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Strain competition restricts colonization of an enteric pathogen and prevents colitis

Aaron Hecht, Benjamin Casterline, Zachary Earley, Youhg Ah Goo, David Goodlett, and Juliane Bubeck Wardenburg

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Editor: Achim Breiling

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 23 March 2016

Thank you for the submission of your research 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 acknowledge the potential interest of the findings. However, all three referees have raised some points to improve the manuscript or to strengthen the data, in particular referee #1 (points 3 and 4) and Referee #3 (point 3). Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns (as detailed in their reports) must be fully addressed in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

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. 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.

Important: All materials and methods should be included in the main manuscript file.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.
I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Overall interesting paper with several clear colonization phenotypes.

Figure 2B and C: Aren't these redundant with Figure 1? Maybe Figure 1 and 2 and could be combined and S4 brought into Figure 3.

In the results text it is not clear if Figure 3C is in SPF or gnotobiotic animals.

The ability of BF9343_1927 to provide immunity to T6SS is demonstrated in the case of co-colonization in Figure 2, but is not shown in any of the cases of sequential colonization. This data is important because it is the main evidence that the reason the mutant has colonization defects is because of its inability to inject an effector.

Can one observe killing in vitro that supports colonization data? Currently in the paper it seems there are no examples of this, as in cases where T6SS is shown to kill B. theta and B. vulgatus, there is no matching colonization phenotype. Does it kill other strains in vitro? If not, is it because of a lack of expression? Is T6SS induced in vivo? Is it induced by the presence of an invader in vivo?

Referee #2:

This study demonstrates the role of type vi secretion in host colonization resistance against pathogenic and non-pathogenic B. fragilis strains. It also identifies a putative type Vi secretion immunity protein in B. fragilis. Very interesting/solid work. I only have a few minor concerns: Figure 3 A and B; it seems that strain E1 colonizes to the same levels as strain N1 when introduced together. It is confusing that only N1 is labeled. Also, how this compares with experiment presented in figure 2B where co-colonization of wild type N1 and E1 strains results in significantly lower levels of colonization of E1.

It was not clear how levels strains were quantified in invasion experiments where the same strain was introduced.

Referee #3:

In this manuscript Hecht et al. very elegantly examine the role of Bacteroides fragilis type VI secretion system in a competitive setting in the mammalian gut. In particular they looked at the commensal B.f. (N1) activity against the toxigenic B.f. (E1) when the commensal E1 either expresses or not the tssC effector protein. Also, they describe the presence of a possible gene encoding for an immunity protein in the N1 genome, which when expressed in E1 confers the ability to E1 to persist in the gut. They further show that the T6SS is important for developing colonization resistance against exposure of E1 when mice were previously colonized with N1. Moreover, they explored how the T6SS would affect a broader community of Bacteroidetes in a competitive setting, highlighting the complexities of microbe-microbe interactions in vivo.

Overall, this manuscript provides strong evidence for the importance of T6SS in competition within closely related bacteria species and strains. Most importantly, this work highlights the significance of investigating the role of bacterial competition strategies in a complex environment such as the mammalian gut. I have a few suggestions below:

1) The authors speculate that niches that are spatially distinct between different Bac strains could explain the differences in the T6SS effect against diverse strains (as shown in Figure 3 and S4). It would be nice if the authors could visualize the differential distributions of these communities, if tissue samples are available and specific FISH probes can be used.

2) Some questions that the authors may want to consider are the following: is there a differential timing expression of T6SS? Is the T6SS expression in vivo depended upon the presence of a competitor? What happens in more complex communities?

3) The therapeutic side of administration of a commensal B. f. to exclude the toxigenic Bac is very interesting and is potentially clinically relevant. Is there a mouse model in which the authors could test this idea further?

1st Revision - authors' response 29 May 2016

We thank the referees for their insightful and helpful comments, which have enabled significant improvements to our manuscript regarding the competition between non-toxigenic and enterotoxigenic strains of *Bacteroides fragilis*. We now find type VI secretion-dependent competition between *B. fragilis* strains *in vitro* that is congruent with the *in vivo* data shown in the initial submission, further illustrating the direct interaction between non-toxigenic and toxigenic strains. In the initial submission, we identified an immunity gene that, when heterologously expressed in enterotoxigenic *B. fragilis*, protects from type VI secretion killing *in vivo*. To further demonstrate that this effector-immunity pair is critical for this interaction, we now show that mutation of the cognate effector in the non-toxigenic strain phenocopied the type VI secretion mutant. Finally, we now demonstrate that the non-toxigenic strain acts as a probiotic through type VI secretion, protecting the host from the enterotoxigenic strain by reducing exposure to toxin and decreasing intestinal damage in a mouse model of colitis. Given the data added to the manuscript, we have changed the title accordingly to more accurately reflect the central findings of the work.

Referee #1:

Overall interesting paper with several clear colonization phenotypes.

Hecht *et al.* Reply: We appreciate the referee's interest in our study.

Figure 2B and C: Aren't these redundant with Figure 1? Maybe Figure 1 and 2 and could be combined and S4 brought into Figure 3.

Hecht *et al*. Reply: We thank the referee for noting the redundancy of Figures 1 and 2 in the initial submission and the suggestion for rearrangement. In the revised manuscript, the redundancy of the previously labeled Figure 2C has been eliminated and replaced with new data on deletion of the

effector BF9343_1928 (now named Bte2). To emphasize Figure S4, we have denoted this as an Extended View Figure, now Figure EV2.

In the results text it is not clear if Figure 3C is in SPF or gnotobiotic animals.

Hecht *et al*. Reply: Figure 3C was performed in SPF mice, which is now noted in the figure legend and the results section.

The ability of BF9343_1927 to provide immunity to T6SS is demonstrated in the case of co-colonization in Figure 2, but is not shown in any of the cases of sequential colonization. This data is important because it is the main evidence that the reason the mutant has colonization defects is because of its inability to inject an effector.

Hecht *et al*. Reply: To provide further evidence for effector injection as a mechanism of strain competition, we now show that mutation of the cognate effector of BF9343_1927, BF9343_1928 (now named 'Bte2' in recent publication)[1,2] causes a loss of E1 killing both *in vitro* and *in vivo* (Figs 2C and E and EV1I). We additionally find that heterologous expression of BF9343_1927 (now named 'Bti2a') protects E1 *in vitro* as support for the *in vivo* data presented upon initial submission (Fig EV1I). This is further confirmed by recent work demonstrating the importance of this effector-immunity pair in *B. fragilis* strain competition [1,2]. We therefore conclude that the effector Bte2 is injected by N1 through T6S into E1 both *in vitro* and *in vivo*, causing a competitive phenotype that can be mitigated upon heterologous expression of the immunity protein Bti2a in E1. At present, we have not explored the role of Bti2a in sequential colonization. N1 is the only strain in our studies encoding Bte2/Bti2a, however this strain does not exclude E1 from secondary colonization (Fig 3A and B). Therefore, the sequential model is not optimal for testing the involvement of this effector-immunity pair in strain competition.

Can one observe killing in vitro that supports colonization data? Currently in the paper it seems there are no examples of this, as in cases where T6SS is shown to kill B. theta and B. vulgatus, there is no matching colonization phenotype. Does it kill other strains in vitro? If not, is it because of a lack of expression? Is T6SS induced in vivo? Is it induced by the presence of an invader in vivo?

Hecht *et al*. Reply: In our revised manuscript, we present evidence of *in vitro* killing that is congruent with co-colonization. Plate competition assays produce significant killing of E1 by N1, which is relieved upon mutation of the T6SS (Fig EV1I). Moreover, deletion of the effector Bte2 in N1 or heterologous expression of Bti2a in E1 similarly reduces the killing phenotype. Thus, *in vitro* and *in vivo* competition between N1 and E1 are consistent with one another, further emphasizing the importance of T6S and this effector-immunity pair. This data suggests that T6S is active both *in vitro* and *in vivo*, supported by work published since our initial submission [1,2]. The conditions of T6SS expression have not been explored in *Bacteroides*. Induction of T6S in the presence of a T6S-encoding competitor is a known phenomenon in *Pseudomonas aeruginosa* [1-3], but remains to be studied in *B. fragilis*. While we find these topics to be of great interest, we believe they fall outside the scope of the current manuscript.

Referee #2:

This study demonstrates the role of type vi secretion in host colonization resistance against pathogenic and non-pathogenic B. fragilis strains. It also identifies a putative type Vi secretion immunity protein in B. fragilis. Very interesting/solid work. I only have a few minor concerns: Figure 3 A and B; it seems that strain E1 colonizes to the same levels as strain N1 when introduced together. It is confusing that only N1 is labeled. Also, how this compares with experiment presented in figure 2B where co-colonization of wild type N1 and E1 strains results in significantly lower levels of colonization of E1.

Hecht *et al*. Reply: We thank the referee for her/his interest in our work and apologize for the confusion regarding Figure 3A and B. In this experiment, primary colonization with N1 (closed squares, upper lines) eliminates secondary challenge with the same N1 strain (closed squares, lower lines). However, primary colonization with N1 (open squares, upper lines) is unable to completely

eliminate E1 secondary challenge (open squares, lower lines). In this experiment, both sets of mice are initially colonized by N1, thus only N1 is labeled in the top lines. To be clear, in this experiment there is no co-colonization of N1 and E1; the inoculation of the two strains is separated by time. Thus, these data are not contradictory with Figure 2B. To better convey this point, Figure 3A and B have been slightly altered, labeling the arrow at the point of inoculation with the strains used.

It was not clear how levels strains were quantified in invasion experiments where the same strain was introduced.

Hecht *et al*. Reply: Sequential colonization using the same challenge strain as the primary colonizing strain was accomplished through expression of differential plasmid-encoded antibiotic resistance markers. The utility of this technique has been previously published [4] and was confirmed in our work through PCR-based genomic identification of the primary and secondary strains (Appendix Figure S1). This technique is noted in the revised text in the results and methods sections.

Referee #3:
In this manuscript Hecht et al. very elegantly examine the role of Bacteroides fragilis type VI secretion system in a competitive setting in the mammalian gut. In particular they looked at the commensal B.f. (N1) activity against the toxigenic B.f. (E1) when the commensal E1 either expresses or not the tssC effector protein. Also, they describe the presence of a possible gene encoding for an immunity protein in the N1 genome, which when expressed in E1 confers the ability to E1 to persist in the gut. They further show that the T6SS is important for developing colonization resistance against exposure of E1 when mice were previously colonized with N1. Moreover, they explored how the T6SS would affect a broader community of Bacteroidetes in a competitive setting, highlighting the complexities of microbe-microbe interactions in vivo.
Overall, this manuscript provides strong evidence for the importance of T6SS in competition within closely related bacteria species and strains. Most importantly, this work highlights the significance of investigating the role of bacterial competition strategies in a complex environment such as the mammalian gut. I have a few suggestions below:

1) The authors speculate that niches that are spatially distinct between different Bac strains could explain the differences in the T6SS effect against diverse strains (as shown in Figure 3 and S4). It would be nice if the authors could visualize the differential distributions of these communities, if tissue samples are available and specific FISH probes can be used.

Hecht *et al*. Reply: We thank the referee for her/his detailed analysis of our studies. We concur that imaging of spatially distinct niches would be of considerable interest and represents a substantial advance in the field, however, technology for differential detection of strains *in vivo* is currently lacking. While FISH has been used successfully to image *B. fragilis*, this was accomplished with FISH probes against 16S rRNA [5]; the similarity in sequence between *B. fragilis* strains in this study makes this approach intractable. We have attempted to image *B. fragilis* through overexpression of fluorescent reporters, but to date, these have failed to produce a significant signal. Future work to develop such technology will be required to successfully accomplish these studies.

2) Some questions that the authors may want to consider are the following: is there a differential timing expression of T6SS? Is the T6SS expression in vivo depended upon the presence of a competitor? What happens in more complex communities?

Hecht *et al*. Reply: We likewise find interest in possible timing and induction conditions of T6S during colonization and in complex communities. T6SSs characterized in *Vibrio cholerae* and *P. aeruginosa* require specific environmental conditions for optimal transcription, and the presence of competitors enhances firing of the system [3]. Our work demonstrates that the *B. fragilis* T6SS is active both *in vitro* and *in vivo*. Recent studies show that additional complexity in bacterial communities *in vivo* dampen the number of *B. fragilis* competition events as a result of T6S [1]. Future work will be required to determine the role of T6SS expression in these contexts and to identify induction conditions.

3) The therapeutic side of administration of a commensal B. f. to exclude the toxigenic Bac is very interesting and is potentially clinically relevant. Is there a mouse model in which the authors could test this idea further?

Hecht *et al*. Reply: We thank the referee for her/his insightful comments on the potential therapeutic implications of the work. In the revised manuscript, we have taken four approaches to determine the health impact of competition between non-toxigenic and toxigenic *B. fragilis* strains. First, we determined the quantity of *bft* mRNA present in the feces during competition of E1 with N1 WT or N1 Δ*tssC* (Fig 5A). We find that N1 WT is able to significantly reduce the toxin expressed relative to the T6S-deficient mutant. Second, to determine if this increased toxin expression manifests as a concomitant host response, we examined the quantity of anti-BFT IgG in the mouse serum after colonization (Fig 5B). We show that N1 WT significantly decreases the anti-BFT IgG compared to N1 Δ*tssC*, suggesting that the host exposure to toxin is modulated by T6S. Third, using a mouse model in which susceptibility to colitis is elicited by dextran sodium sulfate (DSS) treatment, we examined the gross intestinal morphology after infection with E1-only or competition with N1 (Figs 5C and EV4A). Cecum size and architecture changed dramatically in the E1-only inoculated mice, demonstrating shrinking, overt blood and decreased weight. These signs of inflammatory injury were ameliorated by competition with N1 WT. While N1 Δ*tssC* provided partial relief from E1-induced damage, cecal weight remained significantly lower than N1 WT, showing that T6S is partially required for protection. Fourth, we examined the histopathology of the ceca and colons from these mice and found that while the E1-only condition produced significant ulceration in both tissues, including loss of crypts, denudation of the epithelium and inflammatory infiltrates, competition with N1 WT completely restored these phenotypes to normal (Figs 5D-F and EV4B-D). Therefore, we conclude that *B. fragilis* strain competition provides significant beneficial effects via suppression of host exposure to BFT, and may provide a framework for targeted therapeutic probiotics. Associated with these changes, the following text was added to the results section:

Results: ETBF colonization is associated with IBD and colitis in humans, experimentally validated in mouse model systems [6-8]. We hypothesized that strain competition may favorably alter the health of the host through reduced exposure to toxigenic organisms. Co-colonization of E1 with WT N1 reduced BFT transcript present in the feces by approximately 100-fold relative to competition with N1 Δ*tssC*, congruent with the difference in E1 fecal CFU (Fig 5A; compare to Fig 1A, B and D). A concomitant decrease in mouse anti-BFT serum IgG was observed, reminiscent of observations in ETBF-exposed humans (Fig 5B) [8]. Infection of mice with ETBF exacerbates a BFT-dependent, IBD-like colitis induced by dextran sodium sulfate (DSS) administration [7]. Colonization with E1 in this model causes significant intestinal injury, indicated by the presence of visible blood in the ceca, substantial tissue contraction and reduced cecal weight relative to sham-inoculated animals (Figs 5C and EV4A). We hypothesized that N1 competition would reduce the disease burden in this model, consistent with suppression of the E1 burden by WT N1 (Fig 1A). Indeed, co-colonized mice did not exhibit evidence of cecal injury (Figs 5C and EV4A). Competition with N1 Δ*tssC* also reduced inflammation associated with E1 colonization, but not as effectively as N1 WT co-colonization, indicating that T6S is required for full protection (Fig 5C). Examination of both cecal and colonic histopathology revealed severe ulcerations in mice mono-colonized with E1, demonstrated by loss of crypts, epithelial denudation, and the presence of inflammatory cell infiltration (Figs 5D-F and EV4B-D). Competition of E1 with N1 WT provided complete protection from damage throughout the length of the cecum and colon. These data demonstrate that the N1 T6SS affords the host significant protection from E1 colonization and BFT-induced injury.

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3. Basler M, Ho BT, Mekalanos JJ (2013) Tit-for-Tat: Type VI Secretion System Counterattack

during Bacterial Cell-Cell Interactions. *Cell* **152**: 884–894.
4. Lee SM, Donaldson GP, Mikulski Z, Boyajian S, Ley K, Mazmanian SK (2013) Bacterial colonization factors control specificity and stability of the gut microbiota. *Nature* **501**: 426– 429.
5. Huang JY, Lee SM, Mazmanian SK (2011) The human commensal *Bacteroides fragilis* binds intestinal mucin. *Anaerobe* **17**: 137–141.
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8. Sears CL, Islam S, Saha A, Arjumand M, Alam NH, Faruque AS, Salam MA, Shin J, Hecht D, Weintraub A, et al. (2008) Association of enterotoxigenic *Bacteroides fragilis* infection with inflammatory diarrhea. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* **47**: 797–803.

2nd Editorial Decision 13 June 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the enclosed reports on it. As you will see, all three referees find the manuscript suitable for publication in EMBO reports. Before we can proceed with the formal acceptance of your manuscript, I would like to ask you for some very minor revisions.

For a short report, we usually require that the results part and the discussion are combined to one section (Results and Discussion). Could you please do so? We also require a conflict of interest statement, which should be included after the acknowledgements. Please also insert page numbers to your manuscript. Finally, it appears that the third panel (N1 WT, E1 WT) of Fig. EV4A is cut at the right end. Maybe you can replace this?

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have thoughtfully and thoroughly addressed my concerns. I have no more comments and look forward to seeing the paper published.

Referee #2:

my concerns were addressed.

Referee #3:

I confirm my very positive opinion on this work. The authors have also provided additional data that further improved the manuscript. This is a novel and important study, which will be of great interest to the field.

2nd Revision - authors' response 18 June 2016

Thank you for the email – we are delighted on the favorable reviews of our paper! I have attached the revised word doc as you note below, as well as the revised figure.

3rd Editorial Decision 21 June 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.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Juliane Bubeck Wardenburg
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42282V1

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?	Manuscript draft P. 13, Methods section
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C- Reagents

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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.	Manuscript draft P. 13, Methods section
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Manuscript draft P. 13, Methods section
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.	Manuscript draft P. 13, Methods section. We confirm compliance with the recommended guidelines.

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	Manuscript draft P. 12, Methods section
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