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Negative regulation of Type I IFN signaling by phosphorylation of STAT2 on T387

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(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 16 June 2016

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see from the comments below there is an interest in the study. However the referees also find that the analysis should be extended in order to consider publication here. Referees #1 and 2 bring up some suggestions for how to do so. The suggestions raised are very reasonable and I suspect you have given some thoughts to this already. Should you be able to extend the analysis along the lines indicated below then I would be happy to consider a revised manuscript. I realize that you might need some additional time to carry out the revisions and I can extend the revision time to 6 months should that be helpful.

I am also happy to discuss which issues to focus on. Maybe you can go ahead and send me a point-by-point response with what can be done within a reasonable timeframe.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

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

REFEREE REPORTS

Referee #1:

The paper by Yuxin Wang et al., identifies a secondary modification of Stat2, phosphorylation of threonine residue 387, which exerts a negative effect upon the formation of ISGF3 and the expression of its target genes upon type I IFN induction. The authors show that Stat2 is constitutively phosphorylated on T387 in a number of cell lines in culture. The introduction of a T387A variant of Stat2 into U6A cells leads to an enhanced IFN induction of target genes when compared to cells expressing the wt form of Stat2. T387A Stat2 also improves the protection against virus infection and enhances the IFN mediated cell growth inhibition. They suggest that a member of the CDK family is responsible for the phosphorylation of Stat2 and that the simultaneous treatment with CDK Inhibitors and type I IFN might become beneficial for a number of indications.

The paper reports an original observation, the experiments are plausible and the manuscript is well written. It is of value for the field of Stat function and IFN action. The paper would gain significantly in impact if some of the implications mentioned in the discussion, would have been supported by additional experiments in animal models.

Referee #2:

In this study, the authors identify a novel phosphorylation site on the STAT2 protein (T387) which appears to negatively impact STAT2 function. They show that most STAT2 is phosphorylation on T387 in untreated cells, although the proportion of phosphorylated to non-phosphorylated protein varies among different human cell lines in culture. Expression of a T387A or T387D form of STAT2 causes cells to respond more robustly to IFN stimulation of virus infection. Many genes are induced to higher levels in cells expressing mutant STAT2, particularly after short IFN treatments, and they also exhibit greater resistance to virus infection and are more growth inhibited by IFN. Mammary epithelial cells grown in synthetic medium express phosphorylated STAT2, triggered by the presence of glucocorticoid supplements in the medium. Phosphorylation of T387 was found to be sensitive to pharmacologic inhibitors of CDK enzymes, and CDK9 was found to associate with STAT2. Mechanistically, it appeared that phosphorylated STAT2 preferentially dimerized with STAT1 in the absence of tyrosine phosphorylation, but its interaction with pTyr STAT2 was only modestly affected. However, phosphorylated STAT2 bound DNA less robustly than the mutant protein. The authors conclude that STAT2 activity, through its ability to bind DNA and induce gene expression, may be negatively regulated by T387 phosphorylation. Surprisingly, T387 phosphorylation was triggered by short stimulations with IFN at times of maximum gene expression. The mechanism of T387 phosphorylation in response to IFN and the biological significance of the negative regulation of its function during gene induction by IFN remain to be determined.

This study presents interesting and novel data concerning the phosphorylation of STAT2. The data support the authors' conclusion that mutation of T387 in wild type STAT2 yields a more active protein. However, it remains unclear if this phosphorylation event is regulated in a physiologically significant manner, what kineases and signaling pathways are involved, or why phosphorylated STAT2 remains less active.

Minor points:

1. The data on CDK9 association with STAT2, shown only in supplemental table, is insufficient to allow independent evaluation.
2. The phosphorylation site is described as a threonine-phenylalanine motif. The authors probably mean threonine-proline.
3. Whether the changes in STAT2 phosphorylation across different cell lines represents differential IFN responsiveness of different tissues remains to be defined.
4. It should be possible to test the authors' model that U-STAT1/U-STAT2 association explains the impact of pT387 by examining the response of WT and mutant STAT2 homodimers formed in response to IFN in the absence of STAT1.

5. It will be of interest to determine if glucocorticoid treatment impacts IFN gene regulation in mammary epithelial cells in a T387-dependent manner. Similarly, the effect of CDK inhibitors on IFN gene expression could be examined in the context of T387 phosphorylation.

Referee #3:

Wang et al. investigate the transcription factor STAT2, which plays a central role in type 1 interferon signalling. They searched for posttranscriptional modifications of this protein and discovered a novel modification, namely phosphorylation of threonine 387. They go on to characterize the consequences of loss-of-phosphorylation for type 1 interferon signalling and conclude that the modification of T387 results in the suppression of type 1 interferon responses. This is a straightforward and very interesting study with potentially important consequences for the use of interferons in the clinic. I do not have major concerns about this work and its conclusions. In the following I will list several points mainly of editorial nature and to clarify some experimental details.

Figure 1B shows MS results to quantify STAT2 modification at T387. The method description is not entirely clear about how this was done, in particular whether quantitative assessments were done in the absence of ion trapping.
The animal species included in Figure 1D are not sufficiently clear; the Latin names should be given.

On page 4 the authors state that stably transfected HME cells express comparable levels of STAT2 variant proteins, and refer to Fig. 3A. However, Fig. 3A does not show the STAT2 expression level. I think this should be shown, since these cells are an important reagent in this study. Moreover, on page 5 the authors state that Fig. 3A shows that "the expression of T387A inhibited M protein expression compared to cells expressing wild-type STAT2". It would be helpful to add that this effect occurs only in the presence of interferon. And finally, the legend to Fig. 3A should mention that both M protein and STAT2 were detected.

The authors link T387 phosphorylation of STAT2 in HME cells to glucocorticoid signalling (Fig. 4C). Is this a general phenomenon that, for example, can also explain the exceptionally high STAT2 T387 phosphorylation level in Hek cells? In the description of Figure 4C, it would be helpful to refer to individual lanes. Figure 4D is not explicitly mentioned in the text. The text describing Fig. 4E refers to a pan-CDK inhibitor but its name is not given there or in the respective figure legend.

There is a discrepancy between the text describing Fig. 5B and the actual figure. The authors refer to 5B, center, as showing binding partners of STAT2, yet this part shows binding partners of IRF9. Binding partners of STAT2 are shown in 5B, right panel, however, the stated increase in STAT1 binding to the mutated STAT2 is not obvious from the data shown. There appears to be increased co-precipitation of STAT1 and mutant STAT2 with IRF9 (central panel), but the effects are rather moderate. The authors conclude this section by stating that "These results indicate that the phosphorylation of T387 favors the formation of U-STAT1/U-STAT2 heterodimers at the expense of tyrosine-phosphorylated heterodimers and ISGF3." While the first part of this statement is fully justified by the data, and while phosphorylation of T387 inhibits the biological activity of ISGF3, I cannot really see that T387 phosphorylation diminishes the formation of pS1:pS2 heterodimers or pISGF3. My assessment is supported by Fig. 5C, which shows identical heterodimerisation of WT and mutant STAT2 upon interferon treatment. I therefore think that their more cautious statement at the end of the results section is more appropriate at present ("We conclude that the amount of ISGF3 capable of binding to an ISRE sequence is greatly enhanced by the T387A mutation of STAT2, either because less ISGF3 is formed when T387 is phosphorylated, or because the threonine-phosphorylated ISGF3 has a lower affinity for DNA, or both).

In the discussion, the authors mention residue F172 as the potential binding site in STAT1 for the phosphorylated T387 of STAT2, but no justification is given for this assumption. Some elaboration would be helpful for the reader.

Some additional suggestions:
FigS2 (lower part) is somewhat mangled and there seems to be a discrepancy between the figure and

the legend.
In several instances the authors state either the duration or the concentration of interferon treatment. I think both should be given in all cases. In addition, in several figures the lanes are numbered and the content of the lane is given in the legend. It would make the reading easier if this information was provided with the figure labelling.

1st Revision - authors' response 16 August 2016

Referee #1:

1. The paper would gain significantly in impact if some of the implications mentioned in the discussion, would have been supported by additional experiments in animal models.
Performing additional experiment in an animal model is a very good idea, and we are in the process of making T387A STAT2 knock-in mice, which can be studied in a very large number of ways, including responses to various infectious agents, ability to avoid hyper-inflammatory diseases, and the effect of mis-regulation of IFN signaling in tumorigenesis. Because of the broad effects of IFNs, it is far beyond the scope of the current paper to investigate these aspects.

Referee #2:
1. It remains unclear if this phosphorylation event is regulated in a physiologically significant manner, what kinases and signaling pathways are involved, or why phosphorylated STAT2 remains less active.
This question will be answered by #6 listed below.

2. The data on CDK9 association with STAT2, shown only in supplemental table, is insufficient to allow independent evaluation.
We performed a new experiment employing U6A cells expressing FLAG-tagged wild-type or T387A STAT2. The cells were treated with IFN-β (100 IU/ml) for 4 h or were untreated. Whole-cell lysates were used for immunoprecipitations of IRF9 and Flag-STAT2. We observed strong interaction of CDK9 with STAT2 and IRF9 (revised figure 5B). There was little to no effect of IFN or the TA mutation on the binding of CDK9.

3. The phosphorylation site is described as a threonine-phenylalanine motif. The authors probably mean threonine-proline.
As the reviewer pointed out, the "TP" motif should be threonine-proline. Thanks for correcting this error.

4. Whether the changes in STAT2 phosphorylation across different cell lines represents differential IFN responsiveness of different tissues remains to be defined.
Many mechanisms in addition to T387 phosphorylation can affect IFN responsiveness among different tissues, including the expression level of the ISGF3 components, the abundance of IFN receptors, the activity of kinases that affect IFN-dependent signaling, etc. We agree that the studies mentioned by the reviewer will be valuable, and we think that they would best be carried out in T387A knock-in mice.

5. It should be possible to test the authors' model that U-STAT1/U-STAT2 association explains the impact of pT387 by examining the response of WT and mutant STAT2 homodimers formed in response to IFN in the absence of STAT1.
Thanks for this excellent suggestion, which has led to an additional experiment that is reported in the manuscript. We compared ISG induction (Fig. S13) and STAT2-IRF9 interaction (revised Fig. 5D) in STAT1-defecient U3A cells expressing WT or T387A STAT2 with or without IFN-beta treatment. We found that the induction of ISGs and the interaction of STAT2 with RF9 are enhanced in by the T387 mutation. This result indicates that T387 phosphorylation also affects the STAT2-IRF9 interaction.

6. It will be of interest to determine if glucocorticoid treatment impacts IFN gene regulation in mammary epithelial cells in a T387-dependent manner. Similarly, the effect of CDK inhibitors on IFN gene expression could be examined in the context of T387 phosphorylation.

We investigated further ISG induction by IFN-beta following pretreatment with CDK inhibitors or hydrocortisone, with the new results incorporated into the manuscript. As shown in the revised figure 4D, inhibition of ISG induction by hydrocortisone is seen, in a dose-dependent manner. However, the pan-CDK inhibitor not only inhibited T387 phosphorylation but also ablated ISG induction, making interpretation of this part of the experiment impossible

Referee #3:

1. *Figure 1B shows MS results to quantify STAT2 modification at T387. The method description is not entirely clear about how this was done, in particular whether quantitative assessments were done in the absence of ion trapping.*
We agree that the quantitative method was not clear from the description. We added some additional information to the methods section. The quantitative analysis performed involved a targeted analysis of both the unmodified and pT387 forms of three different chymotryptic peptides. These targeted experiments involve trapping of the ions in the instrument, followed by fragmentation in the ion trap. We used the presence of known fragments from each peptide to plot chromatograms and the peak areas of these chromatograms were used for the phosphopeptide quantitation.

2. *The animal species included in Figure 1D are not sufficiently clear; the Latin names should be given.*
Corrected as the reviewer suggested.

3. *On page 4 the authors state that stably transfected HME cells express comparable levels of STAT2 variant proteins, and refer to Fig. 3A. However, Fig. 3A does not show the STAT2 expression level. I think this should be shown, since these cells are an important reagent in this study.*
Corrected as the reviewer suggested.

4. *Moreover, on page 5 the authors state that Fig. 3A shows that "the expression of T387A inhibited M protein expression compared to cells expressing wild-type STAT2". It would be helpful to add that this effect occurs only in the presence of interferon. And finally, the legend to Fig. 3A should mention that both M protein and STAT2 were detected.*
Corrected as the reviewer suggested.

5. *The authors link T387 phosphorylation of STAT2 in HME cells to glucocorticoid signalling (Fig. 4C). Is this a general phenomenon that, for example, can also explain the exceptionally high STAT2 T387 phosphorylation level in Hek cells?*
Cells have different responsiveness to glucocorticoid ligand such as hydrocortisone. Interestingly, HEK293 cells do not express the endogenous glucocorticoid receptor. It is possible that abnormal glucocorticoid signaling contributes to high T387 phosphorylation in HEK293 cells.

6. *In the description of Figure 4C, it would be helpful to refer to individual lanes. Figure 4D is not explicitly mentioned in the text. The text describing Fig. 4E refers to a pan-CDK inhibitor but its name is not given there or in the respective figure legend.*
Corrected as the reviewer suggested.

7. *The authors refer to 5B, center, as showing binding partners of STAT2, yet this part shows binding partners of IRF9. Binding partners of STAT2 are shown in 5B, right panel, however, the stated increase in STAT1 binding to the mutated STAT2 is not obvious from the data shown.*
Corrected as the reviewer suggested.

8. *The authors conclude this section by stating that "These results indicate that the phosphorylation of T387 favors the formation of U-STAT1/U-STAT2 heterodimers at the expense of tyrosinephosphorylated heterodimers and ISGF3." While the first part of this statement is fully justified by the data, and while phosphorylation of T387 inhibits the biological activity of ISGF3, I cannot really see that T387 phosphorylation diminishes the formation of pS1:pS2 heterodimers or pISGF3. My assessment is supported by Fig. 5C, which shows identical heterodimerisation of WT and mutant STAT2 upon interferon treatment. I therefore think that their more cautious statement at the end of the results section is more appropriate at present.*

As crucial and unique components of pISGF3, we considered the interaction of IRF9 with STAT2 as reflecting the integrity of pISGF3. To further justify this point, we have now provided data for the STAT2-IRF9 interaction in STAT1-null U3A cells expressing WT or T387A STAT2 (revised figure 5D). The interaction of IRF9-STAT2 is clearly inhibited by T387 phosphorylation, with or without STAT1.

9. FigS2 (lower part) is somewhat mangled and there seems to be a discrepancy between the figure and the legend. In several instances the authors state either the duration or the concentration of interferon treatment. I think both should be given in all cases. In addition, in several figures the lanes are numbered and the content of the lane is given in the legend. It would make the reading easier if this information was provided with the figure labelling.
Corrected as the reviewer suggested.

10. In the discussion, the authors mention residue F172 as the potential binding site in STAT1 for the phosphorylated T387 of STAT2, but no justification is given for this assumption. Some elaboration would be helpful for the reader.
We employed U3A cells expressing WT or T387A STAT2. Following transient transfection of WT or F172W STAT1, the cells were treated with IFN-beta for 4 hours and total RNAs were analyzed by real-time PCR. ISG induction was enhanced in U3A cells expressing F172W STAT1. Expression of T387A STAT2 and F172W STAT1 synergistically enhanced the expression of some genes, for example, OAS1, but not that of other genes, for example, IFIT1. From the immunoprecipitation experiment, we did not observe significant reduction of U-STAT1/U-STAT2 binding in cells expressing F172W STAT1. At this point, we do not have a clear conclusion about whether F172 in STAT1 is part of a binding site for the phosphorylated T387 residue of STAT2. We have deleted the sentence raising this possibility from the current version of the manuscript.

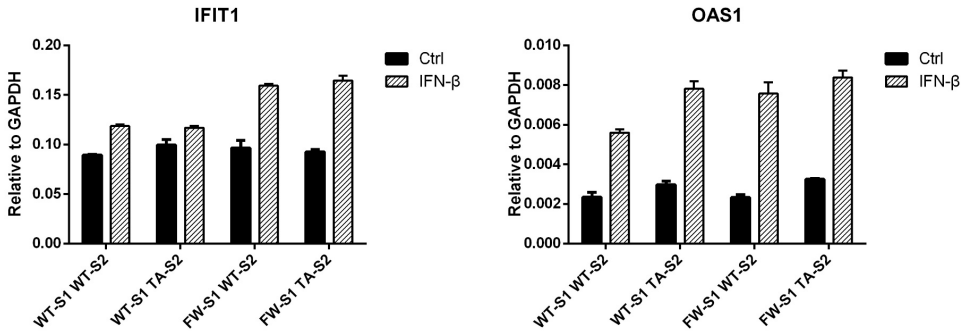

Figure. U3A cells expressing WT or T387A STAT2. The cells were treated with IFN-beta for 4 hours, following transient transfection of WT or F172W STAT1, and total RNAs were analyzed by real-time PCR.

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Corresponding Author Name:	Jinbo Yang and George Stark
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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- 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.
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  - are tests one-sided or two-sided?
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Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

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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).	Please check manuscript pages 12 and 15.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

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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
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The mass spectrometry data from this publication have been submitted to the PRIDE database and assigned the project accession PXD005008. The microarray data from this publication have been submitted to the GEO database "http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE87090" and assigned the GEO accession number GSE87090.
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G- Dual use research of concern

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