Molecular basis of crosstalk between oncogenic Ras and the master regulator of hematopoiesis GATA-2

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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.)

Editor: Esther Schnapp

1st Editorial Decision 23 April 2014

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of referee reports on your study that is copied below.

As you will see, the referees acknowledge that the findings are interesting and the data overall convincing. However, both referees 2 and 3 point out that the (patho-)physiological consequences of GATA-2 phosphorylation/hyperactivity by p38 in response to Ras and/or GATA-2 T354M hyperphosphorylation remain unclear and should be examined. Referee 3 further indicates that it should be analyzed whether GATA-2 T354M hyperphosphorylation decreases GATA-2 function and referee 1 would like to know whether the T354M mutation has an effect in trans on wt GATA-2. Referees 1 and 2 also remark that it should be investigated whether GATA-2 is phosphorylated by other MAP kinases, such as ERK and JNK.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.
Revised manuscripts should be submitted within three months of a request for revision. Please contact us if a 3 months time frame is not sufficient for the revision. Also, the revised manuscript may not exceed 30,000 characters (including spaces, references and figure legends) and 5 main plus 5 supplementary figures, which should directly relate to a corresponding main figure. The current manuscript text largely exceeds our limits, and the text therefore needs to be shortened. Commonly used materials and methods can be moved to the supplementary information, but please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file. Please also change the reference style to the numbered EMBO reports style, which will further help in reducing the overall character count.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. mean +/- SEM, SD) and the statistical tests used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

In this interesting paper the authors establish a mechanistic link between Ras and Gata2, via p38. While the two have been linked functionally before, the mechanistic basis for this has been obscure. Acting downstream of Ras, p38 is shown here to phosphorylate a number of sites in Gata2, of which S192 is the most critical. This phosphorylation results in activation of the transcriptional regulation of Gata2, along with alteration of its nuclear localisation.

This paper makes a very interesting advance and clarifies the relationship between Ras and Gata2. The following considerations may further strengthen the paper.

In figure 2, the p38 inhibitor SB203580 is shown to reduce Gata2 hyper phosphorylation. However, the effect is not complete. Looking at the phosphorylation sites identified in figure 3, some of these could be targets for other MAP kinases. It would be informative to test the effect of inhibition of other MAP kinase enzymes in this context, most notably ERK and JNK.

The relationship between T354M and wt Gata2 remains somewhat unclear. Does the presence of T354M Gata2 within a cell alter the phosphorylation state of wt Gata2 in the same cell? In other words, could the T354M mutation be having an effect in trans on wt Gata2, as well as having an effect in cis?

Referee #2:

The manuscript describes a series of clearly designed, thoroughly controlled experiments showing the importance of phosphorylation in the regulation of the transcription factor GATA2, as well as identifying the responsible kinase and describing the impact of oncogenic Ras on this process. The manuscript is concisely written, the conclusions are justified.

I have one major and some minor points.

The major point concerns the biological consequences of GATA2 phosphorylation by p38 in response to Ras - testing them in an appropriate model system would greatly enhance the significance of the paper.

Minor points:
1) the endogenous hyperphosphorylated GATA2 band is really difficult to see in Figure 1. This is a
very important point and the experiment should therefore be improved.

2) on page 6, line 6 from the top, "GATA-2 expression" should probably be "GATA-2-driven expression"

3) page 7, it is unclear why the authors have started using the p38 inhibitor in the first place. This should be introduced more clearly

4) page 8 and Figure 3, a phosphoetnet analysis of the region containing S192 returns not only p38, but also JNK and ERK as potential kinases. Given that all these kinases can be regulated by Ras, it would be good to determine the effect of specific inhibitors on Ras-driven GATA-2 phosphorylation. Phosphorylation by different kinases may increase both the robustness and the plasticity of the system, and it would be good to have this information

Referee #3:

The present study provides evidence that the GATA2 T354M mutant, associated with immunedeficiency, is hyperphosphorylated and hypofunctional, exhibiting decreased chromatin occupancy, and decreased target gene regulation. Proteomic analyses together with site-directed mutagenesis indicate the importance of S192 in GATA2 T354M hyperphosphorylation. In parallel, the authors show that GATA2 is phosphorylated by p38 and by oncogenic Ras, via S192. This phosphorylation is dependent on the level of p38 activity, and controls chromatin occupancy and the degree of target gene activation.

Both phosphorylation studies and genetic mutations are well documented, and the demonstration of the importance of S192 downstream of p38 and of oncogenic Ras for GATA2 transcriptional activity is convincing. The evidence that S192 is important for GATA2 T354M hyperphosphorylation is also convincing. Transcription factor post-translational modifications downstream of signaling pathways are not well characterized and the manuscript brings new informations in this regard. Several issues remain nonetheless to be clarified.

The authors argue that decreased chromatin binding and decreased function did not contribute to hyperphosphorylation of GATA-2 T354M. Whether or not the reverse is true, i.e. whether hyperphosphorylation of the GATA2 T354M mutant contributes to decreased GATA2 function could be more directly demonstrated using the double mutation.

Furthermore, how are these modifications relevant to the physiological or patho-physiological function of GATA-2?

Minor comment: Fig.5D illustrates 2 doses of GATA-2 but the results do not show dose-dependency in target gene expression. It is preferable to show a dose-dependency or illustrate a single dose.

1st Revision - authors' response 27 May 2014

Reviewer 1:

The reviewer indicated that our paper "makes a very interesting advance and clarifies the relationship between Ras and GATA2."

Query 1 – “It would be informative to test the effect of inhibition of other MAP kinase enzymes in this context, most notably ERK and JNK.”

Response – In the prior version of the manuscript, we provided evidence that p38 inhibition, both chemically and genetically, reduced GATA-2 phosphorylation. We had tested additional signaling inhibitors, but this data was not included in the submission. We repeated these experiments using a range of concentrations of ERK and JNK inhibitors and conducted semi-quantitative analysis to assess whether the inhibitors influenced the S192-dependent hyperphosphorylation. By contrast to the p38 inhibitor (and p38 knockdown), ERK and JNK inhibitors did not significantly affect GATA-2 phosphorylation. This new data is presented as Fig. S2A and B and discussed on page 6, line 16.
Query 2 – “Does the presence of T354M within a cell alter the phosphorylation state of wild type GATA-2 in the same cell? Could the T354M mutation be having an effect in trans on wt GATA-2, as well as having an effect in cis?”

Response – If T354M influences the phosphorylation state and therefore function of wild type GATA-2 in the same cell, it should influence the capacity of wild type GATA-2 to regulate endogenous target genes. We tested this by co-transfecting expression vectors encoding wild type GATA-2 and T354M and quantitated endogenous target gene expression. Increasing amounts of the efficacious T354M expression vector, which was used extensively in the manuscript, did not significantly impact GATA-2-mediated endogenous target gene regulation. This new data was incorporated into Fig. S1B and discussed on page 6, line 8.

We thank the reviewer for the instructive comments.

Reviewer 2:

The reviewer indicated that our paper “describes a series of clearly designed, thoroughly controlled experiments showing the importance of phosphorylation in the regulation of the transcription factor GATA-2, as well as identifying the responsible kinase and describing the impact of oncogenic Ras in this process. The manuscript is concisely written, the conclusions are justified”.

Query 1 - “The major point concerns the biological consequences of GATA-2 phosphorylation by p38 in response to Ras – testing them in an appropriate model system would greatly enhance the significance of the paper”.

Response - Our manuscript describes the discovery of a Ras-p38-dependent pathway that controls GATA-2 activity through targeting specific phosphorylation sites, which had not been previously implicated in GATA-2 function. Our study utilized a powerful discovery system, G1E cells, which have a normal proerythroblast phenotype (Weiss et al. MCB, 2007; Welch et al. Blood 2004), express endogenous GATA-2, and have GATA-2 chromatin occupancy patterns (Fujiwara et al. Mol. Cell 2009) resembling GATA-2 chromatin occupancy in primary murine bone marrow hematopoietic precursors (Li et al. Nat. Immunol. 2011). Thus, we believe that this new regulatory mechanism operational in a biological relevant system represents the first mechanistic advance in understanding how Ras influences GATA-2 activity. The reviewer requested that we further explore the biological consequences of the paradigm that we established in the G1E system and associated molecular studies in 293 cells and in cell-free extracts.

In new experimentation, we made a discovery that confirms and extends the significance of the Ras-p38-GATA-2 axis. In the manuscript, we had proposed that the low-level phosphorylation of wild type GATA-2 might result from the need to suppress the hyperphosphorylated state, which would restrict GATA-2 activity. We explored this mechanism and discovered that treatment of G1E cells with the phosphatase inhibitor okadaic acid leads to major accumulation of the phosphorylated form of expressed wild type GATA-2. The ~6 fold enhancement of wild type GATA-2 phosphorylation mimicked that seen with the T354M mutant. In addition, we demonstrated that okadaic acid induced endogenous GATA-2 to adopt the slow mobility phosphorylated band and importantly also induced endogenous GATA-2 target genes (Hdc and Gfi1). This new data, which was incorporated into Fig. 2I and J, provides strong evidence that cellular phosphatases restrict wild type (expressed and endogenous) GATA-2 phosphorylation. This data further supports the biological importance of the paradigm that we developed in the manuscript. It will be interesting to test this paradigm in diverse biological and pathological contexts, but we believe this is beyond the scope of this phase of the discovery work, given that nearly nothing was known about the mechanistic consequences of GATA-2 phosphorylation and nothing was known regarding how Ras might alter GATA-2 activity.
Minor point 1 – “The endogenous hyperphosphorylated GATA-2 band is really difficult to see in Figure 1”.

Response – Under electrophoretic conditions on large-format 8% SDS-PAGE that effectively separate hyperphosphorylated isoforms of expressed wild type GATA-2 and T354M, endogenous G1E cell GATA-2 migrates as a fuzzy band (Fig. 1B, left panel). Upon lambda phosphatase treatment of cell extracts, this band adopts a considerably faster mobility (Fig. 2D). In our new analysis with okadaic acid, we increased the acrylamide concentration to 10% and subjected the proteins to a longer period of electrophoresis. Under these conditions, one also sees the heterogeneity of the endogenous GATA-2 band, and okadaic acid treatment of cells results in accumulation of the slow mobility, phosphorylated band.

Minor point 2 – “GATA-2 expression should probably be GATA-2-driven expression”

Response – This phrase was eliminated in the process of truncating text to conform to the < 32,000 character/space limit.

Minor point 3 – “It is unclear why the authors started using the p38 inhibitor in the first place. This should be introduced more clearly.”

Response – On page 6, line 16 we added the statement: “Screening signaling pathway inhibitors revealed”.

Minor point 4 – “It would be good to determine the effect of specific inhibitors on Ras-driven GATA-2 phosphorylation”.

Response - In the prior version of the manuscript, we provided evidence that p38 inhibition, both chemically and genetically, reduced GATA-2 phosphorylation. We had tested additional signaling inhibitors, but this data was not included in the submission. We repeated these experiments using a range of concentrations of ERK and JNK inhibitors and conducted semi-quantitative analysis to assess whether the inhibitors influenced the S192-dependent hyperphosphorylation. By contrast to the p38 inhibitor (and p38 knockdown), ERK and JNK inhibitors did not significantly affect GATA-2 phosphorylation. This new data is presented as Fig. S2A and B.

We thank the reviewer for the instructive comments.

Reviewer 3:

The reviewer indicated, “both phosphorylation studies and genetic mutations are well documented and the demonstration of the importance of S192 downstream of p38 and of oncogenic Ras for GATA-2 transcriptional activity is convincing. The evidence that S192 important for T354M hyperphosphorylation is also convincing. Transcription factor post-translational modifications downstream of signaling pathways are not well characterized and the manuscript brings new information in this regard”

Query 1 – “Whether hyperphosphorylation of the GATA-2 T354M mutant contributes to decreased GATA-2 function could be more directly demonstrated using the double mutant”.

Response – If hyperphosphorylation contributes to decreased GATA-2 T354M function, one prediction is that mutating S192, in the context of T354M, would restore activity to regulate endogenous target genes. We tested the double mutant (S192A/T354M) for its capacity to regulate an endogenous target gene, and it had little to no activity, analogous to T354M. This new data was added as Fig. S3E and described on page 8, line 2.
Query 2 – “How are these modifications relevant to the physiological or pathophysiological function of GATA-2?”

Response - Our manuscript describes the discovery of a Ras-p38-dependent pathway that controls GATA-2 activity through targeting specific phosphorylation sites, which had not been previously implicated in GATA-2 function. Our study utilized a powerful discovery system, G1E cells, which have a normal proerythroblast phenotype (Weiss et al. MCB, 2007; Welch et al. Blood 2004), express endogenous GATA-2, and have GATA-2 chromatin occupancy patterns (Fujiiwa et al. Mol. Cell 2009) resembling GATA-2 chromatin occupancy in primary murine bone marrow hematopoietic precursors (Li et al. Nat. Immunol. 2011). Thus, we believe that this new regulatory mechanism operational in a biological relevant system represents the first mechanistic advance in understanding how Ras influences GATA-2 activity. The reviewer requested that we further explore the biological consequences of the paradigm that we established in the G1E system and associated molecular studies in 293 cells and in cell-free extracts.

In new experimentation, we made a discovery that confirms and extends the significance of the Ras-p38-GATA-2 axis. In the manuscript, we had proposed that the low-level phosphorylation of wild type GATA-2 might result from the need to suppress the hyperphosphorylated state, which would restrict GATA-2 activity. We explored this mechanism and discovered that treatment of G1E cells with the phosphatase inhibitor okadaic acid leads to a major accumulation of the phosphorylated form of expressed wild type GATA-2. The ~6 fold enhancement of wild type GATA-2 phosphorylation mimicked that seen with the T354M mutant. In addition, we demonstrated that okadaic acid induced endogenous GATA-2 to adopt the slow mobility phosphorylated band and importantly also induced endogenous GATA-2 target genes (Hdc and Gfi1). This new data, which was incorporated into Fig. 2l and J, provides strong evidence that cellular phosphatases restrict wild type (expressed and endogenous) GATA-2 phosphorylation. This data further supports the biological importance of the paradigm that we developed in the manuscript. It will be interesting to test this paradigm in diverse biological and pathological contexts, but we believe this is beyond the scope of this phase of the discovery work, given that nearly nothing was known about the mechanistic consequences of GATA-2 phosphorylation and nothing was known regarding how Ras might alter GATA-2 activity.

Query 3 – “Fig 5D illustrates 2 doses of GATA-2, but the results do not show dose-dependency”.

Response – We repeated the experiment using an additional concentration of expression vector. The new data (Fig. 4D) illustrates the dose dependency of GATA-2, in the presence of oncogenic Ras, to enhance endogenous target gene expression.

We thank the reviewer for the instructive comments.

2nd Editorial Decision 18 June 2014

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it. Referee 2 still has two concerns that I would like you to address before we can proceed with the official acceptance of your manuscript.

The current character count of the manuscript text still somewhat exceeds our limits. Can you may be shorten the text a little more? You could move the Immunofluorescence section in the Materials and Methods to the supplementary methods, as this is a standard procedure.

Can you please specify the tests used to calculate p-values in the respective figure legends? This information is currently missing and must be provided in the figure legends (1C,E; 2B,E,H,I,J;
Since January this year, EMBO press papers are accompanied online by 1) a short summary of the findings and their significance, 2) 2-3 bullet points highlighting key results and 3) a synopsis image. Can you please send us this information, and may be use the model in figure 5 for the synopsis image, which needs to be exactly 211x157 pixels large? Please note that this is rather small and that not too much information can be included, and that text needs to be readable at the final image size. I think that only the right upper part of the model would fit well. If you want, you can also provide a larger image that is 550 pixels wide and up to 400 pixels high. For selected papers, we have larger synopsis images, but I will need to check first that we still have free slots for larger synopses.

I look forward to seeing a new revised version of your manuscript as soon as possible. Please let me know if you have any questions.

REFEREE REPORTS:

Referee #2:

The manuscript by Katsumura et al. has been improved by the addition of new data showing the presence of a hyperphosphorylated wt GATA-2 in okadaic acid-treated cells, whose appearance correlates with increased expression of GATA-2-dependent genes. This does not quite address my original point, i.e. what are the biological consequences of GATA-2 phosphorylation by p38 in response to Ras, since okadaic acid is very pleiotropic and might affect gene transcription in general, or even viability. Details of the treatment should be given, i.e. how long were the cells treated, and was viability affected. More importantly, since phosphatases, and particularly PP2A, are known to regulate gene transcription, a gene whose transcription is independent of GATA-2 should be shown as a control to rule out that the authors are reading a general effect of the inhibitor on transcription in these cells.

One comment to the authors' response to query 2 of Reviewer 1:

In the experiments shown in revised Figure S1B, the amount of the mutant transfected is lower than, or at best equal to, the amount of GATA2 transfected. It would seem that to assess a potential dominant negative effect the experiment should have been performed in cells expressing lower levels of GATA2 than T354M. An immunoblot showing the amount of GATA2 proteins expressed must be included, particularly because the reviewer's query was about the possibility that the mutant would alter the phosphorylation state of wild type GATA-2 in the same cell and therefore an effect in trans on wt GATA-2.

Referee #3:

The authors have fully addressed my comments.

2nd Revision - authors' response 19 June 2014

Editor's Comments:

1. As instructed, we moved the Immunofluorescence methods into the supplementary file. We also deleted a few redundant and/or non-essential words throughout the manuscript.

2. At the end of each figure legend, we incorporated a statement conforming to EMBO Reports style stating the mode of statistical analysis.

3. We generated the short summary of findings and significance, bullet points highlighting key results, and a synopsis image (part of Fig. 5, as recommended).
Reviewer 2 Comments:

1. We have incorporated details on the time of the okadaic acid treatment in the figure legend on page 15. The okadaic acid treatment did not significantly impact cell viability nor did it impact global RNA Polymerase II transcription. Regarding the latter point, we added a graph to Fig. 2J demonstrating that Gapdh expression is insensitive to okadaic acid. The Hdc and Gfi1 data are normalized to 18S rRNA, which also is insensitive to okadaic acid.

2. The reviewer commented on our response to the query from Reviewer 1 regarding whether T354M might influence phosphorylation and therefore function of wild type GATA-2. The reviewer indicated that we should include a Western blot demonstrating protein expression. We utilized the same vectors used in multiple other figures of the manuscript, and these figures documented the utility of the vectors. T354M and wild type GATA-2 mobility overlap, and one cannot simply use anti-HA or anti-GATA-2 antibodies to simultaneously detect (and compare levels of) the two proteins in the same sample. This would require re-engineering vectors to generate a size difference via mutation or by incorporating tags of different sizes, and the engineered proteins would need to be put through various assays to assess functionality. We do not believe this type of work will be productive, nor should it be required to interpret the rigorous data presented in the manuscript. The reviewer also commented that we should study the problem when T354M is expressed at a higher level than GATA-2. However, we do not believe that this artificial molecular biological scenario would have physiological and/or pathological relevance. MonoMAC is characterized by one wild type GATA2 allele and one mutant allele, and there is no reason to think that T354M level would exceed that of wild type GATA-2. In our very extensive analyses of these two proteins, we have never accrued evidence that T354M accumulates at higher levels than GATA-2.

We thank the editor and reviewer for instructive comments.

3rd Editorial Decision 23 June 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

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