

## A pathogenic role for cystic fibrosis transmembrane conductance regulator in celiac disease

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Editor: Daniel Klimmeck

### Transaction Report:

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

1st Editorial Decision

18th Jul 2018

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Thank you for the submission of your manuscript (EMBOJ-2018-100101) to The EMBO Journal. Your manuscript has been sent to three referees, and we have received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential high interest and novelty of your work, although they also express a number of major issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. While referee #2 is overall more positive, referee #1 states that the molecular details of the mode of inhibition of CFTR by gliadin-peptide P31-43 as well as CFTR agonist VX-770's peptide blocking function are not sufficiently resolved, which undermines the impact of your findings in his/her view (ref#1, pts.1,2,4). Referee #3 agrees in that there are conceptual inconsistencies in the suggested peptide -CFTR interaction model, and questions the physiological relevance of this interaction (ref#3, pts.2). In addition, the referees point to issues related to experimental design, documentation of methodologies and statistics as well as missing controls that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are in principle happy to invite you to revise your manuscript experimentally to address the referees' comments. I agree that adding mechanistic insights into the functional relationship between CFTR, the peptide and VX-770 would be required to achieve a conclusive study.

REFEREE REPORTS:

Referee #1:

In this very interesting study Vilella et al examined the potential role of CFTR function in celiac disease by measuring the effects of gliadin and the gliadin peptide P31-43 on cfr-dependent and other cell functions. The overall conclusion illustrated in the model of Figure 6 is that gliadin peptides, in particular P31-43, interacts with CFTR to inhibit its function, thereby triggering several cellular and tissue events resulting in the disease. Treating cell models and CD mice with CFTR potentiators somehow prevents the reduction in CFTR function and ameliorate the disease.

Although the studies are exhaustive significant uncertainties remain, in particular with respect to the actual effect(s) of P31-43 on CFTR activity and how VX-770 prevents the effects of P31-43.

1. It is not clear how P31-43 inhibits CFTR function. As the authors demonstrate here and reported in many previous studies, P31-43 has many rapid effects on cellular functions, from inhibition of trafficking, aggregation of PKA, protein ubiquitination, to interaction with CFTR to inhibit NBD1 ATPase activity. Demonstrating binding of P31-43 to NBD1 and inhibition of ATPase activity cannot be taken to indicate that P31-43 inhibits CFTR activity. A simple experiment is to measure whole cell CFTR current either in CaCo2 cells or expressed CFTR while including P31-43 in the pipette solution and determine whether P31-43 indeed inhibits CFTR current and how fast after establishing the whole cell configuration. These simple experiments should significantly enhance the studies and main conclusion.
2. It is somewhat puzzling that the potentiators prevent the effect of P31-43. Does VX-770 actually activate inhibited CFTR or does it prevent inhibition by the peptide? Again, the use of current measurement is needed to address this.
3. Considering all the data, to explain the effect of VX-770 it is necessary to assume that P31-43 does not interact with activated CFTR. This should be tested directly by some more direct experiments, at least with the CaCo2 cells. For example, does stimulation of CFTR with Forskolin/IBMX prior to and during treatment with P31-43 prevents inhibition of CFTR current. CFTR current can be assayed as the Cl<sup>-</sup> current inhibited by one of the available specific CFTR inhibitors.
4. Considering CFTR ubiquitination, reduction in bend C and degradation by ubiquitination treatment, it is surprising the authors did not test the effect of CFTR correctors, at least in the cell line. These types of experiments should discriminate between effects of P31-43 on CFTR activity and CFTR expression.
5. The results in Supplementary Figure 7 are quite important to the overall effects of P31-43. I suggest moving this data to the main text. In this respect, Figure 7C lack the control images.

Minor comments:

1. The term % folds in several Figures is strange. Data should be shows either as fold relative to control or as % change. As shown, they are all % change rather than % fold. Please correct.
2. In several blots it is not possible to see the actual level of CFTR due to overexposure. This is important as the authors claim, and show clearly in other blots, that treatment with P31-43 reduces the lever of CFTR and of bend C. This needs to be evident in all Figures, including 3b, 3d, 3i.
3. p7, 16: change nuclear to nucleotide for NBDs.
4. p8, 15: change 2i to 2J and bold like others.
5. ref Zeng et al, 2017 is not complete.

6. Page 12: It is not clear how the mice were treated with VX-770. Was that a single injection of VX-770 15 min prior to treatment with gliadin or was VX-770 injected prior to each challenge over the 4 weeks treatment? Was VX-770 injected daily? If not, what are the biological half-lives of VX-770 and gliadin in mice? Patients are treated daily with VX-770 and thus it is surprising that what appears to be a single injection was sufficient to prevent the effects of 4 weeks treatment with gliadin. Please clarify.

Referee #2:

In this paper, Vilella et al. report a novel and unexpected role for CFTR in celiac disease (CD). CFTR is an anion channel whose dysfunction is associated with cystic fibrosis, and the finding that it is also associated with CD is of considerable interest. Using a combination of molecular, biophysical, cellular and physiological approaches the authors provide evidence that gluten-induced inhibition of CFTR is an environmental stress signal for epithelial cells that constitutes an important step in the inflammatory response leading to CD. More specifically, they show that a gluten-derived peptide inhibits CFTR function, which is sufficient to cause epithelial stress and inflammatory signaling. They also report on the underlying mechanism by which the peptide inhibits CFTR function. Importantly, they also show that pharmacological activation of CFTR limits inflammation and restores tolerance to gluten in mouse models and ex vivo models using patient derived tissues and cells.

The manuscript is well-written and provides a massive amount of data that are thoroughly analysed. The results are straightforward and support the conclusions that are made. The findings are highly novel and of interest for the wider audience of the journal. The fact that already FDA-approved activators of the CFTR can limit inflammation in CA models also illustrates that the work has important clinical implications. I have only some minor concerns:

- Fig. 1B: the activation of caspase-1 (by showing formation of p10) is not very convincing and also the corresponding full blot shown in suppl fig 12 is not supporting caspase-1 activation very well. A better experiment should be provided. Moreover, the authors should analyze IL-1beta maturation as a readout for caspase-1 activation.
- Fig. 2B: The signal for CFTR is hyperexposed and does not allow to show that IP of CFTR is equal in all lanes (which is essential for the conclusion of this experiment). A similar comment applies for Fig. 3 B and D.
- The authors use several CFTR potentiators, including an FDA approved drug. However, the use of another drug, genistein (suppl fig 2), is in my opinion not very relevant as this compound is known as a non-specific Tyr kinase inhibitor that may have various effects on cells.
- The authors show that CFTR potentiators were not able to reverse the inhibitory effect of the gluten peptide P31-43 on CFTR function when given to the cultures after peptide challenge (suppl fig 2I). This means that the approach would not work in a therapeutic setting and is worth taken up in the general discussion of the manuscript.
- Page 14, line 8: Reference to fig.6A,B should be deleted

Referee #3:

This is a very extensive study that proposes that CFTR is a critical target of gliadin-derived peptides and that it participates in the pathophysiology of celiac disease. The studies are rooted in compelling epidemiology that indicate that CF is a risk factor for celiac disease. Moreover, a series of experiments show nicely that decreased CFTR function in animal models is associated with immune changes in the intestinal mucosa that are consistent with those seen in celiac disease. Moreover, a series of experiments also show that gliadin-derived peptides can inhibit CFTR. One compelling element of the story is the demonstration that CFTR potentiators can reverse many of these changes. While the story has all of these positive elements, which drive my enthusiasm, several points work against the story in its present form.

The major concerns that I have with the present manuscript are the following:

- 1.- Rigor: this paper is packed with data, and in many places it is hard to judge how the data was derived. In this regard, some examples include:

- a) Regarding the individual blots that were aggregated to derive the bar graphs shown in Figure 1, the methodology is unclear. It seems that each lane represents 5 animals pooled. Apparently, the samples were run in triplicate: it is unclear if these are the same samples or triplicates of independent pooled samples. Moreover, it is unclear why individual samples were not run instead. That would have been preferable.
- b) The data supporting direct binding of gliadin-derived peptides binding to CFTR is at times of low technical quality, such as Fig 2B (the CFTR blot is uninterpretable).
- c) Many of the immunoblot-based data are reportedly based on replicates, and graphs are presented based on these replicates. However, the replicates are missing in the main figures or in the Suppl Figs (e.g., Fig 4A).

2.- The paper advances the notion that gliadin-derived peptides bind to an intracellular domain of CFTR, the NBD1 domain. While evidence of binding between recombinant proteins in vitro is presented, it is unclear how in vivo this peptide would gain access to the intracellular side of CFTR to bind to this domain. The notion that this domain is indeed involved in binding should be tested in vivo by introducing mutant versions of CFTR and demonstrating that NBD1 residues are indeed required. Absent this evidence, several of the in vitro experiments are of unclear in vivo significance.

3.- Several aspects of paper seem tangential to the main thrust of the paper and contribute only to make the paper very difficult to follow at times. For example, the studies pertaining effects of Gliadin peptides on the Vps34 complex, or on SIgA, do not even make it to the abstract, so it is unclear that are necessary in this paper.

4.- In the co-culture experiments presented, it is implied that VX-770 acts on the epithelium, which then acts secondarily on the immune cells present in the system. It would be important to confirm that this is the case (and not some off target effect) using Caco-2 cells that lack CFTR (through CRISPR for example).

Minor concerns:

5.- IL17 and IFN $\gamma$  data in Fig 1F (protein) and Suppl Fig 1D (mRNA) are not consistent. Please explain.

6.- The in vivo experiments presented in Fig 5 are particularly important. Regarding the data pertaining IL-10 and TGF- $\beta$  it is stated: "VX-770 restored the impaired IL-10 and TGF- $\beta$  production in gliadin-sensitive mice". Rather, the data indicate that VX-770 caused induction of these factors to a higher degree than in any other group.

1st Revision - authors' response

27th Sep 2018

*We thank the editor and the reviewers for their comments that have contributed to improve the quality of our manuscript. We have performed new experiments and have addressed all the reviewers' concerns. Moreover, we have edited the text according to the reviewers' suggestions and the editorial requirements.*

*In this revised version, we have included new technical approaches to better characterize the CFTR inhibitory effect of P31-43, as well as to better characterize the positive effects of VX-770. In this context, we included 3 additional co-authors (Y-K. Chao, C. Grimm and A. Luciani) who performed patch clamp experiments and proximity ligation assays.*

*In particular:*

*- we have included in this revised manuscript new experiments as requested by the **Reviewer 1**. By using both Ussing chambers and patch clamp technology we demonstrate that the inhibitory effect of P31-43 on CFTR function occurs within a few minutes and that VX-770 is able to prevent P31-43 mediated CFTR inhibition (new Expanded View Figure EV2).*

*- we have demonstrated that P31-43 is not able to interact with, and to inhibit the activity of, CFTR after forskolin stimulation (shown in Appendix Figure S2B).*

- In addition, we demonstrate that VX-770 is poorly active if it is added after P31-43 addition (Appendix Figure S2B).

Moreover, we provide molecular details on the preventive effects of VX-770. Thus, we performed co-immunoprecipitation and proximity ligation assays to show that VX-770 prevents the interaction between P31-43 and CFTR (shown in the new Figure 2B).

- Following the suggestions of **Reviewer 2**, we have removed the experiments with genistein to focus the in vivo study on the protective effect of established CFTR potentiators, such as the FDA-approved compound VX-770 or the investigational agent Vrx-532. Moreover, we have added a sentence in the Discussion in which we discuss the potential therapeutic use of CFTR potentiators in celiac patients.

- We have analysed IL-1 $\beta$  protein levels as a readout of caspase-1 activation.

- We have performed new experiments to address the concerns of **Reviewer 3**. In particular we responded to the question as to whether the binding of P31-43 to NBD1 CFTR domain could be relevant in the cellular context. We used intestinal epithelial Caco-2 cells in which we exchanged wild type CFTR by mutant versions (in the NBD1 domain) of CFTR. Then, we showed that the same mutations in NBD1 residues that reduced binding of P31-43 in cell-free assays also reduced the interaction of P31-43 and CFTR in the cellular context (shown in the new Expanded View Figure EV3).

- we added new experiments in the bidimensional co-culture model in which we used Caco-2 cells lacking CFTR to confirm the on-target effects of VX-770;

- in addition, we have moved some data to the new Expanded Figures or the Appendix.

According to the suggestions of **all Reviewers**, we have improved the quality of several blots, addressed all the technical concerns and detailed several methodological issues.

We have highlighted in red the relevant changes we have made in the text.

We have introduced the following changes into Figures, new Expanded View Figures and Appendix Supplementary Figures:

#### Figures:

- Figure 1B: the immunoblots relative to pro-caspase 1 and caspase 10 have been improved;

- Figure 2B: the immunoprecipitation blots of Streptavidin and CFTR have been improved;

- Figures 2E and 2I have been moved to new Fig 3 (now Fig 3A and 3B of the revised manuscript).

- New Figures 3C, 3F and 3K (previously Fig 3B, 3D and 3I, respectively): the immunoprecipitation blots have been improved;

- We have moved prior Supplementary Fig S6H to new Fig 4H, as suggested

- We have moved prior Fig 4H-L to the new Expanded View Figure EV4A-E;

- Fig 5L: we added new experiments with Caco-2<sub>CFTR-KO</sub> cells in the upper compartment of the bidimensional co-culture model;

- Fig 6: We have modified the schematic view of celiac disease pathogenesis.

#### New Expanded Figures:

We have added 5 new Expanded View Figures, according to the editorial suggestions, and moved new data or panels previously shown in Supplementary Figures to them.

- Fig EV1A-C: previously Supplementary Fig S2A-C;

- Fig EV2A and B: new experiments in Ussing chamber (A) or patch clamp (B) models;

- Fig EV3A: new experiment using the proximity ligation assay

- Fig EV3B: previously Supplementary Fig S3A. Moreover, the immunoprecipitation blots have been improved;

- Fig EV3C: new set of experiments with Caco-2<sub>CFTR-KO</sub> cells transfected with CFTR bearing mutant NBD1;

- Fig EV3D-F: previously Supplementary Fig S3C-E-F.

- Fig EV4A-E: previously panels 4H-L of Fig 4;

- Fig EV4F: previously Supplementary Fig S7G. Moreover, the immunoprecipitation blots have been improved;

- Fig EV5A-C: previously Supplementary Fig S8A-B and E;
- Fig EV5D-G: previously Supplementary Fig S10A-D.

Appendix Figures:

- Appendix Fig S1A-E: previously Supplementary Fig S1A-E; S1A left is new set of experiments of IL1 $\beta$ .
- Appendix Fig S2A: previously Supplementary Fig S2D;
- Appendix Fig S2B: new set of experiments on the effects of P31-43 in Ussing chambers;
- Appendix Fig S2C-H: previously Supplementary Fig S2E-I;
- Appendix Fig S3, S4 and S5: previously Supplementary Fig S4, S5 and S6, respectively;
- Appendix Fig S6: previously Supplementary Fig S7 (except for S7G which has been moved in Fig EV4F, as said above);
- Appendix Fig S7A and B: previously Supplementary Fig S8C and D;
- Appendix Fig S8A and B: previously Supplementary Fig S9A and C.
- Appendix Fig S9: previously Supplementary Fig S11.

The uncropped gels of the previous Supplementary Fig S12 have been moved to the file "source data".

We added the "synopsis" together with a synopsis image, according to the editorial requests.

### Point by point responses to the Reviewer 1

In this very interesting study Vilella et al examined the potential role of CFTR function in celiac disease by measuring the effects of gliadin and the gliadin peptide P31-43 on cftr-dependent and other cell functions. The overall conclusion illustrated in the model of Figure 6 is that gliadin peptides, in particular P31-43, interacts with CFTR to inhibit its function, thereby triggering several cellular and tissue events resulting in the disease. Treating cell models and CD mice with CFTR potentiators somehow prevents the reduction in CFTR function and ameliorate the disease.

Although the studies are exhaustive significant uncertainties remain, in particular with respect to the actual effect(s) of P31-43 on CFTR activity and how VX-770 prevents the effects of P31-43.

### Responses

*We thank this reviewer for his/her comments.*

*We have followed his/her suggestions and performed a new set of experiments to address all his/her concerns, as indicated below in the response to each of the points raised by the reviewer.*

1. It is not clear how P31-43 inhibits CFTR function. As the authors demonstrate here and reported in many previous studies, P31-43 has many rapid effects on cellular functions, from inhibition of trafficking, aggregation of PKA, protein ubiquitination, to interaction with CFTR to inhibit NBD1 ATPase activity. Demonstrating binding of P31-43 to NBD1 and inhibition of ATPase activity cannot be taken to indicate that P31-43 inhibits CFTR activity. A simple experiment is to measure whole cell CFTR current either in CaCo2 cells or expressed CFTR while including P31-43 in the pipette solution and determine whether P31-43 indeed inhibits CFTR current and how fast after establishing the whole cell configuration. These simple experiments should significantly enhance the studies and main conclusion.

### Responses

*In the submitted manuscript, we showed that P31-43 inhibits CFTR activity in Caco-2 and T84 cells by measuring the forskolin-inducible chloride currents upon P31-43 challenge (either in the presence or absence of pre-treatment with VX-770).*

*In the revised manuscript, we have carefully followed the reviewer's suggestions and performed a new set of experiments to better characterize "the actual effect(s) of P31-43 on CFTR activity". To this aim, we directly added P31-43 to the solution for a few minutes in either Ussing chambers or patch clamp systems.*

*Briefly, to measure forskolin-stimulated  $I_{sc}$ , the solution was first supplemented with 100 $\mu$ M amiloride. Then the solution was supplemented with P31-43 or the control peptide (100 $\mu$ M each) for a few minutes (5 min) followed by forskolin (20 $\mu$ M) (5 minutes of observation) and finally by the specific CFTR inhibitor CFTRInh-172 (10 $\mu$ M). Using this experimental setup, we show that the P31-43 but not the control peptide, highly reduced forskolin-induced chloride currents within a few minutes. These new data are shown in the new Expanded View Figure EV2A.*

*We also used an electrophysiological approach (patch clamp) to demonstrate the capacity of P31-43 to inhibit the CFTR channel activity. These new data are shown in the new Expanded View Figure EV2B.*

2. It is somewhat puzzling that the potentiators prevent the effect of P31-43. Does VX-770 actually activate inhibited CFTR or does it prevent inhibition by the peptide? Again, the use of current measurement is needed to address this.

### **Responses**

*We thank the reviewer for his/her comment.*

*Indeed, VX-770 prevents the inhibition of CFTR activity induced by P31-43.*

*To address this issue, we followed the reviewer's suggestions and performed a new set of experiments, by measuring the preventive activity of VX-770 on "the actual effect(s) of P31-43 on forskolin-induced chloride currents" both in Ussing chambers and patch clamp systems, as described in the responses to Point No. 1.*

*Briefly, after amiloride, the solution was supplied with VX-770 (10 $\mu$ M) for 5 minutes and then pulsed with p31-43 (100 $\mu$ M) followed by forskolin (20 $\mu$ M). We show that in both models, VX-770 was highly effective in preventing the negative effects of P31-43 on forskolin-stimulated chloride currents (Expanded View Figure EV2).*

3. Considering all the data, to explain the effect of VX-770 it is necessary to assume that P31-43 does not interact with activated CFTR. This should be tested directly by some more direct experiments, at least with the CaCo2 cells. For example, does stimulation of CFTR with Forskolin/IBMX prior to and during treatment with P31-43 prevents inhibition of CFTR current. CFTR current can be assayed as the Cl<sup>-</sup> current inhibited by one of the available specific CFTR inhibitors.

### **Responses**

*We agree with the reviewer's comment that our data suggest that P31-43 does not interact with activated CFTR. In the revised manuscript, we have performed new experiments to confirm this hypothesis by directly adding P31-43 for a few minutes to the solution before or after forskolin stimulation.*

*In the first set of experiments, (see responses to point 1) the solution was supplied with 100 $\mu$ M amiloride and then with P31-43 (100 $\mu$ M) for a few minutes before adding forskolin (20 $\mu$ M) (5 minutes observation) and finally CFTRInh-172 (10 $\mu$ M). In these experimental conditions, P31-43 highly reduced forskolin-induced  $I_{sc}$  (Expanded View Figure 2B of the revised manuscript).*

*In other experiments, P31-43 (100 $\mu$ M) was supplied for 5 min after forskolin stimulation, followed by the addition of 10 $\mu$ M CFTRInh-172. In this experimental setting, P31-43 was unable to affect the  $I_{sc}$  traces, while CFTRInh-172 was still effective (Appendix Figure S2B of the revised manuscript).*

*In other experiments the solution was supplemented with VX-770 (10 $\mu$ M) after P31-43 (100 $\mu$ M) addition, followed by 10 $\mu$ M CFTRInh-172. In this experimental setting VX-770 was poorly active in reversing the effects of P31-43 (Appendix Figure S2B of the revised manuscript).*

*We added the CFTRInh-172 in all experiments to demonstrate that the forskolin-stimulated Cl<sup>-</sup> currents are truly mediated by CFTR.*

*Moreover, we have added new experiments including co-immunoprecipitation and proximity ligation assays to demonstrate that a short pretreatment of Caco-2 cells with VX-770 abrogated the interaction between P31-43 and CFTR.*

*Altogether, these results support the hypothesis that P31-43 does not interact with activated CFTR (Fig 2B of the revised manuscript).*

4. Considering CFTR ubiquitination, reduction in bend C and degradation by ubiquitination treatment, it is surprising the authors did not test the effect of CFTR correctors, at least in the cell line. These types of experiments should discriminate between effects of P31-43 on CFTR activity and CFTR expression.

#### **Responses**

*Regarding CFTR ubiquitination, we should note that we are dealing with the plasma membrane pool of wild-type CFTR and not with misfolded CFTR mutants that are not capable of reaching the cell surface. Indeed, we have previously demonstrated (Vilella VR et al, Cell Death & Differ 2013, 20: 1101-1115) that the disposal of wild-type CFTR from the plasma membrane is consequent to the inhibition of CFTR activity by CFTRInh-172, indicating that CFTR is a protein that must be fully functional to avoid its own premature plasma membrane disposal and degradation. In that paper, we showed that the ubiquitination of wild-type CFTR and its subsequent plasma membrane disposal are quite late events (occurring after 6 and 24 h following CFTR inhibition, respectively) that are secondary to the autophagy inhibition resulting from the inhibition of CFTR function and subsequent accumulation of SQSTM1/p62 at the plasma membrane. Notably, SQSTM1/p62 is required for targeting ubiquitylated CFTR to lysosomal degradation. In the present work, we demonstrate that this is also the case for CFTR inhibition by P31-43, so that the late (24 h) CFTR disposal secondary to autophagy inhibition may enhance the detrimental effects of P31-43.*

5. The results in Supplementary Figure 7 are quite important to the overall effects of P31-43. I suggest moving this data to the main text. In this respect, Figure 7C lack the control images.

#### **Responses**

*We have added the control image to this figure (now Appendix Fig S6C of the revised manuscript). We have moved the Supplementary Fig 7G in the new Expanded View Figure EV4F.*

#### **Minor comments:**

1. The term % folds in several Figures is strange. Data should be shows either as fold relative to control or as % change. As shown, they are all % change rather than % fold. Please correct.

#### **Responses**

*We have corrected % fold in % change, as suggested*

2. In several blots it is not possible to see the actual level of CFTR due to overexposure. This is important as the authors claim, and show clearly in other blots, that treatment with P31-43 reduces the lever of CFTR and of bend C. This needs to be evident in all Figures, including 3b, 3d, 3i.

#### **Responses**

*We have performed new experiments and have changed the blots in all these figures.*

3. p7, 16: change nuclear to nucleotide for NBDs.

4. p8, 15: change 2i to 2J and bold like others.

5. ref Zeng et al, 2017 is not complete.

#### **Responses**

*We have made these corrections in the text*

6. Page 12: It is not clear how the mice were treated with VX-770. Was that a single injection of VX-770 15 min prior to treatment with gliadin or was VX- 770 injected prior to each challenge over



the 4 weeks treatment? Was VX- 770 injected daily? If not, what are the biological half-lives of VX-770 and gliadin in mice? Patients are treated daily with VX-770 and thus it is surprising that what appears to be a single injection was sufficient to prevent the effects of 4 weeks treatment with gliadin. Please clarify.

#### **Responses**

*The mice were treated with daily injections of VX-770 15 min prior to each challenge with gliadin over the 4 weeks treatment. This information has been added to the Materials and Methods of the revised paper.*

#### **Point by point responses to the Reviewer 2**

In this paper, Vilella et al. report a novel and unexpected role for CFTR in celiac disease (CD). CFTR is an anion channel whose dysfunction is associated with cystic fibrosis, and the finding that it is also associated with CD is of considerable interest. Using a combination of molecular, biophysical, cellular and physiological approaches the authors provide evidence that gluten-induced inhibition of CFTR is an environmental stress signal for epithelial cells that constitutes an important step in the inflammatory response leading to CD. More specifically, they show that a gluten-derived peptide inhibits CFTR function, which is sufficient to cause epithelial stress and inflammatory signaling. They also report on the underlying mechanism by which the peptide inhibits CFTR function. Importantly, they also show that pharmacological activation of CFTR limits inflammation and restores tolerance to gluten in mouse models and ex vivo models using patient derived tissues and cells. The manuscript is well-written and provides a massive amount of data that are thoroughly analysed. The results are straightforward and support the conclusions that are made. The findings are highly novel and of interest for the wider audience of the journal. The fact that already FDA-approved activators of the CFTR can limit inflammation in CA models also illustrates that the work has important clinical implications.

#### **Responses**

*We thank this reviewer for his/her comments and suggestions.*

*We have followed his/her comments and have performed a new set of experiments to address all the reviewer's concerns.*

I have only some minor concerns:

- Fig. 1B: the activation of caspase-1 (by showing formation of p10) is not very convincing and also the corresponding full blot shown in suppl fig 12 is not supporting caspase-1 activation very well. A better experiment should be provided. Moreover, the authors should analyze IL-1beta maturation as a readout for caspase-1 activation.

#### **Responses**

*We performed new experiments and showed a new blot of caspase-1 in the revised version (new Fig 2B).*

*Moreover, we have analyzed IL-1beta, as readout of caspase-1 activation, as suggested by this reviewer. The experiments, reported in Appendix Fig S1A of the revised manuscript, demonstrate a major increase in IL-1beta protein levels.*

- Fig. 2B: The signal for CFTR is hyperexposed and does not allow to show that IP of CFTR is equal in all lanes (which is essential for the conclusion of this experiment). A similar comment applies for Fig. 3 B and D.

#### **Responses**

*We performed new experiments and changed the blots of Fig. 2B, 3 B, D, I (now 2B, 3C, F and K), as requested.*

- The authors use several CFTR potentiators, including an FDA approved drug. However, the use of another drug, genistein (suppl fig 2), is in my opinion not very relevant as this compound is known as a non-specific Tyr kinase inhibitor that may have various effects on cells.

#### **Responses**

*We agree with this reviewer that genistein is not a selective compound, although it can be experimentally used as CFTR potentiator. Thus, we have removed the experiments with genistein in the revised manuscript to better focus on more specific CFTR potentiators, including the FDA-approved VX-770 or the widely used investigational Vrx-532 compound.*

- The authors show that CFTR potentiators were not able to reverse the inhibitory effect of the gluten peptide P31-43 on CFTR function when given to the cultures after peptide challenge (suppl fig 2I). This means that the approach would not work in a therapeutic setting and is worth taken up in the general discussion of the manuscript.

#### **Responses**

*We thank this reviewer for his/her comment. Indeed, we have added new experiments in which we show, by co-immunoprecipitation and proximity ligation assay, that VX-770 is highly effective in preventing the interaction between P31-43 and CFTR, thus protecting the cells from the inhibitory effects of P31-43 on CFTR activity (new Fig 2B and Expanded View Fig EV3A).*

*Moreover, we have added a sentence to the Discussion in which we speculate that galenic formulations of CFTR potentiators favoring their release in the intestine after gastric passage could be administered before gluten-containing meals to avoid the binding of gliadin-derived peptides to CFTR at the enterocyte surface, thus preventing the detrimental effects of gluten in celiac individuals.*

- Page 14, line 8: Reference to fig.6A,B should be deleted

*We apologize for the mistake. We deleted the reference to Fig 6A,B.*

### **Point by point responses to the Reviewer 3**

This is a very extensive study that proposes that CFTR is a critical target of gliadin-derived peptides and that it participates in the pathophysiology of celiac disease. The studies are rooted in compelling epidemiology that indicate that CF is a risk factor for celiac disease. Moreover, a series of experiments show nicely that decreased CFTR function in animal models is associated with immune changes in the intestinal mucosa that are consistent with those seen in celiac disease. Moreover, a series of experiments also show that gliadin-derived peptides can inhibit CFTR. One compelling element of the story is the demonstration that CFTR potentiators can reverse many of these changes. While the story has all of these positive elements, which drive my enthusiasm, several points work against the story in its present form.

#### **Responses**

*We thank this reviewer for his/her comments and suggestions.*

*We have followed his/her comments and have performed a new set of experiments to address each of the reviewer's concerns listed below.*

The major concerns that I have with the present manuscript are the following:

1.- Rigor: this paper is packed with data, and in many places it is hard to judge how the data was derived. In this regard, some examples include:

a) Regarding the individual blots that were aggregated to derive the bar graphs shown in Figure 1, the methodology is unclear. It seems that each lane represents 5 animals pooled. Apparently, the samples were run in triplicate: it is unclear if these are the same samples or triplicates of

independent pooled samples. Moreover, it is unclear why individual samples were not run instead. That would have been preferable.

#### **Responses**

*We apologise for not being enough clear. The data shown in the graphs of Fig. 1 for each group of treatment (as well as those reported in the other figures) are triplicates of independent pooled samples from 5 mice. Indeed, the samples were independently run three times for each condition and the data were pooled for the analysis. The blots are representative of one experiment for group of treatment. This has been detailed in the Figure legends of the revised manuscript.*

b) The data supporting direct binding of gliadin-derived peptides binding to CFTR is at times of low technical quality, such as Fig 2B (the CFTR blot is uninterpretable).

#### **Responses**

*We have improved the quality of the blots of Fig. 2B as well as of Figures 3B, 3D, 3I (now 3C, F and K), Supplementary Figure S3A, left and Supplementary Figure S7G (now Expanded View EV3B, left and Expanded View EV4F, respectively)*

c) Many of the immunoblot-based data are reportedly based on replicates, and graphs are presented based on these replicates. However, the replicates are missing in the main figures or in the Suppl Figs (e.g., Fig 4A).

#### **Responses**

*We added the replicates of the immunoblots to the file "source data".*

2.- The paper advances the notion that gliadin-derived peptides bind to an intracellular domain of CFTR, the NBD1 domain. While evidence of binding between recombinant proteins in vitro is presented, it is unclear how in vivo this peptide would gain access to the intracellular side of CFTR to bind to this domain. The notion that this domain is indeed involved in binding should be tested in vivo by introducing mutant versions of CFTR and demonstrating that NBD1 residues are indeed required. Absent this evidence, several of the in vitro experiments are of unclear in vivo significance.

#### **Responses**

*We have shown that CFTR and P31-43 co-immunoprecipitated in clathrin+ EEA1- plasma membrane protein fractions from Caco-2 cells as soon as after 5 min incubation of the cells with P31-43 (now Expanded View Figure 3B of the revised version). Indeed, it is known that CFTR, P31-43 and even TG2 enter the endosomal compartment through clathrin+ vesicles for either recycling or lysosomal degradation (Lukacs et al, 1997; Barone & Zimmer, 2016). Thus, our data support the hypothesis that P31-43 may encounter and bind CFTR (and its NBD1 domain) in cells.*

*In this revised version of the manuscript, we followed the reviewer's suggestions and introduced the mutant version of CFTR (double NBD1 mutant CFTR plasmides pcDNA3.1\_F400A/E403A-CFTR and pcDNA3.1\_P439A/P477A-CFTR, the same NBD1 mutants that lose P31-43 binding in cell-free assays) into Caco-2 cells (that were first rendered CFTR-null by CRISP/CAS9 technology and then transfected with mutant CFTR or WT-CFTR as a control). We found that these CFTR mutants do not co-immunoprecipitate with P31-43, as wild-type CFTR does. These cell-based data support the conclusions obtained in cell-free assays.*

3.- Several aspects of paper seem tangential to the main thrust of the paper and contribute only to make the paper very difficult to follow at times. For example, the studies pertaining effects of Gliadin peptides on the Vps34 complex, or on SIgA, do not even make it to the abstract, so it is unclear that they are necessary in this paper.

#### **Responses**

*We have followed this suggestion and moved these results to Expanded View Figure EV4.*

4.- In the co-culture experiments presented, it is implied that VX-770 acts on the epithelium, which then acts secondarily on the immune cells present in the system. It would be important to confirm that this is the case (and not some off target effect) using Caco-2 cells that lack CFTR (through CRISPR for example).

#### **Responses**

*We thank the reviewer for his/her comment. We have added new experiments in which we placed CFTR depleted (through CRIS/CAS9 approach) Caco2 cells in the upper compartment of the bidimensional co-culture model. We found that, in such a model, VX-770 is no more effective in preventing the increased release of IFN $\gamma$  by celiac PBMNC, thus confirming the VX-770 can modulate the immune response of celiac PBMC through targeting CFTR (Fig 5L of the revised manuscript).*

#### **Minor concerns:**

5.- IL17 and IFN $\gamma$  data in Fig 1F (protein) and Suppl Fig 1D (mRNA) are not consistent. Please explain.

#### **Responses**

*We apologize for the mistake. We have correctly plotted the mRNA data in the new graph shown in the new Appendix Figure S1D of the revised manuscript. The raw data are available in the "source data" file.*

6.- The in vivo experiments presented in Fig 5 are particularly important. Regarding the data pertaining IL-10 and TGF- $\beta$  it is stated: "VX-770 restored the impaired IL-10 and TGF- $\beta$  production in gliadin-sensitive mice". Rather, the data indicate that VX-770 caused induction of these factors to a higher degree than in any other group.

#### **Responses**

*We have modified this sentence, as suggested by the reviewer.*

2nd Editorial Decision

17th Oct 2018

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Your revised study was sent back to the three referees for re-evaluation, and we have received comments from all of them, which I enclose below. As you will see the referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues regarding manuscript formatting, data representation and wording, as outlined below, which need to be adjusted at re-submission.

#### REFEREE REPORTS:

Referee #1:

The authors completely and adequately addressed all my concerns and I do not have any further scientist concerns.

However, it is not clear to me what if the difference between EV figures and the figures shown as Appendix. The Appendix figures contain important information and should be included in the final manuscript one way or another.

Referee #2:

The authors have carefully addressed all my comments (as well as these from the other reviewers) and performed several new experiments. In my opinion this study is ready to be published.

Referee #3:

This is a revised manuscript that I had the pleasure of previously reviewing. As stated before, this is a very extensive study that proposes that CFTR is a critical target of gliadin-derived peptides and that it participates in the pathophysiology of celiac disease. I had specific concerns about data presentation and rigor that have been addressed. Similarly a few added experiments have strengthened the paper further. Therefore, I am delighted to recommend publication of the paper.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: LUIGI MAIURI

Journal Submitted to: EMBO Journal

Manuscript Number: EMBOJ-2018-100101R1

**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.

**1. 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?	It was chosen based on earlier experiments performed in the lab using the same technique. The experiments are repeated thrice. We reported significant or nonsignificant based on the three experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For Mice experiment n=10 for each treatment, based on previous experiments performed in the lab and previous publications of our group. All the data reported are either representative of at least three independent experiments
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples, mice or data points were excluded from the reported analyses.
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.	Mice were randomly divided in different groups
For animal studies, include a statement about randomization even if no randomization was used.	Experimental groups were balanced in terms of animal age, sex and weight. No specific method of randomization was used
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.	Mice were randomly divided in different groups without any bias
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was applied upon harvesting samples after the treatments.
5. For every figure, are statistical tests justified as appropriate?	Yes, statistical tests are mentioned in the figure legends and further described in the Materials and Methods section.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, we performed analysis by prism software
Is there an estimate of variation within each group of data?	Yes, every data is presented as mean +/- standard deviation. Cumulative plots of the data are also provided to show the distribution of the data
Is the variance similar between the groups that are being statistically compared?	Yes, significant differences (* p < 0.05, ** p < 0.01, ***p < 0.001) were indicated in the figure legends.

**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).	All antibodies used in this study are exactly specified in the material and methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cell lines were obtained from ATCC and regularly (every 6 months) tested for mycoplasma contamination.

\* for all hyperlinks, please see the table at the top right of the document

**USEFUL LINKS FOR COMPLETING THIS FORM**

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

#### D- Animal Models

<p>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.</p>	<p>For this study were used this mouse model with:</p> <ul style="list-style-type: none"> <li>- Male and femal BALB/c mice (background BALB/cAnNCr1) were purchased from Charles River (Varese, Italy).</li> <li>- Male and female CF mice homozygous for the F508del-CFTR in the FVB/129 outbred background (Cftrtm1EUR, F508del, FVB/129, abbreviated CftrF508del/F508del) and Wild Type littermates, were obtained from Bob Scholte, Erasmus Medical Center Rotterdam, The Netherlands, CF coordinated action program EU FP6 LSHMCT-2005-018932.</li> <li>- Transgenic KO Cfr mice (B6.129P2-KOCftrtm1UNC, abbreviated Cfr-/-), and Wild Type littermates, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).</li> <li>- In order to obtain TG2 -/- mice carrying F508del-CFTR mutation, C57Bl/6 mice KO for TG2 (obtained from Gerry Melino, Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Rome, Italy) were crossed with 129/FVB mice heterozygous for F508del mutation (abbreviated CftrF508del/del/ TG2 -/-).</li> </ul> <p>All above described mice for the study were aged 10-week-old.</p> <ul style="list-style-type: none"> <li>- Prediabetic NOD (Non-obese diabetic) mice were purchased from Charles River (Varese, Italy). At time 12-13 weeks, female mice with manifested diabetes incidence (&gt;250mg/dl), were used.</li> <li>- Male and female NOD.scid AB0nullDQ8 mice (NOD DQ8tg, transgenic mice that express HLA-DQ8 in an endogenous MHC class II-deficient background were backcrossed to NOD mice for 10 generations and intercrossed to produce congenic NOD AB<sup>+</sup> DQ8 mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA).</li> </ul> <p>At least ten mice per group per experiment were used</p>
<p>9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.</p>	<p>These studies and procedures were approved by the local Ethics Committee for Animal Welfare (IACUC No 583,849, 713, 661, 628) and conformed to the European Community regulations for animal use in research (2010/63 UE).</p>
<p>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.</p>	<p>We confirm compliance</p>

#### E- Human Subjects

<p>11. Identify the committee(s) approving the study protocol.</p>	<p>The Ethics Committee of the Istituto Superiore di Sanità (ISS) approved the protocol (#CE/12/341).</p>
<p>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.</p>	<p>patients or patients' parents signed the informed consent</p>
<p>13. For publication of patient photos, include a statement confirming that consent to publish was obtained.</p>	<p>NA</p>
<p>14. Report any restrictions on the availability (and/or on the use) of human data or samples.</p>	<p>There were no restrictions on the availability or on the use of the human samples</p>
<p>15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.</p>	<p>NA</p>
<p>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.</p>	<p>NA</p>
<p>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.</p>	<p>NA</p>

#### F- Data Accessibility

<p>18: Provide a "Data Availability" section at the end of the Materials &amp; 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'.</p> <p>Data deposition in a public repository is mandatory for:</p> <ol style="list-style-type: none"> <li>Protein, DNA and RNA sequences</li> <li>Macromolecular structures</li> <li>Crystallographic data for small molecules</li> <li>Functional genomics data</li> <li>Proteomics and molecular interactions</li> </ol>	<p>NA</p>
<p>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).</p>	<p>NA</p>
<p>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).</p>	<p>NA</p>
<p>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.</p>	<p>NA</p>

#### G- Dual use research of concern

<p>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.</p>	<p>NO</p>
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