EMBO reports

Manuscript EMBOR-2012-35851

Long-range genomic interactions epigenetically regulate the expression of a cytokine receptor

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Review timeline:

Submission date:	14 February 2012
Editorial Decision:	15 March 2012
Revision received:	19 June 2012
Editorial Decision:	29 June 2012
Revision received:	02 July 2012
Accepted:	05 July 2012

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 15 March 2012

Thank you for the submission of your research manuscript to our editorial office. We have now received the full sets of reviews on your study.

As the reports are pasted below I would prefer not to repeat them here in detail, but to only summarize the main points raised by the referees. You will see that all referees agree on the potential interest of the findings. However, they also agree that more work is needed in some instances to provide stronger evidence for the claims made. Referee 2 points out that additional controls are needed, for example in non-immune cells that do not express IFGNR1. This referee also feels that the ChiP experiments should be extended and that the effects of CTCF depletion on the long-range chromosomal interactions should be investigated. Referee 3 suggests testing whether IFNgamma is expressed in a bi- or mono-allelic way and whether or not demethylation in TH2 cells would lead to co-localization and activation of the IFNGR1 gene.

Overall, given the reviewers constructive comments and the potential interest of the study, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees must be addressed. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will 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; they will otherwise be treated as new submissions. If you feel that this time is not sufficient for a successful resubmission I can extend this period slightly. As indicated in one of my last emails to you, now would be a good time to cut down on the length of the manuscript (it should not exceed roughly 28,000 characters, including references and spaces). Should you find the length constraints to be a problem, you may consider including any peripheral data in the form of Supplementary information. Materials and Methods required for the repetition of the key experiments may, however, not be displayed as supplementary information only. Combining the results and discussion section will also help to shorten the text, as it avoids unnecessary repetition. I would also kindly ask you to identify two of the currently 7 figures that could be moved to the supplementary section, as we can only allow 5 main figures. Finally, in addition to the information on the statistical analysis that you have already given in the manuscript, may I kindly ask you to indicate how many independent times the experiments have been repeated?

I look forward to seeing a revised form of your manuscript when it is ready.

Please do not hesitate to contact me if you have trouble shortening the manuscript. I will be more than happy to help you deal with it.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

This manuscript by Deligianni and Spilianakis investigates the transcriptional regulation of the interferon g receptor 1 (IfngR1) gene during TH1 cell differentiation. The authors report the intriguing observation that IfngR1 colocalizes mostly mono-allelically with the gene coding for its ligand (Ifng) located 1 Megabase away on the same chromosome. It is further shown that mono-allelic co-localization strongly correlates with IfngR1 mono-allelic expression, co-localization also with the RNA polymease II (PolII) but independently of ongoing transcription. Finally, evidence is provided that the long-range interaction process may involve the chromatin organizer protein CTCF. This is a very convincing study and well-written manuscript, which uses up-to date and diverse relevant techniques (DNA/RNA FISH, 3C, Chromatin immuno-precipitation, ChIP-loop assays, etc..), with careful controls performed for all the experiments. The results are original and provide significant insight into molecular mechanisms likely to sustain expression of both a cytokine gene locus and its corresponding receptor during T lymphocyte polarization, and of potential interest to the readership of EMBO reports.

Referee #2:

This paper describes in long range interactions between the Ifng and Ifngr1 loci that may be involved in mediating their co-regulation during T-cell specification. The data are clearly presented and of general interest. Several points in this manuscript require further attention.
1). There are several series of experiments involving DNA and RNA-FISH that have been used to detect the colocalisation between the Ifng and Ifngr1 loci loci. While several control experiments are shown in TH2 cells compared to TH1 cells, it would be appropriate to perform additional control experiments in non-immune cells such as fibroblasts, where these genes are not expressed. One would expect to see no significant colocalisation under these conditions. These same controls in fibroblasts should be performed for the 3C experiments.
2). In Figure 5, the authors look at the colocalisation of the two loci in Pol II 'factories' and conclude that there is colocalisation in absence of transcription and when transcription is blocked by alpha-amanatin. The authors should perform anti-Pol II ChIP experiments to determine whether there is paused Pol II at the Ifng and IfngR1 promoters under conditions where the genes are expressed and not expressed. qPCR with primers at the promoter and further in gene body would reveal whether

the two loci co-localise in 'factories' with Pol II because there is paused Pol II at the promoter even in the absence of their expression, and whether the Pol II is released into elongation. This can be performed also with antibodies specfic for the different phosphorylated Pol II forms.
3). In Figure 6 concerning CTCF, additional control ChIP experiments should be peformed to demonstrate that there is really an enrichement of CTCF at the promoters. Anti-CTCF ChIP should be performed and non-bound regions should be included as negative controls and control ChIP with non-specific antibodies should be included. Further controls of this type are also required for the ChIP loop experiments.
4). The authors postulate that CTCF plays a critical role in the long range interactions between the two loci. To demonstrate that this is the case, the authors should use si/shRNA to silence CTCF and show that the long range association is lost and the transcription of the two genes is down-regulated.
5). The authors mention on several occasions that the Ifngr1 promoter lacks a consensus TATA-box, and that this is somehow related to the fact that there needs to be communication with the Ifng locus. It is not clear to this referee why it is relevant that the Ifngr1 promoter is TATA-less. It is well established that TATA-less promoters are occupied by TBP/TFIID and all the basal machinery. Could the authors be more specific as to what they mean for example on page 16 in the discussion ' could serve as a mechanism for loading or recruiting specific factors.....' What are the specific factors that the authors refer to ?

Referee #3:

This is a very interesting paper that opens a new window on chromatin conformation and regulation of gene expression. The authors provide data that chromatin confirmation brings together the IFN-gamma gene and one of its receptor chain (IFNGR1), thus promoting the monoallelic expression of that receptor gene. The data is convincing and I have only a few comments.

1. There is a general sense (although limited data) that the IFN-gamma gene is transcribed from both alleles. Thus it was surprising that the authors did not look at IFN-gamma RNA-FISH in this study. It would strengthen the paper if the IFN-gamma transcript data was included but such data might raise an interesting conceptual dilemma that the authors should try to address. If IFN-gamma transcripts are indeed biallelic, how do the authors reconcile this with the monoallelic transcription of the receptor chain? It is appreciated that the authors provide data that differential methylation of the genes allow for differential CTCF recruitment and thus monoallelic IFNGR1 expression. Methylation is known to control IFN-gamma gene expression so RNA-FISH data on IFN-gamma would be most interesting.
2. Given the methylation data and the old data that demethylating agents can activate IFN-gamma, have the authors tried treating TH2 cells with demethylating agents to determine if that results in activation of IFNGR1 and co-localization of the chromatin. It is appreciated that this experiment would support the model but is not critical for substantiation of the model.

1st Revision - authors' response 19 June 2012

Response to the referees

First of all we would like to thank the referees for their time in reviewing our manuscript but most importantly for their constructive comments that helped us improve our work. We hope that our responses based on the experiments we have now completed will adequately address their concerns. Below please find a point by point response to the referee's points. (Original comments are in bold).

Referee #1:

This manuscript by Deligianni and Spilianakis investigates the transcriptional regulation of the interferon g receptor 1 (IfngR1) gene during TH1 cell differentiation. The authors report the intriguing observation that IfngR1 colocalizes mostly mono-allelically with the gene coding for its ligand (Ifng) located 1 Megabase away on the same chromosome. It is further shown

that mono-allelic co-localization strongly correlates with IfngR1 mono-allelic expression, co-localization also with the RNA polymease II (PolII) but independently of ongoing transcription. Finally, evidence is provided that the long-range interaction process may involve the chromatin organizer protein CTCF.
This is a very convincing study and well-written manuscript, which uses up-to date and diverse relevant techniques (DNA/RNA FISH, 3C, Chromatin immuno-precipitation, ChIP-loop assays, etc..), with careful controls performed for all the experiments. The results are original and provide significant insight into molecular mechanisms likely to sustain expression of both a cytokine gene locus and its corresponding receptor during T lymphocyte polarization, and of potential interest to the readership of EMBO reports.

We would like to thank the referee for the positive comments. We hope that our revised manuscript including additional experimental controls and data will still convince her/him for the potential interest of our study to the broad readership.

Referee #2:
This paper describes in long range interactions between the Ifng and Ifngr1 loci that may be involved in mediating their co-regulation during T-cell specification. The data are clearly presented and of general interest. Several points in this manuscript require further attention.

1). There are several series of experiments involving DNA and RNA-FISH that have been used to detect the colocalisation between the Ifng and Ifngr1 loci. While several control experiments are shown in TH2 cells compared to TH1 cells, it would be appropriate to perform additional control experiments in non-immune cells such as fibroblasts, where these genes are not expressed. One would expect to see no significant colocalisation under these conditions. These same controls in fibroblasts should be performed for the 3C experiments.

We agree with the reviewer that this is a valid point. As the reviewer pointed out, initially we thought that cells of the TH2 cell lineage would serve as a more conservative control for the analysis performed in T cells. As the reviewer suggested, initially we used mouse NIH 3T3 fibroblasts but by performing DNA-FISH experiments we found that these cells were tetraploid for the loci of interest so we prepared primary mouse embryonic fibroblasts (MEFs) to also conform to the rest of our analysis which was done in mouse primary cells. We used MEFs in the following experiments:

1. DNA-FISH for the *Ifnγ-IfnγR1* loci (Figure 1C,D).
   8% of the cells showed a monoallelic and no biallelic colocalization of the *Ifnγ-IfnγR1* gene loci.

2. 3C for the *Ifnγ-IfnγR1* and *Gapdh* loci (Figure 2B)
   We did perform additional controls for the 3C experiments using MEFs as a template and we did not find any interaction between the *Ifnγ-IfnγR1* gene loci. We also used as additional controls genomic DNA and naked genomic DNA digested with the same restriction enzyme and ligated.

3. RNA-DNA FISH for the *IfnγR1* gene (Figure 3A,D)
   The *IfnγR1* gene was expressed in 9.5% of the cells.

4. RNA-DNA FISH for the *Ifnγ* gene (Figure 3C,D)
   The *Ifnγ* gene was expressed in 6% of the cells.

2). In Figure 5, the authors look at the colocalisation of the two loci in Pol II 'factories' and conclude that there is colocalisation in absence of transcription and when transcription is blocked by alpha-Amanitin. The authors should perform anti-Pol II ChIP experiments to determine whether there is paused Pol II at the Ifng and IfngR1 promoters under conditions where the genes are expressed and not expressed. qPCR with primers at the promoter and further in gene body would reveal whether the two loci co-localise in 'factories' with Pol II because there is paused Pol II at the promoter even in the absence of their expression, and

whether the Pol II is released into elongation. This can be performed also with antibodies specific for the different phosphorylated Pol II forms.

We would like to mention that the experiment regarding the colocalization of the two loci in RNA PolII factories in conjunction with the a-Amanitin treatment was performed in order to try to answer a long standing question in the field as to whether such long range interactions are the cause or the effect of transcription. Specifically, we wanted to address if those interactions involve an active relocalization mechanism prior to transcription in order to facilitate transcriptional gene regulation or if they constitute an epiphenomenon, denoting the colocalization of transcriptionally active genes in the same RNA PolII factory.

Therefore, for the suggested experiments we would like to mention that in the non-differentiated $CD4^+$ cells the *Ifnγ R1* gene is expressed monoallelically so there is an active elongating process, in contrast to the *Ifnγ* gene which is not expressed. TH1 cells express both *Ifnγ* and *Ifnγ R1* genes so again we have an active elongating process for both genes. The question would be if for example TH2 cells and MEFs which do not express the two genes in a high percentage of cells have stalled RNA PolII in their promoters but also these cell types do not show any significant colocalization of the two loci compared to TH1 cells. The aim of our study was the transcriptional regulation of the *Ifnγ R1* gene and we suggest that it could be regulated by the same regulatory elements utilized by the *Ifnγ* gene. Moreover, we believe that RNA or RNA-DNA FISH experiments are informative enough regarding the RNA PolII occupancy since we detect nascent mRNAs and we also get a sense of allelic occupancy.

To summarize, the inhibition of the elongating RNA PolII complexes with the use of a-Amanitin showed that that the long range monoallelic interactions we describe were resistant to transcription blockade. A possible explanation for this would be that once the long range interactions are formed they are stable and other protein factors may exist, which are responsible for the maintenance of the already formed interactions.

3). In Figure 6 concerning CTCF, additional control ChIP experiments should be performed to demonstrate that there is really an enrichment of CTCF at the promoters. Anti-CTCF ChIP should be performed and non-bound regions should be included as negative controls and control ChIP with non-specific antibodies should be included. Further controls of this type are also required for the ChIP loop experiments.

The reviewer is right about this point although we should mention that the data presented for the CTCF chip in the initial submission had been corrected for the rabbit IgG controls we had performed as was indicated in the Methods section. We have now included a control rabbit polyclonal Actin antibody in addition to non-immune rabbit IgG and have analyzed an *Ifnγ R1* promoter region, *Ifnγ R1* intron 3, *Ifnγ R1* intron 6 and a region 20kb downstream the *Ifnγ R1* gene (Revised Figure 4F).

We have now included a rabbit polyclonal Actin antibody in addition to non-immune rabbit IgG in the chip-loop analysis (revised Figure 5D).

4). The authors postulate that CTCF plays a critical role in the long range interactions between the two loci. To demonstrate that this is the case, the authors should use si/shRNA to silence CTCF and show that the long range association is lost and the transcription of the two genes is down-regulated.

We thank the referee for this critical suggestion. Indeed we have been working towards this direction and we were able to complete those experiments. We have constructed 5 retroviral constructs expressing different shRNAs for targeting CTCF. Upon initial experiments (Supplementary Figure 4) we have chosen two of them, produced retroviruses and infected

proliferating TH1 cells. The experiments we performed in the CTCF-knock-down TH1 cells are the following:

1. RT-PCR in TH1 cells for the *Ifnγ R1* gene (Figure 5A).
   We found that *Ifnγ R1* mRNA levels were reduced by 60% by sh1-CTCF and by 46% by sh3-CTCF.

2. DNA-FISH for the *Ifnγ-Ifnγ R1* loci in TH1 cells (Figure 5B).
   - TH1 cells: 50±5.5% monoallelic colocalization, 6±4% biallelic colocalization.
   - TH1/LMP (empty vector control): 49.5±2.12% monoallelic colocalization, 2%±2.8 biallelic colocalization.
   - TH1/sh1-CTCF: 23±2.8% monoallelic colocalization, 2±1.4% biallelic colocalization.
   - TH1/sh3-CTCF: 26±4.5% monoallelic colocalization, 1% biallelic colocalization.

3. RNA-DNA FISH for the *Ifnγ R1* gene in TH1 cells (Figure 5C).
   - TH1 cells: 46.5±6.2% monoallelic expression, 8.5±2.6% biallelic expression.
   - TH1/LMP (empty vector control): 49.5±2.12% monoallelic expression, 4% biallelic expression.
   - TH1/sh1-CTCF: 26±2.82% monoallelic expression, 2±3% biallelic expression.
   - TH1/sh3-CTCF: 33±4.5% monoallelic expression, 2% biallelic expression.

5). The authors mention on several occasions that the Ifngr1 promoter lacks a consensus TATA-box, and that this is somehow related to the fact that there needs to be communication with the Ifng locus. It is not clear to this referee why it is relevant that the Ifngr1 promoter is TATA-less. It is well established that TATA-less promoters are occupied by TBP/TFIID and all the basal machinery. Could the authors be more specific as to what they mean for example on page 16 in the discussion ' could serve as a mechanism for loading or recruiting specific factors.....' What are the specific factors that the authors refer to ?

We agree with the referee on this point and so we have removed from the manuscript such claims. We are sorry that we were not able to deliver a clear message in the initial report. What we meant was that even if we find binding of several factors on a gene, even the basal transcription machinery, it could indicate either direct binding or on the other hand binding or loading of such factors facilitated by another protein which brings together two loci via long range interactions. Since we do not have any data to support such a model we totally agree with the referee and consequently have revised the text.

Referee #3:

This is a very interesting paper that opens a new window on chromatin conformation and regulation of gene expression. The authors provide data that chromatin confirmation brings together the IFN-gamma gene and one of its receptor chain (IFNGR1), thus promoting the monoallelic expression of that receptor gene. The data is convincing and I have only a few comments.

1. There is a general sense (although limited data) that the IFN-gamma gene is transcribed from both alleles. Thus it was surprising that the authors did not look at IFN-gamma RNA-FISH in this study. It would strengthen the paper if the IFN-gamma transcript data was included but such data might raise an interesting conceptual dilemma that the authors should try to address. If IFN-gamma transcripts are indeed biallelic, how do the authors reconcile this with the monoallelic transcription of the receptor chain? It is appreciated that the authors provide data that differential methylation of the genes allow for differential CTCF recruitment and thus monoallelic IFNGR1 expression. Methylation is known to control IFN-gamma gene expression so RNA-FISH data on IFN-gamma would be most interesting.

We thank the referee for the suggestion; it is actually a valid point although not contradicting to our findings. We did perform RNA-DNA FISH experiments to assess the allelic expression profile of the *Ifnγ* gene. *Ifnγ* was indeed specifically expressed in TH1 cells in a biallelic manner (biallelic expression: 72.5±3.53%, monoallelic expression: 18±9.89), compared to non-differentiated $CD4^+$, TH2 cells and MEFs. The data are presented in the revised Figure 3C,D.

Although the *Ifnγ* gene is biallelically expressed and the *IfnγR1* gene is monoallelically expressed our suggestion based on our experiments is that the latter utilizes elements of the *Ifnγ* locus for its transcriptional regulation. The dilemma would be if *Ifnγ* was monoallelically expressed while *IfnγR1* was biallelically expressed. We suggest that there is a monoallelic DNA interaction between the *Ifnγ* and *IfnγR1* gene loci which supports the monoallelic expression of the *IfnγR1* gene. We believe that the differential methylation of the two *IfnγR1* alleles supports the selective recruitment of the CTCF protein in the non-methylated allele which is the one coming in close proximity to the *Ifnγ* regulatory elements.

2. Given the methylation data and the old data that demethylating agents can activate IFN-gamma, have the authors tried treating TH2 cells with demethylating agents to determine if that results in activation of IFNGR1 and co-localization of the chromatin. It is appreciated that this experiment would support the model but is not critical for substantiation of the model.

We have used 5-Aza-2'-deoxycytidine which is an agent causing DNA demethylation or hemi-demethylation and treated differentiating TH2 cells as the referee suggested. The experiments we have performed are the following:

1. RT-PCR for the expression mRNA levels of *IfnγR1* in differentiating TH2 cells treated with increased concentrations of the drug (Figure 5F). Indeed treatment of TH2 cells leads to increased *IfnγR1* mRNA levels.

2. DNA-FISH for the *Ifnγ-IfnγR1* loci in TH2 cells treated with the drug (Figure 5G).
   We observed a two fold increase in the percentage of cells that showed monoallelic *Ifnγ-IfnγR1* colocalization while the percentage of cells that showed biallelic colocalization of the two loci was increased from 1% to 7%.

3. RNA-DNA FISH for the *IfnγR1* gene in TH2 cells treated with the drug (Figure 5H).
   - Monoallelic *IfnγR1* expression: increased from 13.4% to 43% upon treatment with 5-Aza.
   - Biallelic *IfnγR1* expression: increase from 1.5% to 8%.

In conclusion, we found increased colocalization between the *Ifnγ-IfnγR1* loci and increased expression of the *IfnγR1* gene in the 5-Aza treated compared to non-treated TH2 cells, although the ratio of monoallelically interacting loci versus the biallelically interacting loci remained high. One might expect that in the absence of DNA methylation *IfnγR1* alleles would interact with *Ifnγ* loci in a biallelic manner. Our data show that this is not the case for TH2 cells and this could be explained by the presence of other epigenetic marks, apart from DNA methylation, already established in naive $CD4^+$ cells prior to their differentiation into TH2 cells that differentially mark one of the two *IfnγR1* alleles for expression.

2nd Editorial Decision 29 June 2012

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the report below, the referee who was asked to assess the revised version is now positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

1. Please clarify the issue with the p-value of 0.00 in supplementary figure 2 that the referee points out.

2. I appreciate the detailed information on the statistical analysis you provide in the point-by-point response. However, there are some issues that would need to be addressed prior to publication:
(a) it looks as if in some instances, you have calculated mean and standard deviations from triplicates of one experiments (for example in Fig 1B, 5A, 5F). Mean and standard deviations should not be calculated from triplicates of only one experiments, but should be calculated from independent experiments (at least three). Therefore, please redo these calculations where necessary.
(b) in some instances, in which individual cells have been counted and used to calculate the standard deviation (eg Fig 5B, C, and G) I assume that you have calculated the error bars from the individual cells (ie 774 cells in Fig 5B) and not from the two independent experiments. Is this correct? If this is the case, please clearly indicate this in the figure legend, as right now it looks as if you calculated error bars from only two experiments.

If all remaining corrections have been attended to, you will then receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

Yours sincerely

Editor
EMBO Reports

REFEREE REPORTS:

Referee #3:

The authors have done a thorough job addressing my questions and the paper now deserves publication. I have only one minor comment that I add only for clarification. In Supplemental Figure 2, do the authors really mean a p value of 0.00 on some of the panels?

2nd Revision - authors' response 02 July 2012

We have now completed the revision process of our manuscript (EMBOR-2012-35851V2) and we submit the revised version for your consideration. Please find below a detailed response to your comments (in bold).

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the report below, the referee who was asked to assess the revised version is now positive about its publication in EMBO reports. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

1. Please clarify the issue with the p-value of 0.00 in supplementary figure 2 that the referee points out.

We have repeated the calculations for the Supplementary Figure 2 using the “IBM SPSS statistics 19” software (same as for the original submission) and again we do find a *P* value of 0.00 for the Kolmogorov-Smirnov test run for the indicated categories. According to the software guidelines a P value of 0.00 denotes a value of probability for rejecting the *null* hypothesis close to 0.00 with statistical significance $p<0.001$.

2. I appreciate the detailed information on the statistical analysis you provide in the point-bypoint response. However, there are some issues that would need to be addressed prior to

publication:

(a) it looks as if in some instances, you have calculated mean and standard deviations from triplicates of one experiments (for example in Fig 1B, 5A, 5F). Mean and standard deviations should not be calculated from triplicates of only one experiments, but should be calculated from independent experiments (at least three). Therefore, please redo these calculations where necessary.

a) The data of the figures that you indicate are from quantitative RT-PCR results for the expression of a gene generated using a standard curve of samples with known concentrations. Since such calculations for the pg of RNA present in a sample are solely based on the calculations made using the standard curve it is practically impossible to get the same (or even similar) quantity of RNA of a gene in a sample. Therefore taken together the data for the expression of a gene in four different biological replicates would give huge differences (represented in the standard deviation presented) in the quantity of the RNA of a gene. Since the relative fold difference between the different cell types is similar we have chosen to present the data of one representative experiment not giving the standard deviation between the different experiments but the standard error of the mean between triplicates. The figure legends have been corrected as follows:

Figure 1
B. RT-PCR: Results are the mean ±s.e.m from triplicate samples of four independent experiments.
Figure 5
A. RT-PCR for the *IfngR1* gene in TH1 cells infected with 2 different retrovirally transduced shRNAs targeting CTCF. Results are the mean ±s.e.m from triplicate samples from one representative of three independent experiments.

F. RT-PCR for the *IfngR1* mRNA levels in TH2 cells treated with 5-Aza. Results are the mean ±s.e.m from triplicate samples from one representative of three independent experiments.

(b) in some instances, in which individual cells have been counted and used to calculate the standard deviation (eg Fig 5B, C, and G) I assume that you have calculated the error bars from the individual cells (ie 774 cells in Fig 5B) and not from the two independent experiments. Is this correct? If this is the case, please clearly indicate this in the figure legend, as right now it looks as if you calculated error bars from only two experiments.

b) We have calculated the standard errors not measuring individual cells but the percentage of cells expressing a gene for each independent experiment. We have corrected the figure legends as follows:

Figure 5
B. DNA-FISH for the *Ifng-IfngR1* loci in TH1 cells infected with 2 different retroviruses, each of them expressing one distinct shRNA targeting CTCF. Bars are mean values for the percentage of cells ±s.e.m from 2 independent experiments for each treatment. 774 cells have been scored in total.

C. RNA-DNA-FISH for the *IfngR1* locus in TH1 cells infected with 2 different retroviruses, each of them expressing one distinct shRNA targeting CTCF. Bars are mean values for the percentage of cells ±s.e.m from 2 independent experiments for each treatment. 568 cells have been scored in total.

G. DNA-FISH for the *Ifng-IfngR1* loci in TH2 cells treated with 5-Aza. Bars are mean values for the percentage of cells ±s.e.m from 2 independent experiments for each treatment. 1248 cells have been scored in total.

Once more we would like to thank you for your helpful suggestions and hope that you will find our responses satisfactory and accept our manuscript for publication.

3rd Editorial Decision 05 July 2012

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.

Yours sincerely,
Editorial Assistant
EMBO Reports

