EMBO
Molecular Medicine

Manuscript EMM-2016-06690

Sodium permeable and 'hypersensitive' TREK-1 channels cause ventricular tachycardia

Niels Decher, Beatriz Ortiz-Bonnin, Corinna Friedrich, Marcus Schewe, Aytug K Kiper, Susanne Rinné, Gunnar Seemann, Rémi Peyronnet, Sven Zumhagen, Daniel Bustos, Jens Kockskämper, Peter Kohl, Steffen Just, Wendy González, Thomas Baukrowitz, Birgit Stallmeyer, Eric Schulze-Bahr

Corresponding author: Niels Decher, Philipps-University Marburg

Review timeline:		
	Submission date:	09 June 2016
	Editorial Decision:	25 July 2016
	Revision received:	24 October 2016
	Editorial Decision:	24 November 2016
	Revision received:	19 January 2017
	Accepted:	23 January 2017

Editor: Céline Carret

Transaction Report:

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

1st Editorial Decision   25 July 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. Although the referees find the study to be of interest, they also raise a number of concerns that need to be addressed in the next final version of your article.

You will see from the comments pasted below that all referees find the study interesting and novel. While referees 1 and 3 are rather enthusiastic and only have suggestions to improve clarity and conclusiveness, referee 2 is a little more demanding and would like to see more mechanism. We believe that indeed the paper would be much stronger with additional mechanistic insights to explain ventricular arrhythmia in light of the mutation but also provide the additional data to strengthen the translational aspect of the work as underlined by referee 3.

Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible. EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Remarks):

This paper by Decher et al that describes a mutation in TREK-1 as responsible for a common form of ventricular tachycardia is very interesting. This is an original finding as this channel has not yet been involved in a congenital form of cardiac arrhythmia and the functional consequence of the mutation, a loss-of-selectivity, is also unusual. The main weakness is the fact that the mutation concerns only one patient and that family members are not available for genotyping. However, one can anticipate that the publication of this mutation will trigger the research of such mutations in many other patients over the world that will confirm (or not) the involvement of TREK-1 channels in this particular type of cardiac arrhythmia.
The experiments are well designed to address most of the issues.

General comments:
The data are quite interesting but lacking clarity. This paper is hard to follow indeed. The paper may benefit from the correction by a native English speaker to improve the writing. For example, one cannot say, "TREK-1 had a linear relationship" (page 8) or "extracellular Na+ introduces a Na+ permeability and a partial reduction of outward currents in TREK-1 I267T" (page 8).
TREK-1 is highly expressed in the nervous system and in adrenal gland cortex. Surprisingly, no neurological or adrenal disorder is observed in the patient. The hypothesis proposed by the authors, i.e. an heteromerization with TREK-2 is very unlikely in the adrenal gland as TREK-2 expression in adrenal cortex is very low, more than one thousand fold lower than TREK-1 (Choi et al 2011, Science vol 331, Table S4).
As another general concern, the authors may comment on the fact that RVOT-VT arises under ß-adrenergic stimulation, i.e. under elevation of the intracellular cAMP, which is a condition in which TREK-1 should be inhibited. Did the authors test a ß-adrenergic stimulation in HL-1 cells overexpressing the mutant TREK-1?

Specific comments:
Fig 1E: qPCR data. It would be more interesting to compare the level of expression of TREK1 with other classic cardiac K+ channels such as KCNH2, KCNQ1 or KCNA5, etc...As it is, this Fig1E does not present a real interest to appreciate the importance of the TREK-1 channel in the electric activity of ventricular or atrial cardiac cell.

The {section sign} on page 7 should not be entitled "Dominant-negative Loss-of-function". The I267T TREK-1variant produces less current than the WT, but not because of a loss-of-function. This current reduction results from the loss-of-selectivity, and with 140mM external Na+ ions, there is an inward Na+ current that develops together with the outward K+ current, thus making the measured outward current smaller. This cannot be called a loss-of-function. When the external Na+ is replaced by NMDG, the I267T TREK-1 current is not reduced anymore (fig 3D and 3F). By the way, the authors explain this on page 8.

I am not sure that chapter on the structural considerations on the binding of Na+ and K+ ions into the selectivity filter is necessary here (second {section sign} page 9, from "While K+ ions ... to Channel selectivity and conductivity."). It is sufficient to state that the molecular dynamic simulations are in agreement with the loss of selectivity effect of the I267T mutation, the paper is already hard enough to follow without this structural aspect.

The same comment may apply for the large discussion on the stretch-activated gating of TREK-1 (page 13-14). Such a detailed structural discussion may not be appropriate for a journal like Embo Molecular Medicine.

The authors explain the enhancement of EADs in HL1-cells overexpressing the TREK-1 I267T channel by a Ca2+ overload due to the Na+ entry triggering the reverse mode of the Na+/Ca2+ exchanger. This explanation is indeed likely, but cannot be exposed in the abstract as if it was experimentally demonstrated. Ca2+ imaging experiments are necessary to prove that assertion. Therefore, this explanation could be debated in the discussion section, not in the results and certainly not as evidence in the abstract.

Referee #2 (Remarks):

I reviewed the manuscript N. Decher et al. entitled "Sodium permeable and hypersensitive TREK-1 channels cause ventricular tachycardia".

In total 438 patients with various arrhythmias were screened for the presence of a KCNK2 gene mutation. In an over 40 years old patient ventricular tachycardia, emerging from the right ventricular outflow tract, without a morphological substrate was detected. Gene analysis revealed a heterozygous punctual mutation of the K2P potassium channel TREK-1.
Xenopus oocytes as well as HL-1 cells were transduced with equal amounts of TREK-1 and TREK-1l267T cRNA to mimic the heterozygous state.
Transduced oocytes showed reduced K+ currents and leakage for Na+ ions. The authors claim that a NCX mediated intracellular Ca2+ overload may be responsible for the observed arrhythmias.
Using TREK-1 blockers (fluoxetine, verapamil, 2-APB, riluzole, BL-1249) only BL-1249 restored channel function, most likely due to a different binding site, as authors claim.
Mechanical stress, applied via the patch clamp pipette in inside out patches, provoked higher peak currents at less negative pressure and desensitization of the stretch induced currents in TREK-1 cells was much slower than in TREK-1l267T cells.

Comments:
This is a potentially interesting manuscript. However, as it stands, the mechanisms as to how the ventricular arrhythmias are evoked remain unclear and the evidence provided is merely correlational.

1) Is the, for in vitro experiments used TREK-1l267T mutation identical to the TREK-1 mutation found in the patient?

2) It is particularly surprising that the authors are utilizing HL-1 cells as expression model, as this is an atrial tumor cell line. Instead, either hiPS cells or rodent ventricular cells need to be used as expression model to characterize the functional consequences of overexpressing the mutant ion channel.

3) In addition the Na-Ca exchanger and also cytosolic Ca2+ needs to be directly measured to underscore the working hypothesis of the proposed disease mechanism.

4) The stepwise applied mechanical negative pressure of 0 to -80 mmH2O, applied by the patch clamp pipette to a very limited area of the cell membrane, is not comparable to the dynamic stress, applied to the cardiomyocytes of the RVOT during the cardiac cycle. Authors could cultivate TREK-1l267T cells on an elastic membrane, apply physiologic stress to the cellular syncytium and monitor electrophysiological characteristics.

5) Why the clinically observed arrhythmias become present at an age over 40 years and not in younger patients?

Referee #3 (Remarks):

Decher et al

This is a lovely submission that studies a point mutation identified in a patient with RVOT tachycardia in the TREK-1 channel. The study shows that the missense mutation alters the ion selectivity of the channel and modifies its sensitivity to mechanical stretch, two effects that would

be expected to predispose to the arrhythmia observed in the patient. Moreover, a channel activator that might prove to be a lead for therapy is identified. The manuscript is clearly written. There are a small handful of minor issues in the text to address and a two valuable bits of data would make the paper even stronger (points 6 and 8).

1. Page 7. The statement "the mutation is likely to act in a dominant-negative manner" is not well supported at this point in the paper, since decreased current could be due to an additive effect-full dominance with a dimer should decrease in current by 75% and it appears to be down only 50%. A softer assertion here could be followed by a return to the conclusion when they assess other effects that also support the notion (Em changes with substitution, effect of the activator, etc).
2. Page 9. I understand the meaning of the statement that the TWIK-like SF "is in agreement with the sodium permeability observed with TWIK-1" but it is a bit non-specific for the general reader since the change in TWIK takes many minutes to develop under pathological conditions and stated this way it sounds like it is normal TWIK operation to pass sodium.
3. Page 9. "The oxygens of the I267 carbonyl backbone coordinate..." The conclusions should be a bit less definitive since the resolution is low in the structure.
4. Page 3 or 4. Somewhere near the beginning of the paper TREK-1 should be identified with its formal IUPHAR name, K2P2, so the literature is kept clear.
5. Page 10. The nice consideration about sodium overload, calcium overload, and excess Na/Ca exchanger function is stated too much like a conclusion and is in the results. It should be noted to be a hypothesis (and maybe put in the discussion) or else intracellular Na, Ca, and exchanger function should be studied.
6. Page 11. The BL-1249 findings are really quite nice. It would be valuable to show if the activator changes the selectivity of the mutant (and if this explains the increase in current).
7. Page 11. You might cut the word "high" from before the "sensitivity to mechanical stretch" since it overstates the characteristic compared to other stretch sensitive channels and that is not necessary to make any of your strong points.
8. Page 11. It would be valuable to show if stretch changes the selectivity of the mutant (and if this explains the increase in current).
9. Citations. It seems an odd not to cite Thomas et al Neuron 2008 as proof that sodium selectivity is not just seen in the past for TWIK-1 but for TREK-1 as studied here (they showed developmental regulation by ATI produces a truncated version of TREK in the brain that can pass sodium).

1st Revision - authors' response   24 October 2016

Referee #1:

This paper by Decher et al that describes a mutation in TREK-1 as responsible for a common form of ventricular tachycardia is very interesting. This is an original finding as this channel has not yet been involved in a congenital form of cardiac arrhythmia and the functional consequence of the mutation, a loss-of-selectivity, is also unusual. The main weakness is the fact that the mutation concerns only one patient and that family members are not available for genotyping. However, one can anticipate that the publication of this mutation will trigger the research of such mutations in many other patients over the world that will confirm (or not) the involvement of TREK-1 channels in this particular type of cardiac arrhythmia. The experiments are well designed to address most of the issues.

Thank you for reviewing the manuscript and for your positive comments. Please note that we have gained within the process of the revision additional valuable insights into the disease causing mechanism of the TREK-1^{I267T} mutation. (1) RVOT-VTs occur predominantly under sympathetic stimulation. Strikingly, we found that while wild-type TREK-1 currents are almost fully suppressed by ß$_1$-adrenoreceptor stimulation, the TREK-1^{I267T} mutant is not responding in the typical manner, the aberrant Na$^+$ conductivity persists and most importantly there are even more pronounced inward sodium leak currents with an additional depolarization of the cells. Thus, under sympathetic stimulation the depolarizing impact of the mutant is more pronounced as under baseline conditions, an effect that might, in the patient reported, contribute to episodes of VTs under stress conditions.
(2) In addition, we have included more electrophysiological data using HL-1 cells, revealing that the TREK-1^{I267T} mutation strongly slows the upstroke velocity of the action potential, an effect that is known to contribute to ventricular re-entry arrhythmias due to conduction velocity reduction and thus a shorter wavelength. (3) We have included a model of the impact of the TREK-1^{I267T} mutant

on the human ventricular action potential. This data is consistent with our electrophysiological findings in HL-1 cells and predicts a secondary Ca^{2+} overload in cardiomyocytes.

General comments:
The data are quite interesting but lacking clarity. This paper is hard to follow indeed. The paper may benefit from the correction by a native English speaker to improve the writing. For example, one cannot say, "TREK-1 had a linear relationship" (page 8) or "extracellular Na^+ introduces a Na^+ permeability and a partial reduction of outward currents in TREK-1 I267T" (page 8).
Thank you for noticing these poor expressions, which were corrected in the revised manuscript. In addition, Prof. Michael Sanguinetti (Salt Lake City, University of Utah) checked the writing, to involve a native speaker, as suggested. Although referee 3 already stated that the manuscript was clearly written, we think that we have strongly improved the writing and are now much more down to the point. Please note that in the revised manuscript the corrections by the native speaker are not highlighted in gray, in order that only the changes to the content of the paper become evident. Only the changes to these two examples you mentioned are marked.

TREK-1 is highly expressed in the nervous system and in adrenal gland cortex. Surprisingly, no neurological or adrenal disorder is observed in the patient. The hypothesis proposed by the authors, i.e. an heteromerization with TREK-2 is very unlikely in the adrenal gland as TREK-2 expression in adrenal cortex is very low, more than one thousand fold lower than TREK-1 (Choi et al 2011, Science vol 331, Table S4).
We agree that the mechanism of heteromerization with TREK-2 are more likely involved in the lack of neuronal than adrenal phenotypes. In the revised Discussion section we now state:
"*As the TREK-2 expression is also relatively low in the adrenal cortex (Choi et al., 2011, PMID: 21311022), other mechanisms must contribute to the lack of an adrenal phenotype, i.e. heteromerization with other K_{2P} channels expressed in the adrenal cortex like TWIK-1 (Hwang et al., 2014, PMID: 24496152; Choi et al., 2011, PMID: 21311022) or dominant-negative splice variants as TREK-1e (Rinné et al., 2014, PMID: 24196565)*".
As an additional note, the expression data provided by Choi *et al.* in Supplemental Table 4 are shown in $\log_2$ scale and not the logarithm to the base 10 (decimal or Briggsian logarithm). Thus, with NM_021161 *KCNK10* = 5.63 and NM_001017425 *KCNK2* = 10.36 we calculate a 26.5-fold higher expression for TREK-1 ($2^{4.73}$) and not a more than thousand fold difference.

As another general concern, the authors may comment on the fact that RVOT-VT arises under ß-adrenergic stimulation, i.e. under elevation of the intracellular cAMP, which is a condition in which TREK-1 should be inhibited. Did the authors test a ß-adrenergic stimulation in HL-1 cells overexpressing the mutant TREK-1?
Thank you, this is an excellent comment. Please note that the original version of the manuscript already contained measurement under ß-adrenergic stimulation in HL-1 cells. The changes in beating frequency observed in the original Fig. 4B were observed in the presence of 100 µM norephinephrine which is part of the original Claycomb media of HL-1 cells, presumably causing a combined activation of adrenergic α- and β-receptors which are expressed in this cell type (Filipeanu *et al.*, 2006, PMID: 16484224; McWhinney *et al.*, 2000, PMID: 11195782). The information that these recordings were performed under sympathetic stimulation is now provided in the revised Figure legend.
We performed additional experiments probing whether ß$_1$-receptor stimulation alters the selectivity of TREK-1^{I267T}. The new data is now included at the end of the Results section and the new Figure 7, which reads:
"*Altered β1-adrenoreceptor modulation in TREK-1^{I267T} channels*
Attacks of RVOT-VTs arise under ß-adrenergic stimulation, therefore we tested how ß$_1$-adrenergic stimulation influences wild-type and TREK-1^{I267T} channels co-expressed with the ß$_1$-adrenoreceptor in oocytes. Strikingly, while wild-type TREK-1 currents are almost fully suppressed by ß$_1$-adrenoreceptor stimulation (Fig 7), the TREK-1^{I267T} mutant is not responding in the typical manner (Fig 7A-C), the aberrant Na^+ conductivity persists (Fig 7B) and most importantly there are even more pronounced inward leak currents (Fig 7D and E) with an additional depolarization of the cells (Fig 7B). Thus, under sympathetic stimulation the depolarizing impact of the mutant is more pronounced as under baseline conditions, an effect that might in our particular patient contribute to episodes of VTs under stress conditions."

Specific comments:

Fig 1E: qPCR data. It would be more interesting to compare the level of expression of TREK1 with other classic cardiac K^+ channels such as KCNH2, KCNQ1 or KCNA5, etc...As it is, this Fig1E does not present a real interest to appreciate the importance of the TREK-1 channel in the electric activity of ventricular or atrial cardiac cell.

Thank you. We agree that the data could in this form only re-confirm that there is a ventricular expression of TREK-1 in the human heart, not more. As in the initial cloning of mouse and human TREK-1 only a poor cardiac expression was reported (Fink *et al.*, 1996, PMID: 9003761; Meadows *et al.*, 2000, PMID: 10784345), these experiments were initially designed to re-confirm the ventricular TREK-1 expression. As in the meantime other groups also found a strong expression of TREK-1 in the human heart (Hund et al., 2014, PMID: 24445605), the data does not provide much news value, and we have removed this data from the manuscript (Figure 1 and Results).

The {section sign} on page 7 should not be entitled "Dominant-negative Loss-of-function". The I267T TREK-1 variant produces less current than the WT, but not because of a loss-of-function. This current reduction results from the loss-of-selectivity, and with 140mM external Na^+ ions, there is an inward Na^+ current that develops together with the outward K^+ current, thus making the measured outward current smaller. This cannot be called a loss-of-function. When the external Na^+ is replaced by NMDG, the I267T TREK-1 current is not reduced anymore (fig 3D and 3F). By the way, the authors explain this on page 8.

Yes, you are right, although we mechanistically revealed at a later point in the manuscript why the TREK-1^{I267T} conducts less outward currents, we did not state this in the first electrophysiological Result section. This was done intentionally, as the experiments to proof that the apparently reduced K^+ outward currents mechanistically result from a gain of inward Na^+ currents, are provided only at a later point in the manuscript. Yet, we understand that the title should be changed to avoid ambiguity. We have re-named the section to "*Reduced outward currents of homomeric TREK-1^{I267T} and heteromeric TREK-1/TREK-1^{I267T}*". The statement of negative dominance was also removed from the title, as suggested by Referee 3. Just a small note, the physiological external Na^+ concentration in these oocyte experiments were 96 mM.

I am not sure that chapter on the structural considerations on the binding of Na^+ and K^+ ions into the selectivity filter is necessary here (second {section sign} page 9, from "While K^+ ions ... to Channel selectivity and conductivity."). It is sufficient to state that the molecular dynamic simulations are in agreement with the loss of selectivity effect of the I267T mutation, the paper is already hard enough to follow without this structural aspect.

Thank you for this suggestion of how to improve the readability of the manuscript. We have followed your request and removed this section. Instead we now placed at the end of the preceding section "*Na^+ permeability in TREK-1^{I267T}*" a statement as suggested: "*In addition, we have performed Molecular Dynamic simulations which are also in agreement with the loss of selectivity effect of TREK-1^{I267T} (Appendix Fig S4).*"

The same comment may apply for the large discussion on the stretch-activated gating of TREK-1 (page 13-14). Such a detailed structural discussion may not be appropriate for a journal like EMBO Molecular Medicine.

The detailed structural discussion is designed to explain the molecular mechanism for the increased stretch-sensitivity of TREK-1^{I267T} which might be a main mechanism causing the disease. Therefore, we think it belongs to the subject of Molecular Medicine. Besides the impact of the altered stretch-sensitivity of TREK-1 for this disorder, the mutant is highly relevant for the understanding of the mechanism of stretch activation in ion channels, information that we really would like to highlight.

The authors explain the enhancement of EADs in HL1-cells overexpressing the TREK-1 I267T channel by a Ca^{2+} overload due to the Na^+ entry triggering the reverse mode of the Na^+/Ca^{2+} exchanger. This explanation is indeed likely, but cannot be exposed in the abstract as if it was experimentally demonstrated. Ca^{2+} imaging experiments are necessary to prove that assertion. Therefore, this explanation could be debated in the discussion section, not in the results and certainly not as evidence in the abstract.

As suggested we have removed this putative explanation towards the mechanisms that is leading to the RVOT-VT arrhythmias from the Abstract and Results section. Although we did not state that HL-1 cells have an enhancement of EADs, we agree that we did not provide experiments or data to proof the idea of a secondary Ca^{2+} overload that can trigger EADs experimentally. Yet, as it is commonly accepted that an increased Na^+ permeability is very likely to induce exactly these

secondary effects, these `speculations´ are now still, but exclusively, placed once at the end of the Discussion section.

To further support our effects observed in HL-1 cells, we have performed computational action potential modeling using a human ventricular myocyte model described by ten Tusscher (Appendix Material and Methods and Appendix Fig S6). These data were consistent with our experimental observations, and a mild depolarization and a strong slowing of the upstroke velocity was observed (Appendix Fig S6). Our action potential modeling provides also some information towards putative changes in the intracellular Ca^{2+} levels by TREK-1^{I267T}. The model was based on findings that TREK-1 in rat ventricular myocytes conducts about 1.5 pA/pF at +30 mV (Bodnár $et\ al.$ 2015, PMID: 25539776) and our observation that TREK-1^{I267T} has a sodium permeability of 21%. According to Bodnar $et\ al.$ when applying the electrodiffusion model developed by Goldman, Hodgkin and Katz (Hille 1992), a ventricular I_{TREK} should have at -15 mV, a potential between the E_{Na} and E_K, an amplitude of about 0.63 pA/pF. A respective sodium inward leak due to TREK-1^{I267T} at -15 mV was assigned with 20% of the amplitude and the opposite polarity (-0.13 pA/pF). The leak current at different potentials was subsequently calculated with a simple background current equation. Using this additional, pathological sodium background current the model predicts an increase in maximum intracellular calcium concentration of 25% (Appendix Fig S6B), maximum sarcoplasmic reticulum calcium concentration of 9% (Appendix Fig S6C), maximum intracellular sodium concentration of 17% (Appendix Fig S6D) and increased outward currents of the sodium calcium exchange current during the systole of 60.9%, reflecting an increased Ca^{2+} uptake in the reverse mode (Appendix Fig S6J). Given the limitations of a mathematical model, including that the precise magnitude of the human ventricular I_{TREK} is still unknown, we use this model only to mechanistically confirm our electrophysiological data obtained with HL-1 cells. Therefore, as described above we have limited the speculations towards a secondary calcium overload to a hypothesis which is positioned only in the last paragraph of the discussion.

Reference: B. Hille, Ionic channels of excitable membranes, Sinauer Associates, 2 edition, 1992.

Referee #2 (Remarks):

I reviewed the manuscript N. Decher et al. entitled "Sodium permeable and hypersensitive TREK-1 channels cause ventricular tachycardia". In total 438 patients with various arrhythmias were screened for the presence of a KCNK2 gene mutation. In an over 40 years old patient ventricular tachycardia, emerging from the right ventricular outflow tract, without a morphological substrate was detected. Gene analysis revealed a heterozygous punctual mutation of the K_{2P} potassium channel TREK-1. Xenopus oocytes as well as HL-1 cells were transduced with equal amounts of TREK-1 and TREK-1^{I267T} cRNA to mimic the heterozygous state. Transduced oocytes showed reduced K^+ currents and leakage for Na^+ ions. The authors claim that a NCX mediated intracellular Ca^{2+} overload may be responsible for the observed arrhythmias. Using TREK-1 blockers (fluoxetine, verapamil, 2-APB, riluzole, BL-1249) only BL-1249 restored channel function, most likely due to a different binding site, as authors claim. Mechanical stress, applied via the patch clamp pipette in inside out patches, provoked higher peak currents at less negative pressure and desensitization of the stretch induced currents in TREK-1 cells was much slower than in TREK-1^{I267T} cells.

Thank you for reviewing our manuscripts and the positive criticism. Just to be mechanistically clear, we did not transduce oocytes, they were injected with needles to ensure that a precise amount of wild-type or a mix of wild-type plus mutant cRNAs are precisely delivered to every individual oocyte. Please also note that we have gained within the process of the revision additional valuable insights into the disease causing mechanism of the TREK-1^{I267T} mutation. (1) RVOT-VTs occur predominantly under sympathetic stimulation. Strikingly, we found that while wild-type TREK-1 currents are almost fully suppressed by ß$_1$-adrenoreceptor stimulation, the TREK-1^{I267T} mutant is not responding in the typical manner, the aberrant Na^+ conductivity persists and most importantly there are even more pronounced inward sodium leak currents with an additional depolarization of the cells. Thus, under sympathetic stimulation the depolarizing impact of the mutant is more pronounced as under baseline conditions, an effect that might, in the patient reported, contribute to episodes of VTs under stress conditions. (2) In addition, we have included more electrophysiological data using HL-1 cells, revealing that the TREK-1^{I267T} mutation strongly slows the upstroke velocity of the action potential, an effect that is known to contribute to ventricular re-entry arrhythmias due to conduction velocity reduction and thus a shorter wavelength. (3) We have included a model of the impact of the TREK-1^{I267T} mutant on the human ventricular action potential. This data is consistent

with our electrophysiological findings in HL-1 cells and predicts a secondary Ca^{2+} overload in cardiomyocytes.

Comments:
This is a potentially interesting manuscript. However, as it stands, the mechanisms as to how the ventricular arrhythmias are evoked remain unclear and the evidence provided is merely correlational.

1) Is the, for in vitro experiments used TREK-1^{I267T} mutation identical to the TREK-1 mutation found in the patient?
Yes! It is the idea of the electrophysiological experiments to mimic exactly the mutational state and channel form present in the patient. The only exception was for the recording of the HL-1 cells which are transfected with a TREK-1 channel that carries an N-terminal EGFP-tag. For the revision we repeated the experiments in HL-1 cells with untagged TREK-1 and TREK-1^{I267T} which however gave basically the same result and thus the data were not included in the Results section of the revised manuscript.

2) It is particularly surprising that the authors are utilizing HL-1 cells as expression model, as this is an atrial tumor cell line. Instead, either hiPS cells or rodent ventricular cells need to be used as expression model to characterize the functional consequences of overexpressing the mutant ion channel.
Thank you for this suggestion, however our experience with hiPSCs was discouraging, as the cells appear to be only in a good condition, when they are in a monolayer and isolated single cells quickly seem to `round up´ and apparently go into apoptosis. Also the electrophysiological fingerprint was somewhere between an atrial and ventricular cell type (incomplete differentiation), so that we did not see the advantage for our study to use hiPSCs. We agree that using ventricular cardiomyocytes might be advantageous, however, adult ventricular cardiomyocytes are very difficult to transfect with an appropriate efficiency and thus would rather demand a viral transduction. The most suitable approach would be using adult ventricular myocytes from a transgenic TREK-1^{I267T} knock-in mouse, however the latter two approaches would represent a complete and separate story by itself. Given all these pitfalls it was the most suitable approach to use the atrial HL-1 mouse cardiomyocyte cell line. In addition, we have good experience with the electrophysiology of the HL-1 cells and can maintain and transfect them with relatively high efficiency, while they remain in the spontaneously beating stage.

3) In addition the Na-Ca exchanger and also cytosolic Ca^{2+} needs to be directly measured to underscore the working hypothesis of the proposed disease mechanism.
As suggested by Referee 1 we have removed this putative explanation towards the mechanisms that is leading to the RVOT-VT arrhythmias from the Abstract and Results section, as we agree that we did not provide experiments or data to proof the idea of a secondary Ca^{2+} overload that can trigger EADs experimentally. Yet, as it is commonly accepted that an increased Na^+ permeability is very likely to induce exactly these secondary effects, these `speculations´ are now still, but exclusively, placed once at the end of the Discussion section.
To further support our effects observed in HL-1 cells, we have performed computational action potential modeling using a human ventricular myocyte model described by ten Tusscher (Appendix Material and Methods and Appendix Fig S6). These data were consistent with our experimental observations, and a mild depolarization and a strong slowing of the upstroke velocity was observed (Appendix Fig S6). Our action potential modeling provides also some information towards putative changes in the intracellular Ca^{2+} levels by TREK-1^{I267T}. The model was based on findings that TREK-1 in rat ventricular myocytes conducts about 1.5 pA/pF at +30 mV (Bodnár *et al.* 2015, PMID: 25539776) and our observation that TREK-1^{I267T} has a sodium permeability of 21%. According to Bodnar *et al.* when applying the electrodiffusion model developed by Goldman, Hodgkin and Katz (Hille 1992), a ventricular I_{TREK} should have at -15 mV, a potential between the E_{Na} and E_K, an amplitude of about 0.63 pA/pF. A respective sodium inward leak due to TREK-1^{I267T} at -15 mV was assigned with 20% of the amplitude and the opposite polarity (-0.13 pA/pF). The leak current at different potentials was subsequently calculated with a simple background current equation. Using this additional, pathological sodium background current the model predicts an increase in maximum intracellular calcium concentration of 25% (Appendix Fig S6B), maximum sarcoplasmic reticulum calcium concentration of 9% (Appendix Fig S6C), maximum intracellular sodium concentration of 17% (Appendix Fig S6D) and increased outward currents of the sodium

calcium exchange current during the systole of 60.9%, reflecting an increased Ca^{2+} uptake in the reverse mode (Appendix Fig S6J). Given the limitations of a mathematical model, including that the precise magnitude of the human ventricular I_{TREK} is still unknown, we use this model only to mechanistically confirm our electrophysiological data obtained with HL-1 cells. Therefore, as described above we have limited the speculations towards a secondary calcium overload to a hypothesis which is positioned only in the last paragraph of the discussion.

Reference: B. Hille, Ionic channels of excitable membranes, Sinauer Associates, 2 edition, 1992.

4) The stepwise applied mechanical negative pressure of 0 to -80 mmH₂O, applied by the patch clamp pipette to a very limited area of the cell membrane, is not comparable to the dynamic stress, applied to the cardiomyocytes of the RVOT during the cardiac cycle. Authors could cultivate TREK-1^{I267T} cells on an elastic membrane, apply physiologic stress to the cellular syncytium and monitor electrophysiological characteristics.

We agree that it is very promising to study how a more physiological stress affects TREK-1^{I267T}. In the progress of the revision we started these experiments, trying to build up an appropriate recording rig, so we can apply cell stretch to HL-1 cells seeded on flexible silicone elastomer membranes. Here, in the three month provided for the revision, we already encountered a first major obstacle, the pre-treatment of these membranes is crucial for adhesion and proliferation of the HL-1 cells, even if all membranes were coated with the usual mix of gelatin/fibronectin. In addition, the transfection efficiency needed to be optimized under these new conditions while on top, the cells needed to keep beating. Despite some progress with the coating and transfection, we only managed to stretch HL-1 wild-type cells which results in reduced action potential frequency (video analyses). Still this new rig will require to be combined with a patch-clamp set up, in order to be able to patch the cells while stretching them. Most importantly, it seems more suitable once we have established a combined stretch/patch clamp recording rig that we also work with native ventricular myocytes as the cell shape, extracellular matrix and other factors matter for the stretch-sensitivity. Obtaining adult ventricular myocytes for which the majority or all cells carry the TREK-1^{I267T} information would demand a viral transfection or a transgenic TREK-1^{I267T} mouse line. We agree to the point you have raised, however, we strongly believe that these experiment demand a long term commitment and thus will be an independent study, which we are also aiming to target in the future.

5) Why the clinically observed arrhythmias become present at an age over 40 years and not in younger patients?

Approximately 60-80% of idiopathic VTs arise from the RV, most commonly from the outflow tract. Symptoms are related to the frequency of PVCs or non-sustained VTs. The age of presentation is usually 30-50 years (range 6-80 years) and women are more commonly affected. Often, it is likely that minor and non-sustained arrhythmias are present, but not perceived by the patient. This is also typical for other arrhythmia forms which show an age-dependence of hormonally influenced disease manifestation (e.g., Brugada syndrome or LQTS). Here, despite of having a SCN5A sodium channel mutation, the disease often remains `hidden´ (asymptomatic or neglected by the patient) until a certain age. Moreover, the diseases are episodic in nature, meaning that often external triggering events are needed in order that a disease becomes symptomatic. The reason why RVOT-VTs and certain arrhythmias occur for the first time with an age over 40 years is still poorly understood and might involve multiple factors like an age-dependent ion channel expression profile or re-modelling, fibrotic changes or even epigenetic factors that need to accumulate as a final trigger for the disease. For RVOT-VTs, the adrenergic tone is the most important trigger; so far, it is unknown whether regional RV innervation may differ between these patients.

Referee #3 (Remarks):

Decher et al

This is a lovely submission that studies a point mutation identified in a patient with RVOT tachycardia in the TREK-1 channel. The study shows that the missense mutation alters the ion selectivity of the channel and modifies its sensitivity to mechanical stretch, two effects that would be expected to predispose to the arrhythmia observed in the patient. Moreover, a channel activator that might prove to be a lead for therapy is identified. The manuscript is clearly written. There are a small handful of minor issues in the text to address and a two valuable bits of data would make the paper even stronger (points 6 and 8).

Thank you for reviewing the manuscript and for your positive comments. Please note that we have gained within the process of the revision additional valuable insights into the disease causing mechanism of the TREK-1^{I267T} mutation. (1) RVOT-VTs occur predominantly under sympathetic stimulation. Strikingly, we found that while wild-type TREK-1 currents are almost fully suppressed by ß$_1$-adrenoreceptor stimulation, the TREK-1^{I267T} mutant is not responding in the typical manner, the aberrant Na$^+$ conductivity persists and most importantly there are even more pronounced inward sodium leak currents with an additional depolarization of the cells. Thus, under sympathetic stimulation the depolarizing impact of the mutant is more pronounced as under baseline conditions, an effect that might, in the patient reported, contribute to episodes of VTs under stress conditions. (2) In addition, we have included more electrophysiological data using HL-1 cells, revealing that the TREK-1^{I267T} mutation strongly slows the upstroke velocity of the action potential, an effect that is known to contribute to ventricular re-entry arrhythmias due to conduction velocity reduction and thus a shorter wavelength. (3) We have included a model of the impact of the TREK-1^{I267T} mutant on the human ventricular action potential. This data is consistent with our electrophysiological findings in HL-1 cells and predicts a secondary Ca^{2+} overload in cardiomyocytes.

1. Page 7. The statement "the mutation is likely to act in a dominant-negative manner" is not well supported at this point in the paper, since decreased current could be due to an additive effect-full dominance with a dimer should decrease in current by 75% and it appears to be down only 50%. A softer assertion here could be followed by a return to the conclusion when they assess other effects that also support the notion (Em changes with substitution, effect of the activator, etc).
Thank you for this comment. The lack of a perfect dominant-negative effect might also result from the problem of finding a linear relationship of `cRNA to current´ which also becomes evident for the experiments mimicking a `haploinsufficiency´. Here with only half the amount of cRNA injected (1.25 instead of 2.5 ng) the currents were only reduced to 0.8 instead of to 0.5. Therefore, as suggested we tempered our statement to "*might act*" in a dominant-negative manner. In addition the title of the section was changed to: "*Reduced outward currents of homomeric TREK-1^{I267T} and heteromeric TREK-1/TREK-1^{I267T}*".

2. Page 9. I understand the meaning of the statement that the TWIK-like SF "is in agreement with the sodium permeability observed with TWIK-1" but it is a bit non-specific for the general reader since the change in TWIK takes many minutes to develop under pathological conditions and stated this way it sounds like it is normal TWIK operation to pass sodium.
This sentence has been re-written to avoid giving the impression that TWIK-1 is sodium permeable under baseline conditions, although we already stated at the beginning of this section that this sodium permeability occurs only under hypokalemic or acidic conditions, also providing the references by Ma *et al.*, 2011, 2012 and Chatelain *et al.* 2012. The mis-leading sentence was rephrased to "*....potentially explaining the high Na$^+$ ion permeability observed in the mutant channel which however already occurs under baseline conditions.*"

3. Page 9. "The oxygens of the I267 carbonyl backbone coordinate..." The conclusions should be a bit less definitive since the resolution is low in the structure.
As suggested by Referee 1 we have removed this section in order to improve the readability of the manuscript. Instead, as also requested by Referee 1, we now only make a brief statement towards these Molecular Dynamics simulations which can be found at the end of the preceding section "*Na$^+$ permeability in TREK-1^{I267T}*". Here we state: "*In addition, we have performed Molecular Dynamic simulations which are also in agreement with the loss of selectivity effect of TREK-1^{I267T} (Appendix Fig S4).*"

4. Page 3 or 4. Somewhere near the beginning of the paper TREK-1 should be identified with its formal IUPHAR name, K2P2, so the literature is kept clear.
Good point, thank you. We now introduce in the Abstract at the first appearance of the `historically based name´ TREK-1 the IUPHAR name K$_{2P}$2.1 together with the gene name *KCNK2*. Also in the Introduction we now provide the IUPHAR name upon the first appearance of TREK-1.

5. Page 10. The nice consideration about sodium overload, calcium overload, and excess Na/Ca exchanger function is stated too much like a conclusion and is in the results. It should be noted to be a hypothesis (and maybe put in the discussion) or else intracellular Na, Ca, and exchanger function should be studied.

Thank you. As suggested by Referee 1 we have removed this putative explanation towards the mechanisms that is leading to the RVOT-VT arrhythmias from the Abstract and Results section, as we agree that we did not provide experiments or data to proof the idea of a secondary Ca^{2+} overload that can trigger EADs experimentally. Yet, as it is commonly accepted that an increased Na^+ permeability is very likely to induce exactly these secondary effects, these `speculations´ are now still, but exclusively, placed once at the end of the Discussion section.

To further support our effects observed in HL-1 cells, we have performed computational action potential modeling using a human ventricular myocyte model described by ten Tusscher (Appendix Material and Methods and Appendix Fig S6). These data were consistent with our experimental observations, and a mild depolarization and a strong slowing of the upstroke velocity was observed (Appendix Fig S6). Our action potential modeling provides also some information towards putative changes in the intracellular Ca^{2+} levels by TREK-1^{I267T}. The model was based on findings that TREK-1 in rat ventricular myocytes conducts about 1.5 pA/pF at +30 mV (Bodnár *et al.* 2015, PMID: 25539776) and our observation that TREK-1^{I267T} has a sodium permeability of 21%. According to Bodnar *et al.* when applying the electrodiffusion model developed by Goldman, Hodgkin and Katz (Hille 1992), a ventricular I_{TREK} should have at -15 mV, a potential between the E_{Na} and E_K, an amplitude of about 0.63 pA/pF. A respective sodium inward leak due to TREK-1^{I267T} at -15 mV was assigned with 20% of the amplitude and the opposite polarity (-0.13 pA/pF). The leak current at different potentials was subsequently calculated with a simple background current equation. Using this additional, pathological sodium background current the model predicts an increase in maximum intracellular calcium concentration of 25% (Appendix Fig S6B), maximum sarcoplasmic reticulum calcium concentration of 9% (Appendix Fig S6C), maximum intracellular sodium concentration of 17% (Appendix Fig S6D) and increased outward currents of the sodium calcium exchange current during the systole of 60.9%, reflecting an increased Ca^{2+} uptake in the reverse mode (Appendix Fig S6J). Given the limitations of a mathematical model, including that the precise magnitude of the human ventricular I_{TREK} is still unknown, we use this model only to mechanistically confirm our electrophysiological data obtained with HL-1 cells. Therefore, as described above we have limited the speculations towards a secondary calcium overload to a hypothesis which is positioned only in the last paragraph of the discussion.

Reference: B. Hille, Ionic channels of excitable membranes, Sinauer Associates, 2 edition, 1992.

6. Page 11. The BL-1249 findings are really quite nice. It would be valuable to show if the activator changes the selectivity of the mutant (and if this explains the increase in current).

Thank you. Yes, BL-1249 is able to rescue the selectivity of TREK-1^{I267T}. These data were already shown in Fig. 5E-F of the original manuscript.

7. Page 11. You might cut the word "high" from before the "sensitivity to mechanical stretch" since it overstates the characteristic compared to other stretch sensitive channels and that is not necessary to make any of your strong points.

Yes, agreed and changed.

8. Page 11. It would be valuable to show if stretch changes the selectivity of the mutant (and if this explains the increase in current).

The experiments on stretch activation were performed in symmetrical K^+ (without Na^+) and therefore a change in ion selectivity as cause for the increased currents seen for the TREK-1^{I267T} mutant upon stretch activation can be ruled out. This strongly suggests that the main effect of the mutant is, as we have proposed, caused by an altered stretch-sensitivity and stretch-desensitization and that these effects involve the selectivity filter which is the common gate for various stimuli in TREK-1 channels (Piechotta *et al.*, 2011, PMID: 21822218; Bagriantsev *et al.*, 2011, PMID: 21765396).

9. Citations. It seems an odd not to cite Thomas et al Neuron 2008 as proof that sodium selectivity is not just seen in the past for TWIK-1 but for TREK-1 as studied here (they showed developmental regulation by ATI produces a truncated version of TREK in the brain that can pass sodium).

Thank you we have included the following information in the Discussion: "*Noteworthy, alternative translation initiation (ATI) can result in a TREK-1 variant with a truncated N-terminus (TREK-1ΔN) for which a permeability to sodium was proposed (Thomas et al., 2008: PMID: 18579077). However, a recent study by Veale et al. leads to the conclusion that TREK-1ΔN is highly potassium selective and that the previously described effects on the reversal potentials are most likely caused by the very poor expression levels of this variant (Veale et al., 2014; PMID: 24509840).*"

2nd Editorial Decision   24 November 2016

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Please reply to referee 3 in a reply-to-the-referee document and amend your main text accordingly.

Please submit your revised manuscript as soon as possible.

I look forward to receiving it.

***** Reviewer's comments *****

Referee #2 (Remarks):

Most of the questions are answered. It would have been better, if authors would have performed calcium imaging experiments instead of the computational model.

Referee #3 (Comments on Novelty/Model System):

Well rationalized in the answers to review 2. I think the work stands well as it is now without transgenic animals or other methods of studying mechanical stretch.

Referee #3 (Remarks):

I remain very impressed by this study. The changes have increased the strength and clarity of the work. I have only one comment requiring attention prior to publication.

They write in the rebuttal:
9. Citations. It seems an odd not to cite Thomas et al Neuron 2008 as proof that sodium selectivity is not just seen in the past for TWIK-1 but for TREK-1 as studied here (they showed developmental regulation by ATI produces a truncated version of TREK in the brain that can pass sodium).

Thank you we have included the following information in the Discussion:
"Noteworthy, alternative translation initiation (ATI) can result in a TREK-1 variant with a truncated N-terminus (TREK-1ΔN) for which a permeability to sodium was proposed (Thomas et al., 2008: PMID: 18579077). However, a recent study by Veale et al. leads to the conclusion that TREK-1ΔN is highly potassium selective and that the previously described effects on the reversal potentials are most likely caused by the very poor expression levels of this variant (Veale et al., 2014; PMID: 24509840)."

However, the Veale work studied TREK-1ΔN only in the presence of the same fenamate compounds (including BL-1249) that you show in Figure 5 restores potassium selectivity to the sodium permeable TREK variant that you study! If you want to discuss Veale in follow-up to Thomas that first reported that TREK channels can pass sodium you need to tie the results into your own exciting new findings with a sentence something like this on page 13:
"Noteworthy, alternative translation initiation (ATI) can result in a TREK-1 variant with a truncated N-terminus (TREK-1ΔN) that is permeable to sodium (Thomas et al., 2008: PMID: 18579077). Whereas a recent study (Veale et al., 2014; PMID: 24509840) suggested that TREK-1ΔN is highly potassium selective, they only studied the channels in the presence of fenamates (including BL-1249) to increase current density, the same agents that we show here restore potassium selectivity to the sodium permeable TREK-1I267T channel (Fig 5e)."

2nd Revision - authors' response   19 January 2017

Referee #3 (Comments on Novelty/Model System):
Well rationalized in the answers to review 2. I think the work stands well as it is now without transgenic
animals or other methods of studying mechanical stretch.
Thank you for reviewing the manuscript and for your very positive comments.

Referee #3 (Remarks):
I remain very impressed by this study. The changes have increased the strength and clarity of the work. I have only one comment requiring attention prior to publication.
....
However, the Veale work studied TREK-1ΔN only in the presence of the same fenamate compounds (including BL-1249) that you show in Figure 5 restores potassium selectivity to the sodium permeable TREK variant that you study! If you want to discuss Veale in follow-up to Thomas that first reported that TREK channels can pass sodium you need to tie the results into your own exciting new findings with a sentence something like this on page 13: "Noteworthy, alternative translation initiation (ATI) can result in a TREK-1 variant with a truncated N-terminus (TREK-1ΔN) that is permeable to sodium (Thomas et al., 2008: PMID: 18579077). Whereas a recent study (Veale et al., 2014; PMID: 24509840) suggested that TREK-1ΔN is highly potassium selective, they only studied the channels in the presence of fenamates (including BL-1249) to increase current density, the same agents that we show here restore potassium selectivity to the sodium permeable TREK-1I267T channel (Fig 5e)."
Thank you. We have included this point with almost the identical wording:
"*Noteworthy, alternative translation initiation (ATI) can result in a TREK-1 variant with a truncated N-terminus (TREK-1ΔN) that is permeable to sodium (Thomas et al, 2008). While a recent study (Veale et al, 2014) suggested that TREK-1ΔN is highly potassium selective, the authors only studied the channels in the presence of fenamates (including BL-1249) to increase current density, the same agents that we show here to restore potassium selectivity in sodium permeable TREK-1^{I267T} channels (Fig 5).*"

EMBO PRESS

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Niels Decher	
Journal Submitted to: EMBO Molecular Medicine	
Manuscript Number: EMM-2016-06690	

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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.

B- Statistics and general methods

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	For the in vitro experiements of our study there were no pre-specified number of experiments
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, we have followed the general strategy as provided in the Material and Methods section on page 22
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, as described in the Methods section (p. 22) we used a Shapiro Wilk test for normality before deciding whether we have to chose a paramteric or non-parametric statisctial test.
Is there an estimate of variation within each group of data?	Yes, equality of variances was tested using either a parametric or non-parametric Levene's test. See methods on (p. 22)
Is the variance similar between the groups that are being statistically compared?	Yes, for almost all the data. If the variances of the groups were different, significance was probed using either Welch's T-test and for not normally distributed data we used Mood's median test.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The HL-1 cells were reported by Prof. W. Claycomb, as cited in the Methods section of the manuscript, see page 20. The cells were after thawing always directly tested on mycoplasma contamination and cells were subsequently held for only a few passages

* 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.	See page 17
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.	This statement is included in the Ethical Approval section of the manuscript, see page 17
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).	Considered
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).	In the Ethical Approval section on page 17 of the manuscript we have stated: "We did not have an explicit consent for depositing the clinical data in the dbGAP and EGA repositories."
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.	For our computational action potential analyses we used the standard ten Tusscher model which is deposited at CellML.org

G- Dual use research of concern

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