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Antisense transcription represses Arabidopsis seed dormancy QTL DOG1 to regulate drought tolerance

Ruslan Yatusevich, Halina Fedak, Arkadiusz Ciesielski, Katarzyna Krzyczmonik, Anna Kulik, Grazyna Dobrowolska, and Szymon Swiezewski

Corresponding author: Szymon Swiezewski, Institute of Biochemistry and Biophysics

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Editor: Achim Breiling

Transaction Report: This manuscript was initially reviewed at *The EMBO Journal* and was invited for submission to *EMBO reports*

(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.)

Decision letter (*The EMBO Journal*) 07 June 2017

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees, whose comments are shown below. As you will see, while the referees all express interest in the work and topic in principle, they all do not offer strong support for publication in The EMBO Journal - at least at the current stage of analysis.

I will not repeat all individual points of criticism here but you will see that while the refs highlight the importance and novelty in linking asDOG1 expression to ABA signaling and drought resistance they also find that the underlying mechanism would need to be substantially expanded. In addition, they raise a number of additional technical points and question the reliance on reporters relative to endogenous expression. Clearly, a very extensive amount of further experimentation - of currently unpredictable outcome - would be required to address the issues raised by the referees and to bring the study to the level of insight and significance required for publication in The EMBO Journal.

However, given the timeliness and interest of your findings, I have taken the liberty to also discuss your manuscript and the referee reports with my colleagues at our sister journal EMBO Reports. The responsible editor there, Achim Breiling, would be happy to consider a revised version of your manuscript, provided that all technical/control points raised by our three referees are addressed. However, EMBO Reports will not ask you to delineate the underlying mechanism and also not require generation of CRISPR lines. If you choose to take this opportunity you should submit the revised manuscript to EMBO reports and Achim will then only go back to the same three referees. Please feel free to contact Achim directly for specific questions about the experiments required for acceptance of the revised manuscript in EMBO Reports.

Should you decide to go a step further and pursue the mechanism of regulation of and by asDOG1 then we would in principle also be willing to consider a revised version for The EMBO Journal. However, given the extensive experimentation and unclear outcome and timeline this would have to be as a new independent submission. Please feel free to contact me with any questions about this.

In light of the comments and recommendations from the referees, I am afraid we are unable to offer further steps towards publication in The EMBO Journal at this stage. However, as outlined above I do hope you will take the

chance to submit a revised version of the manuscript to EMBO reports instead and that you will find our referees' comments helpful.

Authors' point-by-point response (submitted to EMBO reports) 19 July 2017

Point-by-Point Response to Reviewers' Comments

Referee #1:

Yatusevich and colleagues present a study in which they show that the seed dormancy regulator DOG1 acts in drought response. All in all, the authors have presented simple and straightforward experiments to support their claims, and they make a solid case. I kept wondering, however, why the authors kept using genomic fusion constructs throughout their study, instead of referring to CRISPR technology to delete the 3' region in the endogenous DOG1 locus. Drought tolerance of such lines would have given even better support for their model.

We are grateful for a positive assessment of our work. The point raised by the Reviewer is an interesting additional experiment. Essentially the Reviewer suggested using CRISPR to delete the endogenous antisense promoter from *DOG1* locus. Indeed this is an excellent suggestion and we have already started those experiments a while ago. However, those are not straightforward for the following reasons:

- **Lack of suitable PAM site at the position used in the transgene.**
- **Presence of a complicated system of *cis* elements around *DOG1* antisense promoter, some of them enhancing some of them suppressing antisense expression (our unpublished preliminary data).**

In summary, we are grateful for this suggestion we agree that this experiment would give a strong support for our model, but as explained above those are not straight forward experiments and we believe are outside the scope of the current manuscript. In this respect we are grateful for the editor's decision not to request those experiments for current submission.

Given that lncRNAs/asRNAs have been recognized as being responsive to different abiotic stresses in a number of studies, it is a matter of perspective to what extent the present work adds new insights, besides the finding that DOG1 is a regulator of drought and ABA response.

We agree with the Reviewer that the lncRNA/asRNA are recognized as being responsive to different abiotic stresses in a number of studies. We also agree that the major strength of our work is the "finding that DOG1 is a regulator of drought and ABA response". In our opinion the fact that by studying asRNA regulation we discovered novel function of a gene that has been named by others as a "master regulator of plant development" (Kerdaffrec et al., 2016, eLIFE) –*DOG1* provides major justification for our work.

Minor comments:

- Page 4, bottom: "We next analyzed how asDOG1 expression responds to ABA in Col-0 (WT) plants by using RT-qPCR to monitor mRNA levels (Fig. 1C)". This figure reference seems either wrong or obsolete.

This has been corrected to Fig. 1E.

- I have difficulties interpreting the light intensity quantifications in Fig. 1 D and Suppl. Fig. 4C. According to Suppl. Fig. 4 A and B, line 1 has a much stronger decrease in light intensity upon drought treatment than line 2. In fact, if it weren't for the two individuals in the top row of Suppl. Fig. 4B, I would have had difficulties in making out an effect at all. However, when looking at the corresponding quantifications, it seems as if the effect in line 2 was actually stronger than that in line 1. Can the authors make sure that the data are represented correctly, and comment on this discrepancy?

We are grateful to the Reviewer for pointing out this mistake. Indeed the quantification and visual picture of corresponding lines where accidently mixed between the line 1 and line 2. This has now been corrected and graphs were changed in Suppl. Fig 4 and main Fig. 1D.

- All figures: what do the error bars represent in each case?

This was originally described in the methods. We have now added description of error bars to each figure legend.

- I find statements like this one "plants in which expression of the LUC reporter gene inserted at the full length DOG1 locus" misleading, because they suggest that the LUC fusion has been inserted at the exact DOG1 genomic locus. Instead, if I understand the methods section correctly, the authors have performed standard random insertion of the promoter:LUC:DOG1 construct into an unknown genomic locus.

The Reviewer is correct. We used a transgene that integrates into random genomic loci. We have amended the text to highlight the method used.

"A complementary analysis using plants containing the *LUC* reporter gene inserted in the genomic context of the *DOG1* locus - p_{DOG1}*LUC::DOG1*,… "

has been changed to: "A complementary analysis using plants expressing transgene containing the *LUC* reporter gene fused with the full length *DOG1* locus - p_{DOG1}*LUC::DOG1*, …"

Additional suggestions:

I would have been interested in a more elaborate discussion on how asDOG1 regulation of DOG1 could work. Is it via RNA interference? If so, one could test this by looking for locus-specific siRNAs that should be reduced under drought conditions. As is, the authors provide no speculation on a mechanism. Although not necessarily in the scope, it would be tempting to check for epigenetic marks at the antisense promoter. There have been some examples of methylated lncRNA loci in recent studies. I briefly checked in published datasets, and no DNA methylation has been associated with that locus under normal conditions. It would be interesting to see whether this changes under drought, or if there are any histone marks known to be associated with the locus (in particular given the analogy to the FLC locus).

We have extended the discussion in line with the Reviewers suggestion. In fact there is no DNA methylation, neither small RNA on *DOG1* locus suggesting that the potential mechanism will possible not depend on RNAi pathway. We have also included a small passage in the discussion on the *cis* mode of *DOG1* antisense suppression of *DOG1* sense expression.

"We have previously shown that in seeds *asDOG1*/*1GOD* suppresses *DOG1* expression in *cis* but the molecular mechanism of that suppression is currently not clear. Here we show that in response to ABA and drought *asDOG1* levels are reduced releasing *DOG1* expression. The fact that the *asDOG1* deficient lines - p_{DOG1}*shDOG1::LUC* and p_{DOG1}*DOG1*Δ*TATA::LUC* are constitutively highly expressed in the presence and absence of ABA (Fig. 3) suggests that the *asDOG1* originating from the endogenous copy is unable to silence in *trans DOG1* sense expression in leafs as shown before by us in seeds (Fedak et al., 2016). The molecular mechanism of *asDOG1* mediated *DOG1* suppression is currently not clear. Importantly *DOG1* locus is devoid of DNA methylation, small RNA or high H3K9me2 suggesting that the molecular mechanism may not involve RNA interference but maybe based on *cis* acting mechanisms linked more-directly to antisense transcription (Krzyczmonik et al., 2017)."

Referee #2:

Major comments:

1. Page 5, the authors state "Surprisingly, we saw a simultaneous ten-fold increase in the level of the short functional form of the DOG1 mRNA". I don't understand why this is surprising since the authors detected an approximately 80% reduction in asDOG1 transcript levels after ABA treatment and asDOG1 has been shown to negatively regulate DOG1. Wouldn't an increase in DOG1 expression be expected?

We agree with the Reviewer, that given the reduction in *asDOG1* levels this could be anticipated. We would however, like to stress that the Reviewer assumes here that *asDOG1* has the same function in seeds as in seedlings - in fact this had to be verified. To eliminate the potential confusion, as pointed by the Reviewer, we have changed this sentence into: "Simultaneous we saw a ten-fold increase in the level of the short functional form of the *DOG1* mRNA (Fig. 1F)"

2. Page 5, Although the dog1-3 and dog1-4 mutant alleles were published previously and a reference is given in the Materials and Methods, it would be helpful to give abrief description of these mutants to allow the reader to understand what they are and the evidence that they are knock-outs. In line with this description and to better understand this complex locus, I would suggest include a schematic of the DOG1/asDOG1 gene structure in the SupFig 1, showing the promoters, exons, introns, TSS, and the position of the mutations of the lines dog1-3 and dog1-4 all relative to the upstream and downstream adjacent genomic loci including scalebars.

The requested figure is included in the updated version of the manuscript, as Supplementary Figure 1.

3. Page 5, How was drought recovery scored? Was it based on leaf number, fertility or survival just survival as shown in Fig2B because the plants in Fig2A or SupFig4 do not look dead?

The drought recovery was scored based on survival measured as an ability to produce new biomass – be it flowers or leaves. Although some of the plants may look greenish in the pictures as mentioned by the reviewer, none of them survived after re-watering and further growth in the glasshouse. To clarify this point we have added more extensive description to the figure legend.

4. Page 5, What do the authors mean by « indistinguishable» in the phrase « watering was withheld for a short time until both the WT and dog1 mutant plants were indistinguishable »? Because in SupFig4D, some of the dog1 mutant plants look indistinguishable from the WT Col-0 plants, but according to the authors, less than 7% of these mutant plants survive. Could the weaker induction of stress marker genes in the dog1 mutants just be because the dog1 mutant plants are dying from the drought?

The RNA was extracted from WT and *dog1* mutants after a short drought when there are no phonotypical differences between WT and *dog1*. To make this point clear we have corrected this sentence to: "To confirm this observation by other means, watering was withheld for a short time (2.5-3 days) when there is yet no major visible phenotypical difference between WT and *dog1* mutant (Suppl. Fig 4E)…" We also added a figure showing picture of WT and *dog1* plants subjected to short drought treatment to highlight the lack of differences between WT and *dog1* mutants at the stage where the RT-qPCR analysis was performed.

5. Page 6, Were the pDOG1-LUC::DOG1 and pDOG1-shDOG1::LUC lines stably expression a single insert? Was Crispr-Cas9 used to generate these lines? Is the endogenous locus still intact? The description of these lines is not very clear because it insinuates that the LUC fusion was inserted at the endogenous DOG1 locus. Is this indeed the case?

The reviewer is correct the lines are single copy transgenic lines (based on segregation analysis) and were not generated by CRISP-CAS9. This was originally described in the material and methods section. We have now changed the sentence to make clear we used transgenic plants expressing the *DOG1* locus fused with luciferase. "To further characterize the *DOG1* response to ABA we studied plants expressing *DOG1* sense promoter driven full-length *DOG1* transgene fused with *LUC* reporter (p_{DOG1}*LUC::DOG1*) "

6. Page 6, « we found that removal of DOG1 antisense transcription rendered the truncated construct pDOG1shDOG1::LUC insensitive to ABA at all tested stages of development » If I understand the construction of the plant lines correctly, this means that the asDog1 transcript coming from the endogenous locus cannot act in trans to silence the shDOG1::LUC. I think this is a point that should be added to the discussion.

We are grateful for pointing out this omission. The Reviewer is correct. In the recent PNAS manuscript (Fedak 2016) we showed that *asDOG1* acts in *cis* in seeds. The point raised by the reviewer suggests that also in seedlings the *asDOG1* acts in *cis*. This is further supported by similar observation with p_{DOG1}*shDOG1::LUC* transgenic plants (Figure 3) We have added a requested comment in the discussion. "The fact that the *asDOG1* deficient lines - p_{DOG1}*shDOG1::LUC* and p_{DOG1}*DOG1ΔTATA::LUC* are constitutively highly expressed in the presence and absence of ABA (Fig. 3) suggests that the *asDOG1* originating from the endogenous copy is unable to silence in *trans DOG1* sense expression in leaves as shown before by us in seeds (Fedak et al., 2016).."

7. Page 10, Because the authors do not go further into the understanding of the mechanism by which asDOG1 silences DOG1, I think the discussion could benefit from adding a few sentences describing what is known about the DOG1 protein. Are there any types of predicted domains?

Unfortunately the DOG1 protein has no extensive homology to any protein outside plants, nor contain any clear protein domain of known function, and shows only week homology to transcription factors, including bZIP (http://prosite.expasy.org/PDOC51806).
As requested by the Reviewer we have added a brief description of DOG1 protein. We have also added reference to a newly published paper from Wim Soppe characterizing DOG1 protein partners and genetic interaction in seed dormancy control (Nee 2017)
"DOG1 protein is a plant specific protein that has no extensive homology to known proteins outside plants or contains any domain of known function, but its dimerization has been shown to be required for its ability to enforce seed dormancy (Nakabayashi et al., 2015). Recent efforts have also shown that DOG1 protein directly interacts a number of PP2C phosphatases and genetically require PP2C phosphatases to impose dormancy in developing seeds (Née et al., 2017)."

8. Suppl. Fig 1 is of low visual quality. It is very difficult to see the leaves and floral structures. Maybe a white background would help to improve the visualization of the expression. In addition, the color scale in panels A, B and C is not useful because it is does not include the full range.

We use maximum sensitivity and imaging settings to improve resolution and LUC signal, however, those are limited by our LUC camera setup (NightOwl Berthold as described in Methods). We have however tried to increase the noise/background by making the picture darker. We would like to refrain from extensively editing the pictures by changing the background into white colour. We hope that the new figure quality is acceptable now. The scale has been corrected as suggested by the Reviewer.

Referee #3:

Yatusevich and colleagues report that Delay of Germination 1 (DOG1), a protein that controls seed dormancy, also mediates drought tolerance in Arabidopsis thaliana rosettes. Because the manuscript's strength is analysing DOG1 gene regulation, I am concerned that key conclusions are based solely on light intensity from Luciferase expressed under control of pSenseDOG1 or pAntiSenseDOG1 promoters, or of LUC::DOG1 and shDOG1::LUC fusion proteins under control of pDOG1 and pSenseDOG1 promoters, respectively. Clearly, these Luciferase transgenes are central to the work, serving as proxies for sense or antisense DOG1 promoter activity (Fedak et al. 2016). However, except for Figure 1E, the authors provide no data verifying asDOG1 transcript levels expressed from the endogenous DOG1 locus. They assume that endogenous asDOG1 transcription is closely correlated with LUC activity from pAntiSenseDOG1-driven transgenes, which is not obvious to me. Although past results indicate this correlation, it is imperative to confirm the reliability of the reporter transgenes in each new context (e.g., drought) and for later seeds batches.

We fully agree with the Reviewer that relaying on transgenes for drawing meaningful conclusions is very dangerous. To answer this important point we have now included RT-qPCR based validation in Col-0 (WT) plants with no transgenes present for all of the main Figures/conclusions.

This includes:
- Confirmation by strand-specific RT-qPCR of *asDOG1* reduction by drought (Figure 1)
- Confirmation by RT-qPCR of *DOG1* sense induction by drought (Figure 2)
- RT-qPCR based validation of *DOG1* induction by ABA at different developmental stages (Figure 3)
- Strand specific RT-qPCR based validation of *asDOG1* reduction at different developmental stages by ABA(Figure 3)

We are very grateful to Reviewer for pointing out this omission in the original submission and are glade to provide a transgene independent verification of our conclusions.

An expert in stress response pathways might worry that the question of how DOG1 protein mediates drought tolerance remains unresolved here. But I think the discovery of this protein's role in plant ABA-dependent drought responses and the molecular analysis of asDOG1 non-coding RNA function in the process are worthy of publication in EMBO Journal.

Thank you highlighting this point, we believe this work will provide a starting point for experts in the drought field to address the molecular mechanisms of how DOG1 protein controls drought, in our own opinion it will be very interesting to compere DOG1 protein function in drought as well as in dormancy establishment.

Major concern:

On Page 4 the authors state, "the DOG1 antisense promoter was also strongly downregulated in response to 5 days of water withdrawal (Fig 1C, D, Suppl. Fig 4A, B, C)." None of these data are quantifications of endogenous asDOG1. I recommend that the authors perform strand-specific qRT-PCR to show that drought tolerance is indeed correlated with a reduction in endogenous asDOG1 transcript levels.

This has been done and the data has been included as part of Figure 1.

More generally, wherever the authors could provide corroborating strand-specific qRT-PCR data for endogenous DOG1 versus asDOG1 transcripts, this would be a most welcome improvement.

This has been done for Figure 1 , Figure 2 and Figure 3, to validate all of the main conclusions based on the transgenes.

Queries and minor corrections:

1) Have the authors examined changes in histone modifications at DOG1 and asDOG1 endogenous promoter regions under ABA and/or drought conditions (e.g., H3K4 methylation, H3K9 methylation, H3K27 methylation states)? ChIP-seq data for such marks could be an interesting extension of this work, perhaps uncovering additional mechanistic insights.

This is very interesting suggestion that we would clearly like to pursue in the future however currently we believe those points are outside the scope of this work. We have however included a short description in the Discussion of what is known based on highthroughput data about histone and DNA modification at *DOG1* locus.

2) On page 14, under "Hormonal treatments and Luciferase Measurement", the following ages of plants should be indicated as: "10-day-old, 20-day-old and 40-day-old Arabidopsis plants" without the plural 's'.

This has been corrected

3) In Fig 3A, do the asterisks above "10 days" (*) and "40 days" (**) indicate $P<0.05$ and $P<0.01$, respectively, based on the two tail t-test (Materials and Methods)? If so, does the lack of asterisks above "20 days" indicate no such significant difference? Perhaps the statement on Page 6, "This analysis showed that DOG1 transcription was increased at all tested time points..." should be modified to exclude 20 days. However, strand-specific qRT-PCR could be used to resolve this

discontinuity, by verifying that DOG1 mRNAs are indeed more abundant after ABA treatment at 20 days.

This has been done and the stand-specific RT-qPCR data is now included in the same Figure to facilitate comparison. This experiment has reviled that indeed *DOG1* is induced at all tested stages but the effect is strongest in 40 days old plants.

1st Editorial Decision 11 August 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to re-evaluate your study (the same that have assessed the first version of this paper for *The EMBO Journal*), which can be found at the end of this email. As you will see, all three referees support the publication of your study in EMBO reports. Before we can proceed with formal acceptance, I have several editorial requests.

Please refer to our guidelines for preparing your revised manuscript (for formatting of section headers, order of sections, etc.).

Please have the manuscript edited by a native speaker (see also the comment by referee #2).

Please upload all figures as single figure files in high resolution (for main figures and EV figures).

A Supplemental Table 1 is mentioned in the text, but was not submitted in the previous version. Please upload this (or include it into an Appendix).

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

Regarding data quantification and statistics, please add a paragraph describing how this was handled in the manuscript to the methods section.

Please add scale bars to all microscopic or plant images.

REFEREE REPORTS

Referee #1:

In the revised manuscript, the authors have addressed my initial concerns. As the editorial decision is not to request further experimental proof of the role of asDOG1 in regulating drought response, I have no further comments and recommend publication of the manuscript.

Referee #2:

After careful revision of the changes introduced by the authors, I conclude that they have adequately addressed all of my suggestions originally made for their EMBO Journal submission. Although these changes have been made, the manuscript should be proofread for spelling, grammar and punctuation as there are many errors throughout.

Referee #3:

In this manuscript, which is a revision of one previously submitted to EMBO Journal, Yatusevich and colleagues report that Delay of Germination 1 (DOG1) mediates drought tolerance in Arabidopsis thaliana rosettes. Based on the previous submission, I was largely convinced by the proposed mechanism of antisense DOG1 (asDOG1) transcription and its impact on drought tolerance by regulating the DOG1 protein-coding gene in cis. Because the manuscript's strength was dissecting DOG1 gene regulation, however, I suggested that the authors verify asDOG1 transcript levels from the endogenous DOG1 locus to reinforce several of their results that were based on transgenic reporters. The authors' revised manuscript incorporates all these supporting data, so I recommend its publication in EMBO Reports without any further reservations.

1st Revision - authors' response 08 September 2017

The authors made the requested changes and submitted the final version of their manuscript to *EMBO reports*.

2nd Editorial Decision 15 September 2017

I am very pleased to accept your manuscript for publication in the next available issue of *EMBO reports.* Thank you for your contribution, and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Szymon Swiezewski
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44862V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). 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

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We did not pre-specified effect size
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not relevant
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not relevant
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.	No
For animal studies, include a statement about randomization even if no randomization was used.	Not relevant
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.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not relevant
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	No
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://www.selectagents.gov/

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).	Not relevant
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Not relevant

* 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.	Not relevant
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not relevant
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.	Not relevant

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not relevant
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.	Not relevant
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not relevant
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not relevant
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not relevant
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.	Not relevant
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.	Not relevant

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	Not relevant
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).	Not relevant
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).	Not relevant
21. 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.	Not relevant

G- Dual use research of concern

22. 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