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Cancer-associated mutations in *VAV1* trigger variegated signaling outputs and T cell lymphomagenesis

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1st Editorial Decision 29th Mar 2021

Dear Dr Bustelo,

Thank you for the submission of your manuscript (EMBOJ-2021-108125) to The EMBO Journal. Please accept my apologies for the unusual delay with the peer-review of your work due to protracted referee input and detailed discussions in the team. Your manuscript has been sent to three reviewers and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your resource catalogue and functional results on the roles of mutant VAV1 in cancer, although they also express a number of major issues that will have to be conclusively addressed before they can be supportive of publication of your manuscript in The EMBO Journal. In more detail, referee #3 points to substantial concerns on the importance of additional Rho GTPase family members and asks you to address RAC1 function more directly (ref#3, pts 2,4). This referee also states that the methods annotation and data display need improvement (ref#3, pts 1,5,6). In line, referees #1 and #2 find that the manuscript is too dense currently and needs restructuring and suggest to emphasize the trivalent mutant data and related messages more. These experts also point to additional experiments and controls required to resolve data inconsistencies.

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers.

In light of the extensive requests requested by the reviewers i.p. on the manuscript and data organisation, I would appreciate if you could contact me during the next weeks via e.g. a video call to discuss your perspective on the comments and potential plan for the manuscript revision.

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

I this context I also want to point to our adjusted GTA We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic and have therefore extended our 'scooping protection policy' to cover the period required for a full revision to address the experimental issues highlighted in the editorial decision letter. Please contact us at any time to discuss an adapted revision plan for your manuscript should you need additional time, and also if you see a paper with related content published elsewhere.

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

Kind regards,

Daniel Klimmeck

Daniel Klimmeck, PhD

Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
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IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
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Referee #1:

This manuscript presents new data about oncogenic functions of Vav1 mutations and rearrangements in peripheral T cell lymphomas and selected other malignancies. The authors deserve a lot of credit for investigating systematically more than 50 Vav1 mutations identified in patients through a battery of assays that dissect the different pathways operating downstream of Vav1, including pathways that do and do not require the catalytic function of Vav1, as well as a new tumor suppressor pathway operating via Cbl-b/Notch that the authors reported recently. Most relevant is the identification of recurrent genetic events that create what the authors refer to as "trivalent effects" on Rac1 activation and NFAT activation while also increasing Notch activity through decreased Notch degradation. These "triple hit" Vav1 genetic events are highly enriched in ALCL, AITL and PTCL-NOS, but importantly other families of mutations are also identified with more selective effects on downstream pathways, and systematically catalogued here as a resource for the field. The paper is dense but generally well organized and presented. Structural and biochemical considerations are also a major plus in interpreting the impact of individual mutations. Of note, the authors also present a new in vivo assay leading to PTCL in mice with Tfh characteristics by overexpressing a C-terminal truncation mutant of Vav1 in mature CD4+ T cells. Given the paucity of PTCL models in mice, this could also be a useful resource.

Specific comments:

1) In Fig. 1C-D and 2A, the authors choose a heatmap strategy to graphically display a large amount of experimental data about individual Vav1 mutations (with primary data presented in the supplement). This is a clever strategy to provide a synthetic overview of the findings. However, for the JNK+aCD3 and NFAT+aCD3 readouts, the heatmap system documents the increased activity as compared to that of JNK and NFAT readouts alone, but as a result to not clearly display the impact of individual mutations in these conditions. Could the authors consider normalizing the data to that found with the WT Vav1 construct in these conditions? This would better represent changes introduced by individual mutants compared to wild-type Vav1.
2) In Fig. 5, the authors use an adoptive transfer system to transfer mature CD4+ T cells transduced with retroviral constructs expressing GFP, a C-terminal deletion mutant of Vav1 or the same mutant with a point mutation in Vav1's catalytic site. It appears that the system relies on transfer into lymphopenic Vav1/2/3 TKO recipients, a context that could impact the transforming potential of this strategy. I would recommend to disclose more prominently the unique features of this system in the text, figures and legends (rather than only mentioning it in the Methods section).
3) In Fig. 5M and/or Fig. 6, information about expression of canonical Notch target genes would be useful.
4) In the short-term culture assays presented in Fig. 7-8, it is unclear how much Notch signaling can be delivered to mature T cells cultured in the absence of a defined source of Notch ligands. This limitation should at least be acknowledged. Thus, whether the effects of compound E are fully on target remains not entirely clear. Along the same lines of thought, the anti-Notch1 antibodies used in Fig. 7J and 8D are not specific for the cleaved form of Notch1 (which is classically detected by antibodies reactive with a Val1744 epitope revealed by gamma-secretase cleavage. Such a strategy would provide more specific and definitive information about Notch pathway activation in this system.
5) In Fig. 9, a significant limitation is that the analysis is performed in steady-state conditions rather than after an immunization challenge that triggers a strong Tfh response (and ideally a GC reaction) Thus, conclusions about the physiological role of Vav1/3 in the generation of normal Tfh cells may not be definitive.

Referee #2:

EMBOJ- Robles-Valero et al.-2021-108125

Robles-Valero et al. systematically characterized the functional impacts of cancer-associated VAV1 mutations on the three main VAV1 signaling branches. This includes 51 different VAV1 mutations and definition of a classification based on their impact the downstream signaling branches. They demonstrated the most frequent VAV1 mutant subtype with a truncated CSH3 (that alleviates auto-repression and activates RAC1 and NFAT but lacks CBL-B mediated suppressor activity) specifically drives PTCL formation in mice via the cooperation of the polarization, chronic activation, and transformation of follicular helper T cells.
Overall, while the study is of significance and the conclusions are important for understanding peripheral T cell lymphomagenesis, the presented data is difficult to follow. Figures are very packed, with multiple abbreviations and color codding that make the reading very complex. The thinking process and the reason for each experiment are not clearly presented and can seem random at times. For example, there's no clear reason as to why Lung SH2 mutations are presented in figure 1E; 3D structures presented in figure 2C and 4D-E don't seem to add any information or there's no clear explanation as to why it is being shown to the reader. Figure 3 seems like a repetition of figures 1 and 2 with some added information from other tumors, again with no clear reason. Figures 5-9 seem like a completely different manuscript and go from studying VAV1 mutational landscape to validating in vivo the effects of a specific VAV1 mutation.
Overall, the manuscript as currently organized in nearly incomprehensible due to the density of the data and the fact that the results and discussion sections jump from one figure to the other and to supplementary which contributes to the difficulty in reading.
The manuscript would perhaps be better off being split into two separate papers, one with analysis of VAV1 mutational landscape and its functional impact and the other showing in vivo validation of VAV1ΔC in mouse T cells. A general additional suggestion is that the authors should only show the detailed in vitro assays of the T-cell neoplasm-related VAV1 mutations in the main figures and focus the unique features of the trivalent VAV1 mutations (one of which was functionally validated in the following mouse model). Most data in Figure 1, 2, 3 and 4 can either go to the supplemental data or can be used for another manuscript. Then maybe there would be the space to better describe the reasoning and results of each experiment.
Other specific comments:
1. Figure 1D: R678Q was defined as a "LOF" mutation, but still showed activation on SRF assay. It will be informative to verify whether this mutation affects the RAC activity or not by pull-down assay. This information will help to explain whether the activation on SRF assay is RAC1 dependent or not.
2. Figure 5C, did the mice in the Vav1dC+E201A group which died show any PTCL phenotypes?
3. Figure 5M, the authors should also show the gene expression data of EGFP+Vav1dC+E201A control samples.
4. Figure 6L, the authors should focus the overlapping genes between Vav□C-dependent genes and other PTCL models, not the overlapping genes between Tet2 loss and RhoAG17V.
5. Figure 7C, the ectopic expression level of different Vav1 mutant protein is different, and this may cause different biological effects. The authors should address this.
6. Figure 7L, the increased p-Akt in Vav1dC+E201A infected cells shown here is not consistent with the data shown in Figure 5N.
7. Figure 8. The authors should include the EGFP+Vav1dC+E201A as a control for all the inhibitor treatment experiments.
8. Figure 9E, the difference shown here between Vav1-/- and Vav1-/-, Vav2-/-, Vav3-/- samples suggests Vav2 and Vav3 may also play a role in the proliferation, is this due to the differential

expression of Myc?

Referee #3:

This is a comprehensive tour-de-force analysis of 51 VAV1 mutants found in two human cancer types, PTCL and NSCLC, which is a very valuable resource for both cancer researchers and cell signaling researchers. By using a range of complementary assays following expression of the mutants in cultured cells, the authors choose a functional mutation that they then test extensively in a mouse model in T cells in vivo, as well as cultured mouse T cells.
One concern with the interpretation of their results is that they assume that VAV1 DH-PH only activates RAC1, whereas in some circumstances it can act on other Rho GTPase family members. The indirect luciferase assays they are using could equally be activated by multiple other pathways. These assays are useful for high throughput and rapid screening through the mutants, but not so informative about which pathway(s) are feeding in upstream.

The following points need to be addressed in a revised manuscript:
1. Overall, the text and figures are very dense in information; some careful re-writing would help to get the main messages across without getting lost in the detail.
2. The Introduction should mention that VAV1 has also been reported to be a GEF for RHOA and CDC42 under some cellular contexts. This is particularly relevant for RHOA, since frequent mutations in RHOA are found in the same groups of PTCL as have VAV1 mutations (this should be introduced in more detail and discussed more extensively in the Discussion; e.g. Fujisawa et al., Leukemia 2018), and because SRF activation is most frequently linked to RHOA rather than RAC1 activation. Moreover, some of the LOF mutations in VAV1 could equate to similar LOF/DN mutations of RHOA in some PTCLs.
3. There are some mutants that activated JNK but not SRF signaling, or that have a much stronger effect on one or the other, in the luciferase read-outs, which provides additional evidence that the two assays measure different upstream signals and not simply 'RAC activity'. The authors should revise their interpretation of these results throughout.
4. The authors have not measured RAC1 activation directly for any of the mutants. They need to include some example RAC1 activity assays for at least a subset of relevant mutations (and simultaneously test for RHOA activation, see point 1).
5. Fig. 1: panel B - information on how each pathway was assayed should be provided here (e.g. SRE-Luc), because some of the assays are quite indirect (e.g. JNK activity is not being assayed directly); panels C, D; it would help to add asterisks or similar mark to indicate the positive control mutants (Y174 mutants) because these heat maps contain so many different mutants.
6. Fig 3A, B: the color coding in these figures is very complex and not intuitive to follow. It would be easier if all pathways that are unchanged are in green, and pathways that are changed are in a different color (red/pink for up, blue for down). The naming of the subclasses is also difficult to follow in the text - adding a diagram of VAV1 domains again with the subclasses indicated on it would help interpretation here (or just forget all the subclasses).
7. Fig. 5, Fig. 6 and accompanying text: it is not possible to compare the mouse studies carried here with those from a different group using different genetic changes (Zang et al., 2017). This can be put in the discussion but not stated as fact when the authors have not repeated the same experiment for direct comparison. The text and figure legend for Fig. 6 also need to make it clear that they are comparing gene expression results from Zang et al. with their own results. This information is only hidden in the Methods section.
8. This sentence does not make sense: 'For example, it is known that the bivalent F69V GOF

mutation targets a CH residue contributes to the autoinhibited structure of nonphosphorylated VAV1.....'

9. Use g not rpm throughout the methods section (g is the relevant unit; rpm is centrifuge type-dependent).

COMMENTS TO REFEREES

MANUSCRIPT EMBOJ-2021-108125

REVIEWER #1:

General Comment. *This manuscript presents new data about oncogenic functions of Vav1 mutations and rearrangements in peripheral T cell lymphomas and selected other malignancies. The authors deserve a lot of credit for investigating systematically more than 50 Vav1 mutations identified in patients through a battery of assays that dissect the different pathways operating downstream of Vav1, including pathways that do and do not require the catalytic function of Vav1, as well as a new tumor suppressor pathway operating via Cbl-b/Notch that the authors reported recently. Most relevant is the identification of recurrent genetic events that create what the authors refer to as "trivalent effects" on Rac1 activation and NFAT activation while also increasing Notch activity through decreased Notch degradation. These "triple hit" Vav1 genetic events are highly enriched in ALCL, AITL and PTCL-NOS, but importantly other families of mutations are also identified with more selective effects on downstream pathways, and systematically catalogued here as a resource for the field. The paper is dense but generally well organized and presented. Structural and biochemical considerations are also a major plus in interpreting the impact of individual mutations. Of note, the authors also present a new in vivo assay leading to PTCL in mice with Tfh characteristics by overexpressing a C-terminal truncation mutant of Vav1 in mature CD4+ T cells. Given the paucity of PTCL models in mice, this could also be a useful resource.*

Authors' response: We thank this Referee for her/his comments. We also appreciate it very much her/his technical comments, which have helped us improving quite significantly our work.

Specific Point #1. *In Fig. 1C-D and 2A, the authors choose a heatmap strategy to graphically display a large amount of experimental data about individual Vav1 mutations (with primary data presented in the supplement). This is a clever strategy to provide a synthetic overview of the findings. However, for the JNK+aCD3 and NFAT+aCD3 readouts, the heatmap system documents the increased activity as compared to that of JNK and NFAT readouts alone, but as a result to not clearly display the impact of individual mutations in these conditions. Could the authors consider normalizing the data to that found with the WT Vav1 construct in these conditions? This would better represent changes introduced by individual mutants compared to wild-type Vav1.*

Authors' response: Agree. Thanks for pinpointing this issue. To avoid this problem, we have reformatted the heatmaps originally presented in **Figures 1C,D** and **2A** (now, **new Fig. 1F**). In this new version, we have expanded the color scale to avoid the saturation at the extremes. With this change, we believe we have significantly improved both the resolution and the variations seen in the case of the CD3 stimulation conditions. There was another option: removing the data from stimulated cells. However, we believe that it is important to present the effect of the interrogated mutants under both basal and stimulation conditions. This is particularly important to identify the mutants that show full constitutive, phosphorylation-independent activity. However, if this Reviewer feels otherwise, we can eliminate them in the final version of the manuscript.

Specific Point #2. *In Fig. 5, the authors use an adoptive transfer system to transfer mature CD4+ T cells transduced with retroviral constructs expressing GFP, a C-terminal deletion mutant of Vav1 or the same mutant with a point mutation in Vav1's catalytic site. It appears that the system*

relies on transfer into lymphopenic Vav1/2/3 TKO recipients, a context that could impact the transforming potential of this strategy. I would recommend to disclose more prominently the unique features of this system in the text, figures and legends (rather than only mentioning it in the Methods section).

Authors' response: Agree. As indicated in the original **Methods** section, we have used the compound *Vav1*$^{-/-}$;*Vav2*$^{-/-}$;*Vav3*$^{-/-}$ mice as recipient for these T cell adoptive experiments. The rationale for using this mouse strain is that, due to its lymphopenia, it offers a cheaper model than the standard immunocompromised strains to carry out this type of experiments. Indeed, we have shown before that this model is even suited to carry out xenotransplants with human cancer cells (Lorenzo-Martin et al. *Nat Commun* 2020, PMID: 32963234). From a methodological point of view, these mice should not be that different from other immunodeficient mice that are commonly used in this type of experiments.

Having said this, we do agree that the readers require an explanation for the use of this "nonconventional" model. Accordingly, we have modified the text in the **Results** section to better explain the experimental model used (page 16): *"To this end, we infected TCR plus CD28-stimulated mouse splenic CD4*$^{+}$ *T cells with retroviral vectors encoding bicistronically each mutant Vav1 protein and EGFP and, subsequently, introduced them into Vav1*$^{-/-}$;*Vav2*$^{-/-}$;*Vav3*$^{-/-}$ *mice to test the potential development of lymphomas (Fig. 4A). These recipient mice are T lymphopenic (Bustelo & Dosil, 2016; Fujikawa et al, 2003), a feature that facilitates their use in transplantation experiments involving even cells of human origin (Lorenzo-Martín et al, 2020)."* In addition, we have changed the text in the **Methods** section (pages 38-39), which now says: *"… was then introduced by retroorbital injection into 6- to 8-week-old Vav1*$^{-/-}$;*Vav2*$^{-/-}$;*Vav3*$^{-/-}$ *recipient mice (Lorenzo-Martín et al., 2020; Menacho-Marquez et al, 2013). This lymphopenic mouse strain was used to minimize the potential rejection of the transplanted cells due to neoantigen expression (Lorenzo-Martín et al, 2020)."*

Specific Point #3. *In Fig. 5M and/or Fig. 6, information about expression of canonical Notch target genes would be useful.*

Authors' response: Agree. Following the Referee's recommendation, we now have included in the **Figure 5M** (now, **new Fig. 4M**), the expression of canonical Notch1 target genes in Vav1$^{\Delta C}$-transformed cells using qRT-PCR determinations. We have also included in this panel expression data from cells expressing the catalytically dead mutant version of Vav1 (Vav1$^{\Delta C+E201A}$) that has been requested by **Referee #2** (**specific point #3**). As expected, no alterations are observed in this latter case (new **Fig. 4M**). The text of the **Results** section has been changed to include this new set of data (page 17): *"In addition, they show elevated levels of canonical Notch1 target genes such as Hes1, Dtx1 and Ptcra (Fig. 4M, right panel). Most of these transformed cell isolates also display high levels of p-Akt (Fig. 3N and Fig. EV4A) and p-Erk (Fig. 4O and Fig. EV4A) when compared to EGFP*$^{+}$ *lymphocytes. These molecular and signaling features are not observed in cells expressing the catalytically-deficient Vav1*$^{\Delta C+E201A}$ *mutant protein (Fig. 4M)."*

Regarding **Figure 6** (now, **new Fig. 5**), we have already shown using *in silico* analyses that the Vav1$^{\Delta C}$-driven transcriptome is associated with a high enrichment in gene signatures associated with ICN1 signaling according to gene set enrichment analyses (GSEA) (see **Fig. 6H** in the original manuscript, now **Fig. 5G** in the new version of the manuscript). These results agree with the new data presented in the **new Figure 4M** (see above paragraph).

Specific Point #4. *In the short-term culture assays presented in Fig. 7-8, it is unclear how much Notch signaling can be delivered to mature T cells cultured in the absence of a defined source of*

Notch ligands. This limitation should at least be acknowledged. Thus, whether the effects of compound E are fully on target remains not entirely clear. Along the same lines of thought, the anti-Notch1 antibodies used in Fig. 7J and 8D are not specific for the cleaved form of Notch1 (which is classically detected by antibodies reactive with a Val1744 epitope revealed by gamma-secretase cleavage. Such a strategy would provide more specific and definitive information about Notch pathway activation in this system.

Authors' response: Agree. Thank you for raising this important point. We believe that our short-term culture system is fully compatible with adequate Notch1 signaling. For example, previous reports using short-term culture assays have demonstrated that Notch1 expression and ICN1 production are induced upon the stimulation of $CD4^+$ T cells with a combination of antibodies to CD3 and CD28 (Palaga et al. *J Immunol* 2003, PMID: 12960327 & Steinbuck et al. *J Immunol* 2018, PMID: 29288204). A recent report has shown that both Notch1 and some of its ligands become expressed in $CD4^+$ T cells upon TCR stimulation, leading to ICN1 production (Mitra et al. *Front Immunol* 2020, PMID: 32457739). We have included this information in the new version of the manuscript (see **Results** section, page 22): *"The activation of ICN1 under these cell stimulation conditions is consistent with previous data in CD4+ T cells (Mitra et al, 2020; Palaga et al, 2003; Steinbuck et al, 2018)."*

Regarding the second issue indicated by the Referee, we have used in these experiments an antibody that does recognize the active version of Notch1 (mN1A, Cat. No. 552768, BD). In fact, the term ICN1 used in the text refers to the intracellular fragment of NOTCH1. This information is provided in the **Methods** section (pages 39-40): *"For intracellular Tox and ICN1 staining, cells were fixed with Cytofix/Cytoperm (Cat. No. 554714, BD Bioscience) for 10 min and stained with PE-labeled antibodies to Tox (Cat. No. 12-6502-82; eBiosciences; 1:50 dilution) or ICN1 (mN1A, Cat. No. 552768; 1:50 dilution) for 1 hour at room temperature in phosphate-buffered saline solution supplemented with 5% fetal bovine serum and 10% saponin".*

Specific Point #5. *In Fig. 9, a significant limitation is that the analysis is performed in steady-state conditions rather than after an immunization challenge that triggers a strong Tfh response (and ideally a GC reaction) Thus, conclusions about the physiological role of Vav1/3 in the generation of normal Tfh cells may not be definitive.*

Authors' response: Agree. Given that other Referees of this work have complained about the large amount of data contained in this manuscript, we have decided to remove this figure from the new version of the manuscript.

In any case, following the Referee's recommendation, we have performed an immunization experiment to further determine the importance of Vav proteins in T_{FH} differentiation. To this end, we immunized wild-type, *Vav1*$^{-/-}$ and *Vav1*$^{-/-}$;*Vav2*$^{-/-}$;*Vav3*$^{-/-}$ mice using NP-keyhole limpet hemocyanin in combination with complete Freund's adjuvant and, subsequently, analyzed T and B cell numbers in the lymph nodes 12 days later. In agreement with our short-term cultures, these experiments indicate that the Vav-deficient mice are defective in the germinal center response associated with this type of immunization(see **Figure for Referee 1** below). In addition, we have found that Vav-deficient $CD4^+$ T cells express very low levels of ICOS when compared to controls under those conditions. Taken together, these data corroborate and further expand the general implication of Vav1 protein in T_{FH} cell response that was presented in the first version of the manuscript.

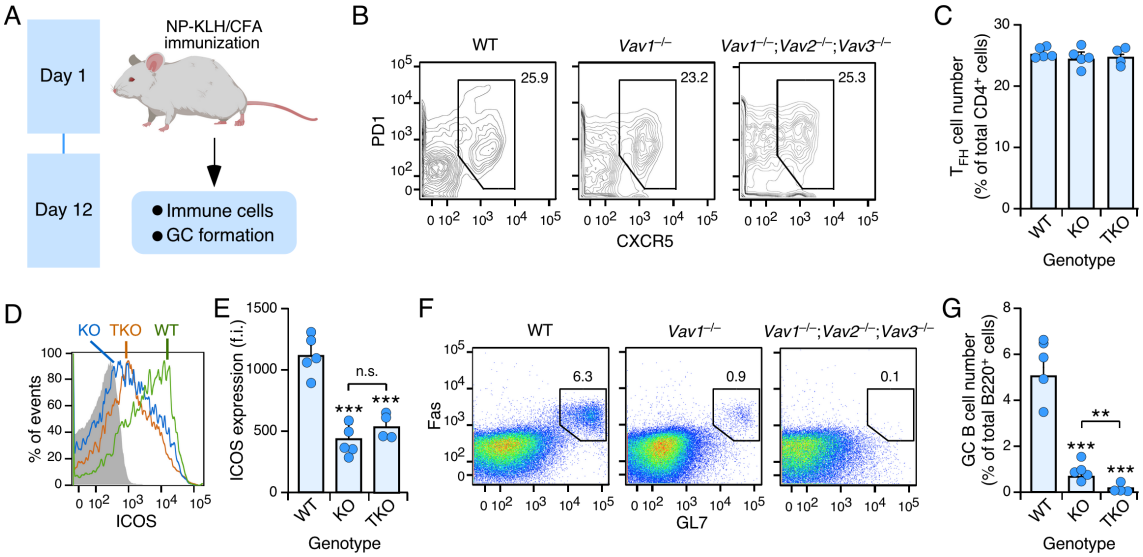

FIGURE 1 FOR REFEREES. ***(A)*** Schematic representation of the experiment used in panels F-J. ***(B)*** Flow cytometry analysis of expression of PD1 and CXCR5 in lymph node-CD4$^+$ T cells from immunized mice of indicated genotypes [(WT (n = 5), *Vav1*$^{-/-}$ (n = 5) and *Vav1*$^{-/-}$;*Vav2*$^{-/-}$;*Vav3*$^{-/-}$ (n = 4)]. Numbers indicate the relative percentage (%) of the cell population selected. ***(C)*** Quantification of the percentage of T_{FH} cell numbers in lymph node-CD4$^+$ T cells from immunized mice. Each point represents the values obtained with a single experimental mouse. n as in B. ***(D*** and ***E)*** Example of the flow cytometry detection (D) and quantification (E) of ICOS expression in T_{FH} cells from immunized mice of indicated genotypes. In E, each point represents the values obtained with a single experimental mouse. n as in B. ***(F)*** Flow cytometry detection of expression of Fas (CD95) and GL7 in B220$^+$-gated lymph node cells from immunized mice of indicated genotypes. Numbers indicate the relative percentage (%) of the cell population selected. n as in B. ***(G)*** Quantification of the percentage of germinal center (GC) B cells in B220$^+$-gated lymph node cells from the indicated mice. Each point represents the values obtained with a single experimental mouse. n as in B.

REVIEWER #2:

General Comment. *Robles-Valero et al. systematically characterized the functional impacts of cancer-associated VAV1 mutations on the three main VAV1 signaling branches. This includes 51 different VAV1 mutations and definition of a classification based on their impact the downstream signaling branches. They demonstrated the most frequent VAV1 mutant subtype with a truncated CSH3 (that alleviates auto-repression and activates RAC1 and NFAT but lacks CBL-B mediated suppressor activity) specifically drives PTCL formation in mice via the cooperation of the polarization, chronic activation, and transformation of follicular helper T cells. Overall, while the study is of significance and the conclusions are important for understanding peripheral T cell lymphomagenesis, the presented data is difficult to follow. Figures are very packed, with multiple abbreviations and color codding that make the reading very complex. The thinking process and the reason for each experiment are not clearly presented and can seem random at times. For example, there's no clear reason as to why Lung SH2 mutations are presented in figure 1E; 3D structures presented in figure 2C and 4D-E don't seem to add any information or there's no clear explanation as to why it is being shown to the reader. Figure 3 seems like a repetition of figures 1 and 2 with some added information from other tumors, again with no clear reason. Figures 5-9 seem like a completely different manuscript and go from studying VAV1 mutational landscape to validating in vivo the effects of a specific VAV1 mutation. Overall, the manuscript as currently organized in nearly incomprehensible due to the density of the data and the fact that the results and discussion sections jump from one figure to the other and to supplementary which contributes to the difficulty in reading. The manuscript would perhaps be better off being split into two separate papers, one with analysis of VAV1 mutational landscape and its functional impact and the other showing in vivo validation of VAV1ΔC in mouse T cells. A general additional suggestion is that the authors should only show the detailed in vitro assays of the T-cell neoplasm-related VAV1 mutations in the main figures and focus the unique features of the trivalent VAV1 mutations (one of which was functionally validated in the following mouse model). Most data in Figure 1, 2, 3 and 4 can either go to the supplemental data or can be used for another manuscript. Then maybe there would be the space to better describe the reasoning and results of each experiment.*

Authors' response: We thank the Referee for considering our work important for understanding peripheral T cell lymphomagenesis. We also appreciate it very much her/his constructive criticisms regarding the complexity of the work and the way in which some data are presented. Those comments have helped us in reformatting the manuscript in a more structured manner. We indicate below our comments on the issues raised by the Referee as well as the changes we have made in the new version of the manuscript to tackle them:

(1) We have eliminated the following panels in the figures (we use here to the numbers used in the first version of the manuscript): **1E** (the distribution of mutations in different tumor types), **1F** (the table with the uncoupling mutants), **2C** (3D structure of the CSH3), **3A** (the scheme of passenger mutations), **4A-C** (scheme and 3D structures), **4E** (3D structures CSH3), **6C** (expression of T_{FH} markers in the transcriptome of $Vav1^{\Delta C}$-transformed cells) and the entire **Figure 9** (role of Vav family proteins in normal T_{FH} differentiation). We have also eliminated the panels **B-G** of the old **Supplementary Figure 1** (now, **new Fig. EV1**) as indicated by the Referee (the 3D structures of all the domains).

(2) We have simplified the information given in **Figure 3** (now, **new Fig. 2**). Specifically, we removed the scheme of the passenger mutations (panel A) and we refer to the names of the tumors following the abbreviations used in the rest of the manuscript.

(3) We have simplified the information given in **Figure 4D** (now, **new Fig. 3A**) (only highlighting this time the residues that are outside the previously described inhibitory interfaces of the CSH3 domain).

(4) We have changed the place where some of the data are presented. Thus, all the data regarding the characterization of the VAV1 mutants is concentrated in **Figure 1** (leading to the elimination of **Figure 2**). In **Figure 4** (**now Fig. 3**), we have transferred panels A-C to the **Figure EV3**.

(5) We have changed some figures to reduce the number of colors used (e.g., **Figure 3**, which is now the **new Figure 2**). The rest of the figures maintain rather uniform color codes. The same applies, we believe, to the extended view and appendix figures.

(6) We have reformatted parts of the manuscript to explain better the rationale of some experiments. This has been mainly done in the section describing the functional characterization of the mutants (which was simplified, shortened, and better integrated with the information contained in former **Figure 3** (now, **new Fig. 2**), see pages 7-9), the explanation of the effect of the mutants in the context of the regulation of the activity of Vav proteins (which was also simplified and abbreviated, see pages 12-15), and the rationale for the *in vivo* experiments (pages 15-16).

(7) We reformatted the manuscript to avoid multiple calls for figures that could distract the readers.

We thank again the Referee for her/his advice on all those issues. It is obvious that we could have done a much better work in all those aspects in our original submission. However, we disagree with some of her/his comments:

(1) It is true that the manuscript has a lot of data and that most figures are composed of many panels. We believe that this is to some extent unavoidable since we aimed at: **(i)** Carrying out an extensive characterization of the mutations found in tumors (and that had to include lung tumors as well given that they have been potential hotspot *VAV1* mutations in them). **(ii)** Cataloguing these mutations in all the downstream pathways regulated by Vav1 (which is far from being monofunctional). **(iii)** Demonstrating that the most relevant mutations do act as oncogenic drivers *in vivo*. **(iv)** Dissecting the specific downstream pathways involved in that process. **(v)** Deciphering whether this transforming activity is due to *ex novo* functions or the exacerbation of the normal physiological role of the protein (this information has been removed from the current version of the manuscript). In addition, we preferred to show the data obtained and do not use "data not shown" throughout the manuscript.

(2) It is possible that we have failed in doing that, but we do believe that the data are presented in a logical way (an issue appreciated by the two other Referees). This logic follows the steps indicated in the above paragraph. Regarding the rational of the tumors used, that has been explained in the Introduction. In any case, we hope that such logic is now more apparent upon the changes made in the manuscript thanks to the input from the Referees of this work.

Specific Point #1. *Figure 1D: R678Q was defined as a "LOF" mutation, but still showed activation on SRF assay. It will be informative to verify whether this mutation affects the RAC*

activity or not by pull-down assay. This information will help to explain whether the activation on SRF assay is RAC1 dependent or not.

Authors' response: Agree. We have carried out in the new version of the manuscript G-LISA assays in both COS1 and Jurkat cells to evaluate the impact of several VAV1 mutants (including R678Q) on the activation of the three main members of the RHO family (RHOA, RAC1 and CDC42). Our data indicate that VAV1^{R678Q} does activate RAC1 in both cell types (see **new Figs. 1E** and **Appendix S9**). This info is given in the new Results section as well (see pages 8-9 of new manuscript version): *"As a complementary avenue to the data obtained using the indirect JNK and SRF assays, we used the G-LISA method to test the direct effect of 3 VAV1 mutants belonging to the bivalent (Y174C, G819S) and signaling branch-specific (R678Q) subsets on the activation of the three main RHO family GTPases in both COS1 and Jurkat cells. As positive controls, we utilized constitutively active versions of VAV1 (Δ1-189 and Δ835-845), RAC1 (Q61L), RHOA (Q63L) and CDC42 (Q61L). When compared to VAV1^{WT}, we found that all the chosen VAV1 mutants could activate the incorporation of GTP onto RAC1 irrespectively of the functional subclass involved (Fig. 1E and Appendix Fig. S9). By contrast, they exhibited much lower activities on RHOA and CDC42 (Fig. 1E and Appendix Fig. S9). This RAC1-specificity is consistent with previous biochemical and cell-based experiments (Aghazadeh et al, 2000; Couceiro et al, 2005; Crespo et al., 1997; Rapley et al., 2008)."*

Specific Point #2. *Figure 5C, did the mice in the Vav1dC+E201A group which died show any PTCL phenotypes?*

Authors' response: Yes, we have analyzed this mouse. We could not see any evidence for the development of any tumor type in that single mouse according to anatomopathological analyses. This information has been included in the legend to this figure (now, new **Fig. 4**) (page 60): *"Note: the mouse transplanted with EGFP-transduced cells that has died in these experiments did not show any sign of tumor development according to anatomopathological analyses (data not shown)."*

Specific Point #3. *Figure 5M, the authors should also show the gene expression data of EGFP+Vav1dC+E201A control samples.*

Authors' response: Agree. We have included the requested data. As expected, we did not detect any differences in the interrogated transcripts in this case when compared to EGFP$^+$ controls (see new **Fig. 4M**). We have also modified the text in the **Results** section to include these data (see pages 17-18): *"Further buttressing the T_{FH} cell-like phenotype of these tumor cells, we found using quantitative reverse transcription-PCR (qRT-PCR) that they express high levels of transcripts encoding typical follicular helper cell markers such as PD1, CXCR6, ICOS, Bcl6 and interleukin 21 (Fig. 4M, left panel). In addition, they show elevated levels of canonical Notch1 target genes such as Hes1, Dtx1 and Ptcra (Fig. 4M, right panel). Most of these transformed cell isolates also display high levels of p-Akt (Fig. 3N and Fig. EV4A) and p-Erk (Fig. 4O and Fig. EV4A) when compared to EGFP$^+$ lymphocytes. These molecular and signaling features are not observed in cells expressing the catalytically-deficient Vav1$^{ΔC+E201A}$ mutant protein (Fig. 4M)."*

Specific Point #4. *Figure 6L, the authors should focus the overlapping genes between VavΔC-dependent genes and other PTCL models, not the overlapping genes between Tet2 loss and RhoAG17V.*

Authors' response: Agree. It is important to note, however, that there is a chronic lack of a good mouse models for these tumors (reviewed in Mhaidly et al. *Oncogenesis* 2020; PMID:

32796826) and, when available, most of them do not have associated genome-wide expression data. Due to this, we have included the following datasets that are available:

(i) The AITL-like condition that develops in the SJL mouse model (Jain et al., *Am. J. Pathol.*, 2015; PMID: 26363366). As seen in the **new Figure EV5D,E**, we could not find any similarity with this transcriptome. This is indicated in two paragraphs in the **Results** section of the new manuscript version (pages 19-20): *"In addition, we have included in these in silico comparisons the transcriptome previously described in the AITL-like condition that spontaneously develops in Swiss Jim Lambert (SJL)/J mice (Jain et al, 2015; Mhaidly et al, 2020). This disease is primarily derived from the exacerbation of IL21 signaling in T_{FH} cells (Jain et al., 2015).* […] *We did not find any statistically significant similarity with the transcriptome previously described in the SJL/J mouse model, indicating that the similarity found between the Vav1$^{\Delta C}$ and the RhoA^{G17V};Tet2$^{-/-}$ model is specific. In fact, in the case of SJL/J mice, we found quite opposite transcriptomal patterns when using GSEAs (Fig. EV5D,E)."*

(ii) Human AITL patients. To this end, we used the two GSE6338 and GSE19069 gene expression microarray datasets that are publicly available (Piccaluga et al., *J Clin Invest*, 2007; PMID: 17304354. Iqbal et al., *Blood*, 2010; PMID: 19965671). This expression datasets are, to our knowledge, the best available in terms of: **(a)** Number of specific AITL samples contained (*n* = 40). **(b)** The presence of data from healthy controls (CD4$^+$ T cells). In this case, we did find a significant level of overlap with the transcriptome of our Vav1$^{\Delta C}$-driven AITL condition (see **new Fig. EV5G-J**). We have included this information in the **Results** section of the new manuscript version (page 21): *"Further in silico analyses indicated that the transcriptome of the Vav1$^{\Delta C}$-driven AITL bears high levels of similarity with the differential expression programs present in a large percentage of AITL patients (Fig. EV5G,H). Such similarity is significantly higher in the case of the Vav1$^{\Delta C}$ upregulated (49.5% of cross-species overlap) than in downregulated (19.2% of cross-species overlap) gene subset (Fig. EV5H). The overlapping transcriptomal subsets are enriched in gene signatures linked to the function of E2F and the NFAT–Tox axis, although SRF-, ICN1-, AP1-, Foxo- and mTORC-related gene expression programs are also observed (Fig. EV5I,J). As expected (Fig. 5J-K), a similar overlap is seen between the transcriptomes of human- and mouse RhoA^{G17V};Tet2$^{-/-}$ AITL samples (Fig. EV5J). However, unlike the case of the Vav1$^{\Delta C}$-driven transcriptome, we could not observe any consistent enrichment in this case in Notch1-related gene signatures (Fig. EV5J, right panel). Collectively, these data indicate significant levels of similarity of the gene expression programs of Vav1$^{\Delta C}$-transformed CD4$^+$ T cells and a significant percentage of human AITL cases."*

Specific Point #5. *Figure 7C, the ectopic expression level of different Vav1 mutant protein is different, and this may cause different biological effects. The authors should address this.*

Authors' response: Disagree. It is true that the mutant versions are expressed at lower levels than the wild-type counterpart, but this is a usual feature that is observed when using truncated versions of Vav proteins in most studies (and independently of the cell model or transfection method used). This is an advantage in this case, since it can be argued that the lack of transformation by the wild-type Vav1 cannot be due to problems associated with low levels of protein expression. The three mutants used are expressed at comparable levels. It can be in fact argued that, given its lower molecular weight, the Vav1$^{\Delta N}$ that shows reduced activity in these assays is expressed at higher levels if we consider molar ratios.

Specific Point #6. *Figure 7L, the increased p-Akt in Vav1dC+E201A infected cells shown here is not consistent with the data shown in Figure 5N.*

Authors' response: Agree. However, we do not consider that these two conditions are strictly comparable. In **Figure 7L** (now, **new Fig. 6L**)**,** we have analyzed $CD4^+$ T cells that have been stimulated with antibodies to CD3 and CD28 in cell culture. By contrast, in **Figure 5N** (now, **new Fig. 4N**), we have analyzed $CD4^+$ T cells directly collected in a steady-state condition from healthy spleens.

Specific Point #7. *Figure 8. The authors should include the EGFP+Vav1dC+E201A as a control for all the inhibitor treatment experiments.*

Authors' response: We respectfully disagree. We have already shown in **Figure 7** (now, **new Fig. 6**) that the catalytically dead mutant does not stimulate any of the signaling readouts used in **Figure 8** (now, **new Fig. 7**). Likewise, the effects of this mutant in cell proliferation are also quite marginal (**Fig. 7F**, now **new Fig. 6F**). Thus, the benefit of including this mutant protein in these experiments is unclear to us. It is also worth noting that repeating again all these experiments would entail the use of extra mice that, given the explanation stated above, is not very appropriate according to institutional animal experimentation procedures.

Specific Point #8. *Figure 9E, the difference shown here between Vav1-/- and Vav1-/-, Vav2-/-, Vav3-/- samples suggests Vav2 and Vav3 may also play a role in the proliferation, is this due to the differential expression of Myc?*

Authors' response: This is an interesting question indeed, although we believe that is not within the scope of the work presented in this manuscript. In any case, and as indicated above (**General Comments** section), we have decided to eliminate this part of the results to shorten the manuscript and keep with the main take-home message (the role of the mutants in tumorigenic processes *in vivo*).

REVIEWER #3:

General Comment. *This is a comprehensive tour-de-force analysis of 51 VAV1 mutants found in two human cancer types, PTCL and NSCLC, which is a very valuable resource for both cancer researchers and cell signaling researchers. By using a range of complementary assays following expression of the mutants in cultured cells, the authors choose a functional mutation that they then test extensively in a mouse model in T cells in vivo, as well as cultured mouse T cells. One concern with the interpretation of their results is that they assume that VAV1 DH-PH only activates RAC1, whereas in some circumstances it can act on other Rho GTPase family members. The indirect luciferase assays they are using could equally be activated by multiple other pathways. These assays are useful for high throughput and rapid screening through the mutants, but not so informative about which pathway(s) are feeding in upstream.*

Authors' response: We thank the Referee for her/his kind comments and for the overall positive view of the results presented. Regarding the role of other GTPases in the context of *VAV1* mutations, we will discuss this issue within the comments to her/his **Specific Points #2 to 4**.

Specific Point #1. *Overall, the text and figures are very dense in information; some careful re-writing would help to get the main messages across without getting lost in the detail.*

Authors' response: Agree. This point of concern was also raised by **Referee #2** as well. To tackle with this problem, we have done the following changes in the new version of the manuscript:

(1) We have eliminated the following panels in the figures (we use here to the numbers used in the first version of the manuscript): **1E** (the distribution of mutations in different tumor types), **1F** (the table with the uncoupling mutants), **2C** (3D structure of the CSH3), **3A** (the scheme of passenger mutations), **4A-C** (scheme and 3D structures), **4E** (3D structures CSH3), **6C** (expression of T_{FH} markers in the transcriptome of Vav1$^{\Delta C}$-transformed cells) and the entire **Fig. 9** (role of Vav family proteins in normal T_{FH} differentiation). We have also eliminated the panels **B-G** of the old **Supplementary Figure 1** (now, **new Fig. EV1**) as indicated by **Referee #2** (the 3D structures of all the domains).

(2) We have simplified the information given in **Figure 3** (now, **new Fig. 2**). Specifically, we removed the scheme of the passenger mutations (panel A) and we refer to the names of the tumors following the abbreviations used in the rest of the manuscript.

(3) We have simplified the information given in **Figure 4D** (now, **new Fig. 3A**) (only highlighting this time the residues that are outside the previously described inhibitory interfaces of the CSH3 domain).

(4) We have changed the place where some of the data are presented. Thus, all the data regarding the characterization of the VAV1 mutants is concentrated in **Figure 1** (leading to the elimination of **Figure 2**). In **Figure 4** (**now Fig. 3**), we have transferred panels A-C to the **new Figure EV3**.

(5) We have changed some figures to reduce the number of colors used (e.g., **Figure 3**, which is now the **new Figure 2**). The rest of the figures maintain rather uniform color codes. The same applies, we believe, to both the expanded view and appendix figures.

(6) We have reformatted parts of the manuscript to explain better the rationale of some experiments. This has been mainly done in the section describing the functional characterization of the mutants (which was simplified, shortened, and better integrated with the information contained in former **Figure 3** (now, **new Fig. 2**), see pages 7-9), the explanation of the effect of the mutants in the context of the regulation of the activity of Vav proteins (which was also simplified and abbreviated, see pages 12-15), and the rationale for the *in vivo* experiments (pages 15-16).

(7) We reformatted the manuscript to avoid multiple calls for figures that could distract the readers.

Specific Point #2. *The Introduction should mention that VAV1 has also been reported to be a GEF for RHOA and CDC42 under some cellular contexts. This is particularly relevant for RHOA, since frequent mutations in RHOA are found in the same groups of PTCL as have VAV1 mutations (this should be introduced in more detail and discussed more extensively in the Discussion; e.g. Fujisawa et al., Leukemia 2018), and because SRF activation is most frequently linked to RHOA rather than RAC1 activation. Moreover, some of the LOF mutations in VAV1 could equate to similar LOF/DN mutations of RHOA in some PTCLs.*

Authors' response: Partially agree. In the **Introduction**, we already indicate that *"VAV1 is a GEF for RHO GTPases, namely RAC1"*. It is true that some reports have indicated connections of VAV1 with RHOA and CDC42. However, if they exist, they must be through a catalytic-independent, indirect signaling mechanism. In this context, we have extensively shown that VAV1 uses RAC1 as main substrate and, to a much lower extent, RHOA (Crespo et al. *Nature* 1997, PMID: 8990121; Couceiro et al. *Exp Cell Res* 2005, PMID: 15950967). This is also in agreement with the biochemical activity shown by the protein *in vitro* (Crespo et al. *Nature* 1997, PMID: 8990121), an issue that has been corroborated by other groups (Rapley et al. *EMBO Rep* 2008, PMID: 18511940). For example, in that latter work, it has been estimated that the exchange rates of VAV1 towards RAC1 are 12.4- and 14.2-fold higher than those for RHOA and CDC42, respectively. This also puts into question the work by Fujisawa et al. (Leukemia 2018, PMID: 28832024), since it is unlikely that the proposed $RHOA^{G17V}$–VAV1 adaptor module could be assembled with such low catalytic affinities. Furthermore, it is known that exchange factors can only bind to GTPases in the nucleotide-free state, not in the GDP-bound conformation in which the $RHOA^{G17V}$ mutant must likely exist in cells. In line with the latter issue, we have in fact a work going on in the lab in which we demonstrate that the leukemogenic activity of $RHOA^{G17V}$ is not VAV1-dependent.

Regarding the activation of the serum response factor (SRF), it is worth noting that this event can occur upon the activation of any RHO GTPase, including of course RAC1 (Hill et al. *Cell* 1995, PMID: 7600583 & Westwick et al. *Mol Cell Biol* 1997, PMID: 9032259). In fact, any factor that would alter the G-actin/ F actin ratios in cells will do the job. Agreeing with this, it has been recently shown that the oncogenic driver function of the $RAC1^{P29S}$ mutant is mediated, at least in part, by the activation of SRF (Lionarons et al. *Cancer Cell* 2019, PMID: 31257073).

In any case, and to satisfy the Referee's request, we have included additional experiments in the new version of the manuscript that have measured the impact of a collection of VAV1 mutants on the activation of the three main members of the RHO family (RHOA, RAC1 and CDC42). Our data, generated both in COS1 and Jurkat cells, indicate again that the main substrate of VAV1 is RAC1 (new **Figs. 1E** and **Appendix Fig. S9**). We have included proper controls to demonstrate that these assays can detect GTP-loaded RHOA and CDC42 in both cell types (new **Figs. 1E** and **Appendix Fig. S9**).

Due to the inclusion of these data, we have incorporated the following text in the Results section of the new manuscript version (pages 8-9): *"As a complementary avenue to the data obtained using the indirect JNK and SRF assays, we used the G-LISA method to test the direct effect of 3 VAV1 mutants belonging to the bivalent (Y174C, G819S) and signaling branch-specific (R678Q) subsets on the activation of the three main RHO family GTPases in both COS1 and Jurkat cells. As positive controls, we utilized constitutively active versions of VAV1 (Δ1-189 and Δ835-845), RAC1 (Q61L), RHOA (Q63L) and CDC42 (Q61L). When compared to VAV1^{WT}, we found that all the chosen VAV1 mutants could activate the incorporation of GTP onto RAC1 irrespectively of the functional subclass involved (Fig. 1E and Appendix Fig. S9). By contrast, they exhibited much lower activities on RHOA and CDC42 (Fig. 1E and Appendix Fig. S9). This RAC1-specificity is consistent with previous biochemical and cell-based experiments (Aghazadeh et al, 2000; Couceiro et al, 2005; Crespo et al., 1997; Rapley et al., 2008)."* The **Methods** section has also been modified to describe this new set of experiments (see page 35).

Specific Point #3. *There are some mutants that activated JNK but not SRF signaling, or that have a much stronger effect on one or the other, in the luciferase read-outs, which provides additional evidence that the two assays measure different upstream signals and not simply 'RAC activity'. The authors should revise their interpretation of these results throughout.*

Authors' response: It is difficult to compare side by side both assays since they are performed using different cell lines and transfection protocols. The cytoskeletal remodeling of these two cell lines is also different, an issue that can influence the SRF readout. In general, it is true that the SRF assays usually gives better signals when using proteins with low activity. This is probably the reason for the discrepancy observed in the color codes of the heatmap between the SRF and JNK data for some VAV1 mutants. In any case, we believe that the new data presented in our work regarding the activation of RAC1, RHOA and CDC42 by VAV1 clearly indicate that these readouts reflect primarily the effects of the mutants on RAC1 activity (new **Figs. 1E** and **Appendix Fig. S9**; see **Specific Point #2** above).

Specific Point #4. *The authors have not measured RAC1 activation directly for any of the mutants. They need to include some example RAC1 activity assays for at least a subset of relevant mutations (and simultaneously test for RHOA activation, see point 1).*

Authors' response: Agree. As indicated in **Specific Point #2**, these data have now been included in the new **Figures 1E** and **Appendix Fig. S9**.

Specific Point #5. *Fig. 1: panel B - information on how each pathway was assayed should be provided here (e.g. SRE-Luc), because some of the assays are quite indirect (e.g. JNK activity is not being assayed directly); panels C, D; it would help to add asterisks or similar mark to indicate the positive control mutants (Y174 mutants) because these heat maps contain so many different mutants.*

Authors' response: Agree. Thank you for raising those points. Following this Referee's advice, we have reformatted the way in which these experiments are indicated in the **new Figure 1B**. In addition, we have added red asterisks to indicate the positive controls used in these experiments in the **new Figure 1C,D**.

Specific Point #6. *Fig 3A, B: the color coding in these figures is very complex and not intuitive to follow. It would be easier if all pathways that are unchanged are in green, and pathways that are changed are in a different color (red/pink for up, blue for down). The naming of the subclasses*

is also difficult to follow in the text - adding a diagram of VAV1 domains again with the subclasses indicated on it would help interpretation here (or just forget all the subclasses).

Authors' response: Agree. Regarding the first part of the Referee's comment, we have changed the colors used in **Fig. 3** (now, **new Fig. 2**). Regarding the second issue, we do believe that it is important to keep the different subclasses to give all the information to the readers. In addition, the Vav1 domains targeted by the mutations are already shown in gray boxes in each subclass. We also mentioned in which tumors these mutations have been found. All this information is indicated in the legend to the **Fig. 3** (now, **new Fig. 2**).

Specific Point #7. *Fig. 5, Fig. 6 and accompanying text: it is not possible to compare the mouse studies carried here with those from a different group using different genetic changes (Zang et al., 2017). This can be put in the discussion but not stated as fact when the authors have not repeated the same experiment for direct comparison. The text and figure legend for Fig. 6 also need to make it clear that they are comparing gene expression results from Zang et al. with their own results. This information is only hidden in the Methods section.*

Authors' response: Partially agree. The information regarding how these analyses were done was already indicated in the **Methods** section. However, we acknowledge that it is perhaps better to emphasize this issue in the main text. We have done that in the new version of the manuscript (page 19). The new text says: *"Given the AITL-like phenotype exhibited by Vav1$^{\Delta C}$-transformed T_{FH} cells, we next decided to investigate the level of similarity of the transcriptome of those cells with the gene expression changes previously seen associated with the deletion of Tet2 and/or the expression of a dominant negative (G17V mutation) version of the GTPase RhoA in CD4^{+} T cells (Zang et al., 2017*)." We have also modified the legend to **Figure 6K** (now, **new Fig. 5J**) as requested (page 62): *"Dot plot of the Vav1$^{\Delta C}$-dependent gene signature fit score in the indicated experimental groups (bottom) that were retrieved from a previous work (Zang et al., 2017).*

Regarding the validity of the comparison, we have used in these analyses the GSEA (Gene Set Enrichment Analysis) algorithm. This is a thoroughly validated method with more than 26,700 citations in scientific literature (Subramanian et al. *PNAS* 2005, PMID: 16199517). This is the gold-standard tool to perform cross-comparisons of transcriptomic data from different datasets.

Specific Point #8. *This sentence does not make sense: 'For example, it is known that the bivalent F69V GOF mutation targets a CH residue contributes to the autoinhibited structure of nonphosphorylated VAV1.....'*

Authors' response: Agree. Sorry about this. We tried to indicate here that this CH residue is involved in the intramolecular inhibition of Vav1 activity. However, it is obvious that it has not been properly written. It has been modified in the new version of the manuscript (which has now been transferred to the **Appendix** file, **Appendix Supplementary Text 1**, page 2). The new sentence says: *"For example, it is likely that the F69V promotes a bivalent GOF effect through the disruption of the interactions that the F^{69} residue establishes with the PH (D^{406}), the DH α_{11} helix (F^{386}) and the first PH β strand (Y^{441})."*

Specific Point #9. *Use g not rpm throughout the methods section (g is the relevant unit; rpm is centrifuge type-dependent).*

Authors' response: Agree. Following the Referee's recommendation, we have made the appropriate changes in the **Methods** section of the new version of the manuscript.

CHANGES MADE IN NEW VERSION

MANUSCRIPT EMBOJ-2021-108125

(A) Main text:

Title page. No changes made.

Abstract. We have made minor changes (indicated in red). Total number of words: 150 words.

Introduction. Minor changes (indicated in red)

Results. They were modified to include all the new experiments, controls and comments suggested by Referees. Main changes can be found in pages 7-10, 13-17 and 19-21 of the new version of the manuscript. All changes have been highlighted in red.

Discussion. It has been modified to accommodate the points raised by Referees. Main changes can be found in page 25 of the new version of the manuscript. Changes indicated in red.

Methods. They were modified to include all the new experimental procedures. Changes indicated in red.

References. It has been modified to include new references.

Figure legends. They have been modified to incorporate the changes and new experimental data requested by the Referees. Changes indicated in red.

(B) Figures:

Old/New Figure 1. We have modified panel B to include the experimental approached used to determine each of the signaling activities of VAV1 (requested by Referee #3, Specific Point 6). We have also colored them to get correlations with each of the pathways depicted in Figure 1A. We have widened the color scale of the heatmaps in panels C and D (requested by Referee #1, Specific Point 1). We have also removed the original panels E and F. Finally, we have included two new panels: panel E (showing the effect of Vav1 mutations on the activation status of RHO proteins using G-LISA assays) and panel F (former panel A in old Figure 2).

Old Figure 2. It has been eliminated. Panel A is now in Figure 1 (panel G). Panel B is now in panel E in Figure EV2. Panel C has been eliminated.

Old Figure 3/New Figure 2. We have removed panel A (bystander mutations) and reduced the number of colors used in the new panel A (former panel B; requested by Referee #3, Specific Point 6). As a result of these changes, this figure now has 3 panels (the old version had 4 panels).

New Figure 3: It has been significantly simplified. The new figure has now 3 panels.

Old Figure 4. It is now presented as the new Figure EV3.

Old Figure 5/New Figure 4: We have included the samples from Vav1$^{\Delta C+E201A}$-expressing tumoral cells in panel M (requested by Referee #2, Specific Point 3). We have also changed panel M to include data of Notch1 target genes (right graph; requested by Referee #1, Specific Point 3). Data of *Trp53* and *Tet2* mRNA expression levels have now been transferred to the Figure EV4 (panel B).

Old Figure 6/New Figure 5: We have eliminated the original panel C.

Old Figure 7/New Figure 6: No changes.

Old Figure 8/New Figure 7: No changes.

Old Figure 9: It has been eliminated from the manuscript

(C) Appendix and Extended View files:

(C.1) Text:

Appendix text 1 (page 2). It has been modified to accommodate all changes made in the new version of the manuscript.
Figure legends. They have been modified to accommodate all changes made in the new version of the manuscript.

(C.2) Figures:

Old Figure S1/New Figure EV1. We have removed panels B-G as requested by Referee #2. As a result of these changes, this figure now has 2 panels (the old version had 8 panels).

Old/New Figures S2-S9/New Appendix Figures S1-S8. No changes made.

New Appendix Figure S9. We have included the raw data for the GTPase activity assays.

Old Figure S11/New Figure EV2. We have included the panel B from old Figure 2 as the new panel E in this supplementary figure.

Old Figure S12/New Figure EV3: We have included here some of the panels originally presented in old Figure 4.

Old Figure S13/New Figure EV4. We have included a new panel (B) originally present in old Figure 5M.

Old Figure S14/New Figure EV5. We have included five new panels showing the *in silico* analyses of the Vav1$^{\Delta C}$-dependent transcriptome with the SJL model (panels D,E) and human AITL samples (panels F-J). We included these new data to accommodate the request made by Referees #2 (point 4). As a result of these changes, this figure now has 10 panels (4 panels in the original submission).

(C.3) Tables:

Appendix Table S1. No changes.

(D). Other datasets:

Old/New SuppDataSet1. No changes.

Old/New SuppDataSet2. No changes.

Old/New SuppDataSet3. No changes.

1st Revision - Editorial Decision 24th Aug 2021

Dear Dr Bustelo,

Thank you for submitting your revised manuscript (EMBOJ-2021-108125R) to The EMBO Journal. Please accept my apologies for the unusual protraction with the processing of your revised manuscript. Your amended study was sent back to the reviewers for re-evaluation, and we have received comments from two of them, which I enclose below.

As you will see, referees #1 and #3 stated that their issues have been comprehensively resolved and they are now broadly in favour of publication, pending minor revision.

Please note that we also had input from referee #2 who doesn't find that the revisions have address his/her concerns. However, we have received further comments on this from referee #1 who found the issues to be satisfactorily addressed.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal.

Please consider the remaining minor issues stated by referee #3 carefully by adjusting the text where appropriate. Further, we need you to consider a number of points related to formatting and data representation as detailed below, which should be addressed at re-submission.

Please contact me at any time if you have additional questions related to below points.

As you might have noted on our web page, every paper at the EMBO Journal now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes a 'model' figure as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide this figure and the bullet points.

Thank you for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to your final revision.

Again, please contact me at any time if you need any help or have further questions.

Kind regards,

Daniel Klimmeck

Daniel Klimmeck PhD
Senior Editor
The EMBO Journal

Formatting changes required for the revised version of the manuscript:

>> Limit the keywords for your manuscript to maximally five

>> Rename the current 'Competing interests' section into 'Conflict of Interest'.

>> Rename the current 'Data Resources ' section into 'Data Availability. Remove the privacy from the GEO microarray data set.

>> Add the funding information 'senior postdoc contract for J.R-V. by the Spanish Association against Cancer ; Salamanca local section of the Spanish Association against Cancer support to L.F.-N' to our manuscript onine system.

>> Nomenclature of the Dataset EV legends needs to be corrected to Dataset EV1 etc. (files and manuscript text). Dataset EV1 has 2 tabs with a Table S2, Dataset EV3 has 2 tabs with a Table S4. These tables are not called out in the manuscript and could be confusing for readers Please rename them to Table A and B to make sure there is no confusion with the regular EMBO Press nomenclature for tables.

>>Please provide source data as one zipped file per figure.

>> Add the current Appendix Supplementary text 1 to the main manuscript.

>> Please consider additional changes and comments from our production team as indicated by the .doc file enclosed and leave changes in track mode.

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 22nd Nov 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors are presenting a revised manuscript that is further improved and adequately addresses key suggestions from the reviewers.

Referee #3:

In this revision, the authors have answered all of my points by adding extra data and changing the text. They have put a lot of effort into improving the clarity of their complex and impressive results by modifications to figures and text. The response to reviewers is also very comprehensive and clear.

I have two remaining suggestions for improving the text:

1. Introduction: 'One of the main functions of VAV1 is the catalysis of the activation step of RHO GTPases, namely RAC1'. Based on their results, I suggest rewording to 'One of the main functions of VAV1 is to catalyse the activation of the RHO GTPase RAC1'.
2. Figure 2: UNCLOUPLING should be UNCOUPLING.

2nd Revision - Editorial Decision 9th Sep 2021

Dear Dr Bustelo,

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. I would thus like to ask for your consent on keeping the additional referee figure included in this file.

Also, in case you might NOT want the transparent process file published at all, you will also need to inform us via email immediately. More information is available here:
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On a different note, I would like to alert you that EMBO Press is currently developing a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:
https://www.embopress.org/video_synopses
https://www.embopress.org/doi/full/10.15252/embj.2019103932

Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

If you have any questions, please do not hesitate to call or email the Editorial Office.

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.

Kind regards,

Daniel Klimmeck

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xosé R. Bustelo
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2021-108125

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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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?	No statistical methods were used to determine sample size. In general, at least three independent replicates were performed in all experiments. For experiments subjected to higher variability, such as animal-based studies, at least five animals per experiment. The sample size used for each experiment is indicated in the appropriate figure legend of the manuscript.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	OK. We have included this statement in Method section.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded.
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.	In all animal studies, groups were allocated randomly.
For animal studies, include a statement about randomization even if no randomization was used.	OK. We have included this statement in Method section
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.	Most animal studies were coordinated by a technician in the lab, who does not have direct knowledge of the experiments and hypothesis tested.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For all animal studies, the investigators were blind to group allocation. Blinding was not applicable to the rest of experiments. We have included this statement in Method section.
5. For every figure, are statistical tests justified as appropriate?	We have included all the statistical tests used in each experiment in Method section: "The number of biological replicates (n), the type of statistical tests performed, and the statistical significance are indicated for each experiment in the figure legends as well as the results section of this document. Parametric and nonparametric distributions were analyzed using Student's t-test and Mann-Whitney test, respectively. Chi-squared tests were used to determine the significance of the differences between expected and observed frequencies. The Tukey's honest significance difference test was used to identify groups showing differential enrichment of the indicated signatures. Statistical analyses of the immunoblot-generated data were carried out using the GraphPad Prism software (version 6.0). In all cases, values were considered significant when $P \leq 0.05$. Data obtained are given as the mean ± SEM."
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We have included all the statistical methods used in each experiment in Method section

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

http://grants.nih.gov/grants/olaw/olaw.htm
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http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Yes. We have included standard deviation in each group of data
Is the variance similar between the groups that are being statistically compared?	Yes. All the groups have a similar variance

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).	In our study we have used commercially-available antibodies that have been validated by the manufacturer for the application (immunoblot, flow cytometry) and species (mouse or human) utilized in our experiments. This information is available at each manufacturer's website and can be obtained through the catalog numbers indicated above. The homemade Vav1 antibody has been validated by us in overexpression, knockdown and knockout experiments.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We have included information about cell lines used in this paper in Method section. All cells were obtained from the ATCC and they are authenticated by the manufacturer. Periodic checkouts were performed at the Genomics Unit of our Center. All cell lines have tested negative for mycoplasma contamination.

* 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.	Mus musculus, C57BL/10 background, 6-8 week old. The genotype (WT and Vav1–/–;Vav2–/–;Vav3–/–) and age of the animals used in each experiment is detailed in the Methods section of the manuscript. Animals were kept in ventilated rooms in pathogen–free facilities under controlled temperature (23ºC), humidity (50%), and illumination (12–hour–light/12–hour–dark cycle) conditions.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	This information is included in Methods, section Ethics Statment: "All mouse experiments were performed according to protocols approved by the Bioethics Committee of the University of Salamanca and the animal experimentation authorities of the autonomous Government of Castilla y León (Spain). We have not utilized patients or patient-derived samples in this work."
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.	We have complied with all relevant ethical regulations and include a statement affirming this in the manuscript.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
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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	Microarray data presented in this paper has been deposited in GEO database (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE165006.
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).	A Source Data and an unprocessed WBs files were provided with this paper.
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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.	NA