discovery that RipA, a cell wall endopeptidase in mycobacteria, is substrate of MarP, a protease important for the survival of M. tuberculosis in the mouse model and under acidic conditions. The authors show that under acidic stress conditions, a MarP mutant exhibits a septation defect that mirrors that seen in a RipAB depletion mutant under normal growth conditions. Furthermore, they show that this septation defect is also seen in a RipA deletion mutant experiencing acidic stress. These observations tie together two aspects of mycobacterial physiology that heretofore have not been linked: cell division and survival under acidic conditions. Overall, the experiments are straightforward and the authors have taken particular pains to quantify septation defects in the experiments described in the manuscript. The main conclusions are supported by the data presented in the work, although there I have a few questions and concerns that I would like the authors to clarify and address.

1. The authors studied marP mutants of both M. smegmatis and M. tuberculosis, and complemented strains of both. However, the M. smegmatis mutant was complemented with the wild type marP of M. tuberculosis and not that of M. smegmatis. I am a bit concerned by this, since in several of the experiments, the ΔmarP/marPTB strain is does not display a fully wild-type phenotype. It's been previously shown that RipA processing may be species-specific, and the M. tuberculosis ripA gene does not complement a M. smegmatis ΔripA mutant, presumably because of inefficient processing of the M. tb. RipA protein by the M. smegmatis MarP protease. I wondered why the M. smegmatis marP gene wasn't used for complementation. I think it would be a good idea for the authors to address the potential species-specific processing of RipA as an explanation for the differences between the wild type M. smegmatis and them. Msm ΔmarP/marPTB.
This is an important point. Following the reviewer's suggestion, we decided to test if $MarP_{smeg}$ rescues the phenotype of the Msm mutant in acidic pH. As shown in Figures 1 & 2, the expression of $marP_{smeg}$ in Msm Δ*marP* complemented all phenotypes displayed by the MarP deletion mutant (i.e. elongation, chain formation and delayed septum turnover) at acidic pH. In fact, $MarP_{smeg}$ complemented more efficiently than $MarP_{TB}$, suggesting that MarP of *M. smegmatis* and *M. tuberculosis* may not be fully redundant. This indicates that MarP may possess some functional species specificity, as suggested by this reviewer.

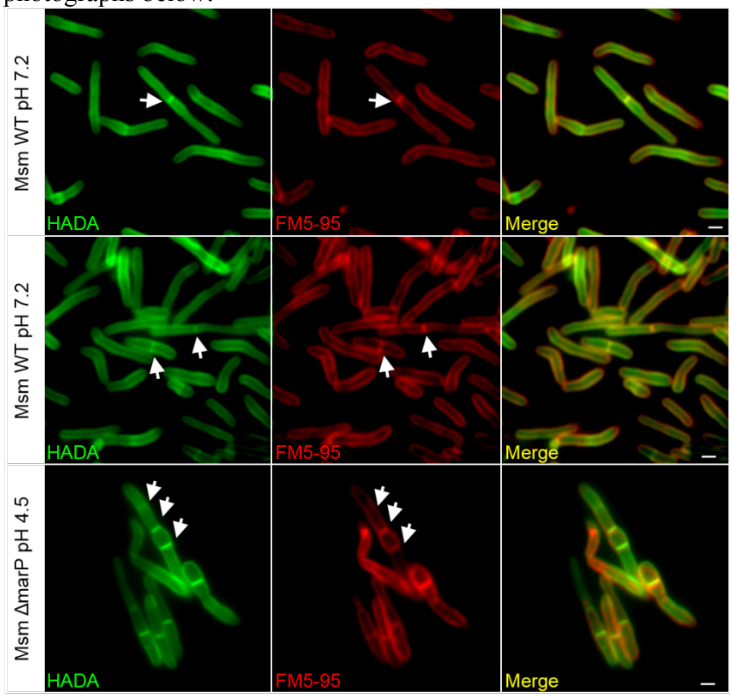
This is particularly important since the form of RipA that is IP'd with MarP in M. smegmatis is the unprocessed form (See Fig. 5).
To avoid artifacts due to species-specific activities, we co-expressed $marP_{TB}$ and $ripA_{TB}$ in Msm Δ*marP* to determine if the two proteins interact. We initially reported that $RipA_{TB}$ was pulled-down with $MarP_{TB}$ when bacteria were exposed to pH 4.5. In the revised manuscript, we added a direct comparison of $MarP_{TB}$ pull-down after incubation at pH 7.2 and pH 4.5. As shown in Figure EV3, MarP co-immunoprecipitated a larger proportion of $RipA_{TB}$ at pH 4.5 than at pH 7.2. This suggests that MarP and RipA specifically interact when bacteria are exposed to acidic pH.

2. The microscopy data appears to be quantified appropriately with statistical analysis, but the images shown in Figs 2B and S2C are a little confusing to me. I had a difficult time figuring out what I was looking for. I also thought that the red NADA staining pattern for M. smegmatis looked very different than that of M. tuberculosis. The former had uniform staining, while the latter had very strong polar staining.
Mycobacteria elongate by addition of new cell wall material at the poles. The poles will thus be the first to be labeled by D-alanine analogs. If cells are pulsed with fluorescent D-alanine analogs for a time shorter than their generation time, labeling will mostly be polar. This explains the polar

staining of Mtb—that divides every 24 hours—incubated for 16 hours with NADA. However, Msm cells have been perfused with FDAAs for 4 or 16 hours, which is longer than the generation time of Msm (~ 3 hours). This explains the uniform labeling of Msm.

3. The differences in HADA/NADA septal staining were interpreted as a delay in septa formation. It seems this stems from the idea that the HADA/NADA D-alanine analogs are incorporated into nascent PG precursors, but it's my understanding that D-amino acids can be incorporated into PG peptides by a variety of mechanisms-mostly depending upon exchange reactions performed by various PG transpeptidases acting on extracytoplasmic PG peptides. Alterations in peptide degradation by modulating RipA activity could potentially affect introduction of the D-alanine analogs into peptides at the septum, which would look like an increase in the number of septa, as opposed to an increase in the ability of septa to be labeled with these reagents. Could the authors explain the labeling mechanism in a bit more detail?

Fluorescent D-alanine analogs incorporate mainly through periplasmic exchange reactions with the muropeptides (Kuru et *al.*, Angew Chem Int Ed Engl. 2012 Dec 7;51(50):12519-23). Depending on the organism, they are incorporated in the stem peptide at the 4^{th} or 5^{th} position by L,D-transpeptidases or D,D-transpeptidases, respectively (Kuru et *al.*, Angew Chem Int Ed Engl. 2012 Dec 7;51(50):12519-23). Fluorescent D-alanine analogs mark sites of active PG synthesis, and for this reason, we used them as chemical tools to follow the dynamics of septum formation and resolution. We interpret the delay in septum resolution observed in the marP mutants as a defect in RipA activation, which impairs PG degradation and further impedes progeny cells separation. However, the formation of the septum is not altered in the mutant. This results in the formation of multiple septa in single cells. Cells are similarly labeled by HADA and FM5-95, as shown in the photographs below.



3. In the discussion, the authors describe the MarP acidic response as something that has evolved in M. tuberculosis for adaptation to its intracellular lifestyle. However, since the MarP acidic response also exists in M. smegmatis, it would seem that it is an older, general mycobacterial adaptation to environmental stress that has been maintained by M. tuberculosis for intracellular survival.

We agree and we edited the text as follows: “Mtb limits its exposure to acidic pH in infected hosts by blocking the fusion of the phagosome with lysosomes. If halting phagosomal maturation fails, Mtb possesses mechanisms by which it can survive in acidified phagosomes.”

4. On page 13 of the discussion pertaining to published studies on the activity of RipA on various PG substrates, the authors mention that the peptide structure of the PG of B. subtilis is the same as that of M. tuberculosis. The peptides are not identical. The D-glutamate residues in the PG of mycobacteria are amidated, and are formally described as D-iso-glutamine. I can't find any reference that states that this same modification occurs on B. subtilis PG peptides.

Thank you for catching this mistake. *B. subtilis* and *M. tuberculosis* PG peptide chains are not identical. Both glutamate and meso-diaminopimelic acid residues are amidated in Mtb's PG. We removed the sentence that stated that PG from *B. subtilis* and *M. tuberculosis* are identical.

5. There is an error in the model in Figure 7. The scissors depicting RipA are shown cutting the PG peptide between the meso-DAP at position 3 and the D-alanine at position 4. However, RipA cuts the peptide between the D-iso-glutamine at position 2 and the meso-DAP. The figure should be modified to show this.
Thank you for pointing that out. The model depicted in the figure 7 has been corrected accordingly.

Referee #3:

In prior work, the Ehrt group identified the protease MarP as being required for acid tolerance of M. tuberculosis and for maintaining the intrabacterial pH of the bacteria. Here through a candidate analysis, they identify the peptidoglycan hydrolase RipA as a substrate of MarP. They convincingly show that MarP cleaves RipA and that these proteins interact. In vitro, they show in Figure 4 B that MarP can cleave RipA at a pH of 7.4 (or so it appeared from reading the methods section). Then, in their in vivo binding assay, they show binding of the two proteins in bacteria grown at pH 4.5. Do MarP and RipA interact in non-acid-stressed cells? (Fig 5)

The authors state in the abstract "Here we used biochemical methods coupled with supravital chemical probes that facilitate imaging of nascent peptidoglycan to demonstrate that - during acid stress - MarP cleaves the peptidoglycan hydrolase RipA, a process required for RipA's activation". Similarly, the heading for the results section for Figure 5 is "RipATB and MarPTB interact in acid stressed cells." Are they implying that this interaction specifically happens during acid stress?
Thank you for raising this question. To assess this point, we performed pull downs of $MarP_{TB}$ after incubation at pH 7.2 and pH 4.5. As shown in Figure EV3, MarP pulled down a larger proportion of $RipA_{TB}$ at pH 4.5 than at pH 7.2. This suggests that MarP and RipA specifically interact when bacteria are exposed to acidic pH.

Or are one or both proteins only expressed during acid stress? (I could not find specific information on this either here or in they're past work.) If so this should be shown.
In the pull-down assay, we constitutively expressed both proteins in Msm and we observed a strong interaction between MarP and RipA at pH 4.5; this interaction is weak when cells have been subjected to pH 7.2. Moreover, *marP* transcription has been shown to be constitutive and is not induced upon acid stress in Mtb (Vandal et al., Nat Med. 2008 Aug;14(8):849-54). To our knowledge, the expression of *ripA* when bacteria are subjected to acidic environment has not been evaluated; Chao and colleagues have shown that the expression of *ripA* in Msm remained stable from exponential growth to stationary phase (Chao et al, PLoS Pathog. 2013 Feb;9(2):e1003197.). Altogether, these observations suggest that the two proteins are regulated post-translationally, and that their interaction is either promoted at pH 4.5, or prevented at pH 7.2. Sonawane et al. reported that MarP is glycosylated, although the authors did not provide experimental evidences (Crit Rev Microbiol. 2012 Aug;38(3):250-66.). The role of posttranslational modifications will be the scope of further work.

Since cleavage appears to be occurring at pH7.4, how do their findings explain the specific or at least exaggerated failure to make and break septa?
We reported that MarP cleaves RipA *in vitro* at pH 7.4, but we also confirmed that the processing occurred *in vitro* at pH 4.5 (data not shown). Other levels of regulation may facilitate RipA processing by MarP *in vivo* in acid stressed cells that are not reconstituted *in vitro*; this includes the likely interaction with other proteins or the role of post-translational modifications.

In summary, this very nice work falls short of getting at the question of why either mutant accumulates septa secondary to failure of division in an acidic environment. In other words, why do these cells fail to divide in acid? Also, we already know from their prior paper that most of the MarP mutant cells die at acidic pH, could that be related to failed cell division attempts?
RipA, along with RipB, is a major PG hydrolase in mycobacteria required for cell division. Our results suggest that MarP does not regulate RipA in neutral conditions. This may be the role of other proteins that have been shown to act in concert with RipA (Hett et al., PLoS Pathog. 2008 Feb

29;4(2):e1000001, Hett et al., PLoS Pathog. 2010 Jul 29;6(7):e1001020). Moreover, RipB can compensate for the lack of RipA at neutral pH (Martinelli and Pavelka, J Bacteriol. 2016 Apr 14;198(9):1464-75). We hypothesize that proteins that regulate RipA at neutral pH are no longer active at acidic pH. In this condition, MarP activates RipA to assure PG hydrolysis. In D*marP* cells, RipA fails to be activated at acidic pH, thus impeding cell separation. Impaired RipA activity culminates in the formation of chains. Whether cell separation itself or discrete PG remodeling is needed to survive acidic stress remains unknown. However, Δ*ripA* cells strictly phenocopy Msm Δ*marP*. This indicates that MarP's mediated protection against acidic stress occurs *via* RipA's activity.

Minor points:

- The way bar graphs in Fig 2C and Fig 2D are presented is confusing. It would be simpler if only three colors were used for each outcome (as was done in Fig 2E). As the data are already separated by genotype, it is unnecessary and confusing to further differentiate by color.
We agree that the Figure 2 was not intuitive. Reviewer 2 also made this point. We revised Figure 2 as described in the response to reviewer 2. Briefly, only the data obtained at pH 7.2 and pH 4.5 are reported, and as suggested by reviewer 3, we used one color per probe, and a third color to represent the combination of the two.

- Fig 2D and 2E need n values.
Thank you for pointing that out. We added the number of bacteria counted on the figure.

- What is the basis in the introduction for implicating MarP in latent infection only? Have they tested the MarP mutant's ability to grow in naïve macrophages? What about the RipA mutant?
We did not mean to imply that MarP is important only in latent/chronic infection. We mentioned in the introduction "The MarP-deficient mutant is attenuated in immunocompetent mice. It did not replicate to the same extent as wild type (WT) Mtb and failed to persist during chronic infection", underlining the importance of MarP to both establish and maintain infection in the mouse. We previously demonstrated that Mtb Δ*marP* replicates to the same extent as Mtb WT in naïve macrophages whereas it is more readily killed by IFNγ–activated macrophages (Vandal et al. Nat Med. 2008). These results reinforce the idea that MarP is important for Mtb to survive in stressful environments such as the phagolysosome, but may be dispensable in less stringent environment such as the phagosome.

To our knowledge, an Mtb RipA mutant has not been generated. Based on the results presented here, we speculate that it would be attenuated in activated macrophages. These experiments are beyond the scope of this paper.

2nd Editorial Decision 21 November 2016

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by two of the original referees, whose comments are copied below. As you will see, referee 2 considers the study significantly improved and now in principle suitable for publication in The EMBO Journal, pending some minor modifications. However, referee 3 is at this stage still not fully satisfied with all responses to their original criticisms, and also retains important reservations regarding interpretation of findings in the literature and of the present results. Before we shall be able to proceed with eventual acceptance for publication, I would therefore kindly like to ask you to carefully consider and address the various issues concerning presentation, writing and discussion raised by both referees. Regarding referee 3's requests for experimental extensions, we do not consider it necessary to directly investigate the question of expression further. However, we do agree with the referee that this article would certainly constitute a stronger and more compelling contribution if some insights into the referee's original question regarding the physiological importance of cell separation and/or peptidoglycan remodeling for survival under acidic conditions would be provided. I would therefore appreciate if you would get back to me to discuss possible experiments that could be done within a reasonable time frame to shed some further light on this question.

I am therefore returning the manuscript to you once more for an additional round of minor revision,

and I look forward to hearing your proposal(s) for possible experiments getting at the one outstanding issue, as well as their feasibility.

REFEREE REPORTS

Referee #2:

The authors did a commendable job answering my concerns of the previous version of this manuscript, and I think that they also answered the concerns of the other reviewers. I have two minor but important points that ought to be modified in the current version:

1. Page 3, the authors write that RipA is a PG hydrolase required for the separation of progeny cells and reference the Hett 2008 and the Martinelli 2016 papers. However, the conclusion of the latter paper is that RipA itself is not required for septation, and that the conclusion of the Hett 2008 paper is faulty since their ripA depletion strain is really a ripAB depletion strain. The authors of this revised manuscript make this distinction later on, so it's bit confusing to see this written this way in the introduction.

2. The proper nomenclature for the M smegmatis delta ripA complemented strain is delta ripA/ripA, not delta ripA::ripA, since the complementing wild type ripA gene is not integrated into the chromosome but rather, is expressed from a replicating plasmid, as described in the Martinelli 2016 reference.

Referee #3:

The authors have improved several aspects of the paper.

- The new pulldown experiment nicely shows that the two proteins interact more in acid-stressed cells.

- They make a good point about other proteins potentially inhibiting MarP-mediated cleavage of RipA at neutral pH, and might think about clarifying this in the discussion.

- With respect to the question about expression, they present some nice mental gymnastics instead of simply doing the experiments!

- As to the broader point about why the mutants seem to have so much trouble specifically in an acidic environment, they make some nice points about PG hydrolysis but I was hoping that they would have come up with a creative experiment to really get at this question, and determine if cell separation and/or PG remodeling is needed to survive acid stress. To me this is the most relevant question and the one that deserves answering, but I recognize that it may be outside of the scope of this work.

- Finally, I would urge the authors to consider the following suggestions to ensure accuracy, fairness, and most importantly a sharper focus on their own findings.

Abstract Line 1:
Mycobacterium tuberculosis (Mtb) can persist in the human host in a latent state for decades, in part because it has the ability to withstand numerous stresses imposed by host immunity.

This statement is misleading or at best a non sequitur. What does this have to do with the paper? Furthermore, the recent paper from Levitte et al. that they cite later in the paper expressly provides evidence that mycobacterial (including Mtb) phagosomes can acidify from early in infection and that MarP the subject of this study affects the ability of mycobacteria to establish infection from the very first step. The authors have cited this paper and this fact but then have perhaps not understood its implications fully.

Introduction, pages 2 and 3:

In resting macrophages, Mtb stalls the maturation of the phagosome. However, Mtb fails to do so once macrophages have been activated by the T-cell-derived cytokine IFN-γ, causing acidification of the phagosomal milieu, a stress with which Mtb must cope to survive (MacMicking et al., 2003; Schaible et al., 1998; Via et al., 1998).

This is not so black and white as a substantial proportion of Mtb are found in acidified phagosomes from the beginning of infection in the innate immune phase. The extensive body of data in support of this is reviewed in a recent paper by Levitte et al. that the authors have cited.

2nd Revision - authors' response 28 November 2016

Thank you for your letter from November 21, 2016 containing your and the reviewers' comments on our revised manuscript. We addressed all of the remaining concerns in this newly revised version.

Why cell separation and/or peptidoglycan remodeling is required for survival in acidic conditions is an interesting question. We are actively investigating this question and listed several ongoing lines of investigation in the response letter. However, answering this question requires developing new, technically challenging methodologies and generating new Mtb mutants, which will take at least nine months of work. We will not be able to complete these experiments and answer the above question conclusively within a predictable time frame for inclusion in the current manuscript. We hope you understand.

A point-by-point reply is included that responds to all comments and questions that were raised by the reviewers.

Referee #2:

The authors did a commendable job answering my concerns of the previous version of this manuscript, and I think that they also answered the concerns of the other reviewers. I have two minor but important points that ought to be modified in the current version:

1. Page 3, the authors write that RipA is a PG hydrolase required for the separation of progeny cells and reference the Hett 2008 and the Martinelli 2016 papers. However, the conclusion of the latter paper is that RipA itself is not required for septation, and that the conclusion of the Hett 2008 paper is faulty since their ripA depletion strain is really a ripAB depletion strain. The authors of this revised manuscript make this distinction later on, so it's bit confusing to see this written this way in the introduction.
We agree that the sentence was equivocal and suggested that RipA is essential. We revised the text in the introduction as follows: "Here, we identified RipA - a peptidoglycan hydrolase (Hett et al., 2008; Martinelli and Pavelka, 2016) - as a MarP substrate."

2. The proper nomenclature for the M smegmatis delta ripA complemented strain is delta ripA/ripA, not delta ripA::ripA, since the complementing wild type ripA gene is not integrated into the chromosome but rather, is expressed from a replicating plasmid, as described in the Martinelli 2016 reference.
Thank you for pointing this out. We have corrected this annotation throughout the manuscript.

Referee #3:

The authors have improved several aspects of the paper.
- The new pulldown experiment nicely shows that the two proteins interact more in acid-stressed cells.
- They make a good point about other proteins potentially inhibiting MarP-mediated cleavage of RipA at neutral pH, and might think about clarifying this in the discussion.
We agree that we had not fully discussed this point in the previous version of our manuscript, and have clarified it in the discussion of its latest revised version.

- With respect to the question about expression, they present some nice mental gymnastics instead of simply doing the experiments!

We demonstrated that the interaction of MarP and RipA is stronger at acidic pH compared to neutral pH when both genes are constitutively expressed. We have analyzed the transcriptome of Mtb at pH 4.5 and not found marP or ripA induced (unpublished and not shown). These unpublished findings are in agreement with published work in which neither gene was found upregulated at pH 5.5 (Rohde et al. Cell Host & Microbe 2007; 2(5):352-64). We hypothesize that the enhanced interaction of the two proteins at acidic pH may depend on post-translational modifications of MarP, RipA or both. In addition, MarP's activity may be inhibited by other proteins at neutral pH or is redundant because of other proteases that activate RipA at neutral pH. We will be investigating these hypotheses in future work.

- As to the broader point about why the mutants seem to have so much trouble specifically in an acidic environment, they make some nice points about PG hydrolysis but I was hoping that they would have come up with a creative experiment to really get at this question, and determine if cell separation and/or PG remodeling is needed to survive acid stress. To me this is the most relevant question and the one that deserves answering, but I recognize that it may be outside of the scope of this work.

We agree that it will be important to determine if and why cell separation, PG remodeling or both are required for mycobacterial survival during acid stress. Numerous enzymes may be involved in both of these processes so that distinguishing one from another may not be trivial.

To address these questions, we are exploring various routes of investigation that, as mentioned by the reviewer, are beyond the scope of this manuscript.

- In collaboration with investigators at the Forschungzentrum Borstel, we are evaluating the structure and composition of PG from Mtb WT and Δ*marP* cells subjected to neutral or acidic pH. The purification of PG from mycobacteria that meets the quality criteria for these analyses is challenging and will require further optimization.
- Our experiments suggest that peptidoglycan remodeling may occur at acidic pH. Using confocal photomicroscopy we observed that the fluorescent signal of D-alanine analogs in Msm cells subjected to a prolonged incubation in pH 4.5 may become cytoplasmic (see Figure 2B). In collaboration with Dr. Rhee at Weill Cornell Medicine, we are employing metabolomics to extract PG fragments from the cell cytoplasm, identify their composition and quantify them. These are challenging experiments, but we anticipate that identifying the nature of these fragments will help us to pinpoint the enzymatic activities required to generate them.
- To further investigate the requirement of PG remodeling and cell separation we are generating additional *M. tuberculosis* mutants and will assay their phenotypes in neutral and acidic pH. These include mutants of penicillin binding proteins, transpeptidases and PG hydrolases, particularly RipA and RipB.
-

- Finally, I would urge the authors to consider the following suggestions to ensure accuracy, fairness, and most importantly a sharper focus on their own findings.

Abstract Line 1:

Mycobacterium tuberculosis (Mtb) can persist in the human host in a latent state for decades, in part because it has the ability to withstand numerous stresses imposed by host immunity.

This statement is misleading or at best a non sequitur. What does this have to do with the paper? Furthermore, the recent paper from Levitte et al. that they cite later in the paper expressly provides evidence that mycobacterial (including Mtb) phagosomes can acidify from early in infection and that MarP the subject of this study affects the ability of mycobacteria to establish infection from the very first step. The authors have cited this paper and this fact but then have perhaps not understood its implications fully.

We believe that our statement is scientifically correct and that it is fully in line with the scope of this work, which investigates how Mtb withstands acid stress imposed by host immunity, both innate and adaptive. We furthermore believe it not to be in conflict with the work by Levitte and colleagues. As demonstrated by Levitte et al., phagosome acidification can occur early during infection, but that does not exclude a role for host immunity. Mtb's ability to withstand phagosome

acidification, including early in infection, allows the pathogen to establish chronic infection in animal models and, perhaps, latent infection in humans. Of note, the Mtb marP mutant is able to replicate and expands by almost 2 orders of magnitude following infection of mice (Vandal et al. Nature Medicine, 2008; 14(8):849-54).

Introduction, pages 2 and 3:

In resting macrophages, Mtb stalls the maturation of the phagosome. However, Mtb fails to do so once macrophages have been activated by the T-cell-derived cytokine IFN-γ, causing acidification of the phagosomal milieu, a stress with which Mtb must cope to survive (MacMicking et al., 2003; Schaible et al., 1998; Via et al., 1998).

This is not so black and white as a substantial proportion of Mtb are found in acidified phagosomes from the beginning of infection in the innate immune phase. The extensive body of data in support of this is reviewed in a recent paper by Levitte et al. that the authors have cited.
We have revised the text as follows: Mtb can stall the maturation of the phagosome until macrophages have been activated by the T-cell-derived cytokine IFN-g, enhancing acidification of the phagosomal milieu, a stress with which Mtb must cope to survive (MacMicking et al., 2003; Schaible et al., 1998; Via et al., 1998). Moreover, Mtb and *M. marinum* have been found in acidic phagolysosomes early during infection of mice and zebrafish larvae (Levitte et al., 2016; 20(2):250-8).

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: Sabine Ehrt
Journal Submitted to: The EMBO journal
Manuscript Number: EMBOJ-2016-95028

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 $\chi 2$ 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).

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?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
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
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For datapresented in Figure 1A, 1B and Figure 6A: Q-Q plot and Kolmogorov-Smirnov test rejects normality. Thus, in order to compare the distributions under two different conditions, we conducted a ranksum test, a non-parametric test which does not require specific distribution shape.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
http://figshare.com	Figshare
http://www.ncbi.nlm.nih.gov/gap	dbGAP
http://www.ebi.ac.uk/ega	EGA
http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

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).	MBL D153-11 (anti-GFP magnetic beads). Clontech 632381 (anti-GFP antibody). sigma F3165-5MG (anti-Flag antibody). Thermofisher Scientific 26183 (anti-HA antibody).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	NA