THE EMBO JOURNAL

Manuscript EMBO-2012-81857

TNFα signals through specialized factories where responsive coding and miRNA genes are transcribed

Argyris Papantonis, Takahide Kohro, Sabyasachi Baboo, Joshua D. Larkin, Binwei Deng, Patrick Short, Shuichi Tsutsumi, Stephen Taylor, Mika Kobayashi, Guoliang Li, Huay-Mei Poh, Xiaoan Ruan, Hiroyuki Aburatani, Yijun Ruan, Tatsuhiko Kodama, Youichiro Wada and Peter Cook

Corresponding author: Peter Cook, University of Oxford

Review timeline:

Submission date:	26 April 2012
Editorial Decision:	20 June 2012
Appeal:	22 June 2012
Editorial Correspondence:	28 June 2012
Editorial Correspondence:	09 July 2012
Author Correspondence:	10 July 2012
Revision received:	11 August 2012
Editorial Correspondence:	13 August 2012
Editorial Decision:	18 September 2012
Revision received:	22 September 2012
Accepted:	24 September 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 20 June 2012

Thank you for submitting your study on TNFalpha-induced transcription factories for consideration by The EMBO Journal. The manuscript has now been seen by three referees, whose comments are shown below.

You will easily recognize that all referees acknowledge the importance of your high-throughput approach to demonstrate association of TNFalpha-responsive genes in discrete transcription factories. However, I had to notice that two of the referees remained rather skeptical about the exact nature and specificity of the transcription factories as visualized by RNA-FISH, and that they are therefore not willing to support publication in The EMBO Journal based on the current dataset. In order to consider your manuscript for publication we would require a substantial characterization of the observed nuclear bodies and evidence for a direct role in transcription of signaling responsive genes.

Given the amount and uncertain outcome of experimentation required to clarify these points, we are currently unable to invite to a single round of timely rather limited revisions, since this would mean to essentially commit to your manuscript. I am therefore sorry to have to return the manuscript to you with the message that I am unable to offer further consideration at this stage, a decision that might also enable you to potentially seek rapid publication in a less demanding venue.

We do however recognize the potential significance of your findings, and in case you were willing and able to expense the necessary experimental efforts to address these critical concerns, I would be happy to re-assess an amended version at a later stage and as new submission to our title.

I am really sorry that I cannot be more encouraging at this stage, but I hope that clear and rapid communication of essential demands by The EMBO Journal may help facilitate your decision of how to proceed with this project. Please do not hesitate to contact me directly in case of necessary further clarifications (preferably via E-mail).

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

In this manuscript Papantonis et al. examined regulation of transcription by TNFa in the context of transcription factories. By using 4C, CHIA-PET and RNA FISH they provided evidence that TNFa induces some colocalization of TNFa-induced transcripts, a result indicative of coexpression in specific regions (transcription factories). The authors also claim that nascent transcripts colocalize with activate nuclear p65 (p65p.
Overall the manuscript address an important issue, that is, whether co-regulated genes are coexpressed in defined nuclear regions. The authors call these regions NF-kB factories and present evidence that genes regulated by intersecting pathways (like TGFb) may share the same factory.
I find these ideas interesting, but the data supporting them, not yet convincing.

1)The authors should also perform DNA fish to check the colocalization of the genes under examination. Otherwise, the nascent RNA colocalization could imply splicing factories and not transcription factories, as they propose.
2)The resolution of the FISH experiments is low and the individual hybridization spots are nearly invisible.
3) How can someone propose the existence of specialized factories when the percentage of shared genes between the SAMD4 and EXT1 is so small? It can't be that there is one factory per couple of genes, because the idea of the factories (coordination in regulation) goes away.
4)The authors state in p11 (around the middle of the page) that the number of contacts made between SAMD4A, EXT1 and miRNAs is proportional to the transcriptional rate. If this is the case then how can someone exclude that the contacts are the mere result of transcription and do not play any role in coordinated regulation?
5) I think that all sections related top miRNAs have nothing to do with this manuscript and should be removed. Also SF3. DF4 and SF5 should be part of the main text.

Referee #2:

This is a very interesting story aimed at extending ideas related to clustering of developmentally regulated genes at specialized "tx factories" to the idea of similar clustering of genes connected by specific signaling pathways.

The Cook laboratory is known for its long term interest and work in the idea of clustering of tx sites and polymerase at "tx factories". The Fraser laboratory over the last 10 years or so, starting from the observation of spatial co-localization of developmentally co-regulated genes, has also pioneered the concept of clustering of active genes at tx factories. Combining cytological and 3C methods, the

Fraser lab has also suggested the idea of clustering of genes regulated by the same tx factor at specialized tx factories enriched in this tx factor.

The functional significance of this clustering of similarly regulated genes has been questioned, with some genes expressed and clustering during erythroblast differentiation in mouse unclustered in human erythrocyte differentiation. The competing concept is that the clustering involves co-localization of active genes at tx factories or nuclear speckles with the degree of clustering dependent on higher order chromatin organization as well as actual level of tx activation, with the most active genes more likely to colocalize.

A second, unsettled issue of interest to the field related to this topic of tx factories is that there seems to be a significant difference in the nature of the "tx factories" identified in the pioneering work of the Fraser and Cook laboratories. Whereas in most fibroblast and epithelial cells studied in tissue culture, thousands of ~90 nm "tx factories" are visualized per nucleus, in the hematopoietic cells taken from mice studied primarily by the Fraser labs with somewhat different IF methods there are just 100-300 of several hundred nm to one micron diameter "tx factories". Therefore there is still uncertainty whether this is simply a cell type difference or whether we are talking about apples and oranges- ie different nuclear bodies.

It is in this context that the current manuscript dives into this very timely research question. Specifically they present data leading them to conclude the clustering of NF-KB responsive genes into specialized tx factories which are enriched in the active p65 tx factor. The manuscript contains compelling data supporting this conclusion. However, there are a number of loose ends and questions that are still left poorly addressed or unresolved in the current manuscript that should be addressed.

A major question is whether the observed associations relate to clustering of highly active genes per se rather than genes which are coregulated by p65. In fact the discussion seems to suggest a model in which at early time points, at least, association begins at generic tx factories which then become specialized NF-KB factories. A second question revolves around the number of clusters and whether these represent the Cook or Fraser type of "tx factory" or something else. Whereas the authors show colocalization of their target genes with p65 IF stained foci, they do not directly address whether these gene associated p65 foci are in fact RNA pol 2 containing "tx factories". For that matter, they also don't formally confirm that the gene contacts inferred by 3C methods exist in situ as they are measuring co-association of intron probes rather than DNA FISH signals. While unlikely there is formally the possibility that the accumulation of intron RNA is displaced from the actual site of gene transcription- this particularly effects the measurements of distances between the two RNA FISH signals and the argument that this due to colocalization to tx factories. A third related question has to do with the actual number of these NF-KB "factories" and how this matches up with the very high ~60% colocalization of their target SAMD4A target gene with just 7 "contact genes". This percentage doesn't change between 30 and 60 mins post-stimulation which seems to argue against the idea of specialization evolving over time. Moreover, it seems hard to reconcile the ~60 colocalization rate observed by RNA FISH with the expected number of tx factories (thousands) or the large number of p65 foci shown in the supplemental figure. Exactly where do these genes colocalize? Are these colocalization sites actual tx factories, are they a subset of p65 foci which also colocalize with tx factories, are they in fact at other types of well defined nuclear bodies such as speckles, PML, or Cajal bodies that would be much smaller in number?

More specifically, I had the following particular questions / issues:

1. Does the "intragenic" contact category also include nearby cis interactions immediately upstream of the gene? So if a contact several kb downstream of the TSS is considered intragenic, what about a contact several kb 5' to the TSS? As it is written now, the intragenic category does NOT include the 5' contacts, yet Fig. 2 A shows frequencies of intragenic contacts much higher than 50% at zero time. I would have suspected a rough symmetry for nearby upstream versus downstream contacts, which would be impossible with the definition of intragenic only referring to downstream contacts.

On a related note, the decrease in intragenic contacts could have multiple explanations besides the gene localizing to a nuclear body- a change in compaction might do the same.

2. In Fig. 2A again, why should the SMAD4A-seq data look so different from the library data? Specifically, the intra-SMAD4A fraction is just a few percent versus close to 40% in the library values.

3. I am confused about the numbers shown for contacts in Fig. 2B. The library data, if I understand the approach correctly, is based on sequencing just ~80 clones and produces something like 30 "contacts". The high throughput sequencing approach in contrast has millions of reads, yet produces only a few fold more "contacts". Why? Does this mean that the number of true contacts is relatively small? Does it mean that the vast majority of reads in the 4C -Seq readout are just hits on the same small number of contacts? Does it mean that the library contacts are primarily the high frequency contacts while the additional contacts revealed by the Seq approach are primarily much lower probability events? What is the expected fraction of contacts that can be expected to be detected by the Seq approach at the given read rate- ie what is the estimated coverage of these contacts or how many are found in two different Seq runs?

4. On a related note, exactly how many TNF- p65 regulated genes are there that show significant upregulation? How do these numbers compare to the number of contacts? How do they compare to the number of phospho-p65 foci?

5. It would be nice to separate the idea of contacts because of co-regulation by the same tx factor versus contacts by chance dictated primarily by the level of tx activity. The current scheme of looking at either TNF responsive OR p65 binding OR pol 2 binding doesn't do that.

For that matter, I am very curious about the significant difference in fraction of non-responsive contacts in the library versus Seq approaches. There is a big jump in non-responsive genes in the Seq approach which makes me think there is a big selection in the library approach that may represent it's contacts corresponding to the highest probability contact events, which may then also represent contacts with high tx activity.

6. Can the authors come up with some scheme that compares level of tx activity with probability of contact? I assume that TNF induction leads to high tx activity of target genes after induction. How do their activities (not sure how you measure this- ideally by run on tx or RNA FISH) compare to typical house-keeping genes? What about a better control list of the most active house-keeping genes in this cell type? How would the top 100 most highly expressed house-keeping genes compare in terms of representation in the contact list versus those with lower activities?

7. Similarly, what about a control for Fig. 4 that involves one of the most highly active but non-responsive housekeeping genes?

8. What fraction of phospho-p65 foci overlap with RNA pol 2 foci (ie tx factories) or Br-UTP foci? How many p65 binding sites are there in the Chip-Seq data? Again how many significantly (by some fold threshold) upregulated genes are there after TNF stimulation?

9. What fraction of RNA FISH signals to intron probes actually colocalize with tx factories? What about RNA-DNA FISH to examine the distance of these intron signals to the genes? Short of that what about two color RNA FISH where they also use exon-intron junction probes or exon probes.

10. Is there strong selection for the most highly expressed genes by the ChIA-PET approach? Numbers of contacts seem to scale disproportionally to levels of tx measured by FISH for the micro-RNAs.

11. My major cognitive dissonance in reading this paper involves the 60% colocalization frequency of the SAMD4A gene with the 7 multiplex contact list. This is really high and seems to point to a relatively small number of foci. If each contact has equal probability, this works out to about 10% rate per contact. Assuming that these contacts are random with regard to the number of NF-KB "factories" doesn't this imply a low number then of about 10 factories per nucleus?

What am I missing? Is it possible that this low number of estimated factories suggests that the colocalization occurs at a nuclear body of lower frequency than tx factories or p65 foci?

I would really like to see costaining of the RNA FISH data with nuclear bodies such as speckles, Cajal bodies, PML bodies of such low frequency.

Alternatively, the choice of this 7 multiplex mixture might be yielding unusually high fractions, and biased by one or two of these contacts having an exceptionally large colocalization frequency. So on that note, where are these 7 contacts relative to the chromosome location of the target SAM4DA start site? Are any in cis and close by, resulting in a very high fraction of colocalization? For instance a contact within 10s of Mbp of the SAM4DA site might show a very high percentage of colocalization because the probability of colocalizing to the same nuclear body is much higher than a contact on a different chromosome. Very crudely, if you divide the genome size by the number of NF-KB factories, then this would give the genomic distance expected for colocalization to the same factory.

Referee #3:

The manuscript by Papantonis et al addresses a very interesting topic: Do transcriptional units responding to inducive cellular microenvironments gather in specialized transcriptional units? This idea has earlier been championed by the senior author, Dr Peter Cook although hard evidence in favour of it was largely lacking. In the current manuscript, however, Dr Cook and colleagues describe results that provide considerable support for this notion. Using state of the art techniques, such as 4C, ChIA-PET and RNA FISH, the authors do a very good job showing that TNFalpha-inducible genes gather in specialized transcriptional units. In an additional novel twist, the authors show that microRNA genes targeting genes being suppressed by TNFalpha likewise are being found clustered together, even from different chromosomes. Although the underlying mechanisms are poorly understood, the current work will be of considerable interest from not only scientists interested in transcriptional regulation, but also from scientist trying to comprehend the inflammatory response.

Appeal 22 June 2012

Thank you for sending our paper out for review. Even though it is not my custom to appeal, I feel I must do so on this occasion.

I respond to the each of the points raised by the referees in the attached (I cannot see a substantive objection raised by any referee that requires extra experimental work or that has not been answered). Finally, your major concern seemed be about the amount of extra experiments required - I hope we have alleviated this concern and that you will reconsider your decision.

With apologies for the bother that I know this request will inevitably cause.

General points made by referees:

The referees found the manuscript '*interesting*','*timely*', and '*addressing an important issue*'. Referee 2 said '*The manuscript contains compelling data supporting this conclusion'*, and referee 3 the '*authors do a very good job showing that TNFalpha-inducible genes gather in specialized transcriptional units'*. There were also a number of misunderstandings, and when additional experiments were suggested, they go well beyond the scope of this manuscript. We can respond to the major points of the referees as follows:

'Referee #1:'

'*1)The authors should also perform DNA fish to check the colocalization of the genes under examination. Otherwise, the nascent RNA colocalization could imply splicing factories and not transcription factories, as they propose.*' FISH experiments have revealed that the contacts detected by 3C are rare events in a cell population; moreover, not all alleles of even highly-active genes are being transcribed at any moment. As DNA FISH detects both active and inactive alleles, RNA FISH applied with intronic probes to well-characterized transcripts is the method of choice because it detects transcripts at just the active alleles – the ones of interest here. [Note that the nascent transcripts we study are well characterized (Wada *et al*., 2009; Larkin *et al*., 2012). For example, we know exactly when a nascent transcript will begin to be produced after stimulation, its half-life (typically ~5 min), and how many alleles are active in the diploid G0 cells that we use (assessed by RNA FISH). We also know that that all splicing occurs co-transcriptionally; for example, we only see an RNA FISH spot if an RNA polymerase is transcribing that region (assessed using ChIP, ChIP-seq, microarrays) and we never see more than two RNA FISH spots per nucleus (seeing a third might hint at residence at a hypothetical 'splicing factory').] I would also add that the RNA FISH experiment is only a supporting experiment (supporting 3 different 3C approaches), and why would we bother to perform the more difficult RNA FISH (compared to DNA FISH) unless we had good reason! Nonetheless, DNA FISH was performed. In the examples shown (Suppl. Fig. S5A) colocalization frequencies are comparable to those seen by RNA FISH.

'*2) The resolution of the FISH experiments is low and the individual hybridization spots are nearly invisible.*' A misreading. Unusually, we use super-resolution localization (i.e., with 22-nm precision – which is well below the diffraction limit, and so our resolution is hardly low!). We agree the spots are '*nearly invisible*'; this is as it should be – our probes are necessarily short, and so cannot carry the number of fluors that give the bright spots seen using conventional probes/microscopy.

'*3) How can someone propose the existence of specialized factories when the percentage of shared genes between the SAMD4 and EXT1 is so small? It can't be that there is one factory per couple of genes, because the idea of the factories (coordination in regulation) goes away.*' The answer is simple (see Discussion). If responsive genes are transcribed in one of many specialized factories, then only rarely will any two specified responsive genes be found together on one factory. We have now strengthened the sentence in the Results section. Note that we also deliberately chose to analyze two genes with distinct interactomes (as they are in different parts of the nucleus – see the legend to Suppl. Fig. S4), yet found that both nevertheless contact other responsive genes.

'*4) The authors state in p11 (around the middle of the page) that the number of contacts made between SAMD4A, EXT1 and miRNAs is proportional to the transcriptional rate. If this is the case then how can someone exclude that the contacts are the mere result of transcription and do not play any role in coordinated regulation?*' This is the case (p.11),

and Fig. S6C(iv) directly shows that the contacts do not result simply from the clustering of active-genes.

'*5) I think that all sections related top miRNAs have nothing to do with this manuscript and should be removed.*' I disagree – they allow us to confirm the principle of specialization using 3 more responsive genes, extend the analysis to down-regulated genes, and establish another principle that some of the factories transcribing responding genes can further specialize to transcribe genes encoding miRNAs/ncRNAs.

'*Also SF3. DF4 and SF5 should be part of the main text.*' We are happy to do this but we are already at your character limit.

'*Referee #2:*'

'*The functional significance of this clustering of similarly regulated genes has been questioned.. competing concept is that the clustering involves co-localization of active genes at tx factories or nuclear speckles with the degree of clustering dependent on higher order chromatin organization as well as actual level of tx activation, with the most active genes more likely to colocalize.*' Agreed. We wish only to demonstrate that responsive genes colocalize – without

resolving all these other issues!

'*A second, unsettled issue ... thousands of ~90 nm "tx factories" ... just 100-300 of several hundred nm to one micron diameter "tx factories".*' This is an important issue, but it is one that is not addressed here (although, in passing, the 'super-resolution' localization gives results consistent with factories in these cells having diameters of ~90 nm).

'*A major question is whether the observed associations relate to clustering of highly active genes per se rather than genes which are coregulated by p65. In fact the discussion seems to suggest a model in which at early time points, at least, association begins at generic tx factories which then become specialized NF-KB factories.*' This point is raised repeatedly by this referee. Fig. S6C (discussed at the bottom of p.11) puts this issue to rest: for example, comparison of panels (i) and (iv) shows that the clustering of responsive genes is not due simply to activity. [Fig. S3A provides additional confirmation.]

'*A second question revolves around the number of clusters and whether these represent the Cook or Fraser type of "tx factory" or something else. Whereas the authors show colocalization of their target genes with p65 IF stained foci, they do not directly address whether these gene associated p65 foci are in fact RNA pol 2 containing "tx factories".'* Agreed, but I think most people are happy using the nascent transcript copied from a protein-coding gene as a marker for an active RNA polymerase II! Nonetheless, we now include data (again using 'super-resolution' localization) showing that the p65 foci colocalize with sites rich in nascent BrRNA (which one could argue is currently the best marker for a transcription factory).

'For that matter, they also don't formally confirm that the gene contacts inferred by 3C methods exist in situ as they are measuring co-association of intron probes rather than DNA FISH signals. While unlikely there is formally the possibility that the accumulation of intron RNA is displaced from the actual site of gene transcription-this particularly effects the measurements of distances between the two RNA FISH signals and the argument that this due to colocalization to tx factories.' This is the issue of RNA v DNA FISH again. While I agree with the formal position as stated, I disagree (see above) that DNA FISH provides a better approach when targeting these particular genes than RNA FISH (as the latter allows us to concentrate on active alleles). Nonetheless, DNA FISH was performed (Suppl. Fig. S5A), and the colocalization frequencies are similar to those seen by RNA FISH.

'*A third related question has to do with the actual number of these NF-KB "factories" and how this matches up with the very high ~60% colocalization of their target SAMD4A target gene with just 7 "contact genes".*' It is difficult to know what a '*very high*' colocalization might be, and so how to respond to this. [Is the >50% statistical chance of two people with the same birthday being present in a room containing 23 people '*very high*'?] But I do agree that it would be nice to know the number of 'NFκB' factories, but – unfortunately – there are too many unknowns to obtain a secure number from existing data. Nonetheless, we now include in the 'Discussion' some estimates (our rationale is thoroughly explained in the Suppl. Methods in the section '*Estimating numbers of "NFκB" factories'*). [A responsive gene like *SAMD4A* can access ~8 such factories, and the upper limit for NFκB-factories in a HUVEC nucleus is ~250 (out of ~2200 for RNA polymerase II in total) – calculated using RNA FISH data, and verified by statistics on genome-wide ChIA-PET data (see Suppl. Fig. S6).] We do, however, stress that these are coarse estimates given the number of assumptions.

'*This percentage doesn't change between 30 and 60 mins post-stimulation which seems to argue against the idea of specialization evolving over time.*' A misunderstanding: the percentage could (and indeed does) remain constant as *different* genes associate with – and dissociate from – one factory.

'*Moreover, it seems hard to reconcile the ~60 colocalization rate observed by RNA FISH with the expected number of tx factories (thousands) or the large number of p65 foci shown in the supplemental figure.* This is the same point again. *Exactly where do these genes colocalize?*' *Are these colocalization sites actual tx factories, are they a subset of p65 foci which also colocalize with tx factories, are they in fact at other types of well defined nuclear bodies such as spec kles, PML, or Cajal bodies that would be much smaller in number?*' We provide Fig. S5 confirming the one (obvious) possibility, in which BrRNA data have now been added. [Note that speckles, PML bodies, and Cajal bodies are all transcriptionally inactive and so would be the negative controls in such a list!]

'*1. Does the "intragenic" contact category also include nearby cis interactions immediately upstream of the gene?'* No (as clearly stated). *So if a contact several kb downstream of the TSS is considered intragenic, what about a contact several kb 5' to the TSS? As it is written now, the intragenic category does NOT include the 5' contacts, yet Fig. 2 A shows frequencies of intragenic contacts much higher than 50% at zero time. I would have suspected a rough symmetry for nearby upstream versus downstream contacts, which would be impossible with the definition of intragenic only referring to downstream contacts.*' This is an interesting observation. We attribute this to the (low) transcriptional activity of *SAMD4A/EXT1* before induction, and the ChIA-PET data confirms the clear asymmetry. [Such asymmetry is seen in many other 3C experiments.]

'*On a related note, the decrease in intragenic contacts could have multiple explanations besides the gene localizing to a nuclear body-a change in compaction might do the same.*' It could, but we apply Occam's razor and choose the simplest interpretation.

'*2. In Fig. 2A again, why should the SMAD4A-seq data look so different from the library data? Specifically, the intra-SMAD4A fraction is just a few percent versus close to 40% in the library values.*' As we clearly state, each method has its own particular bias. For example, the 4C-seq data was produced by size-selecting a small window of ~300 bp from the same libraries used for 'old-fashioned' sequencing, so it is not surprising that most intra*SAMD4A* contacts are no longer seen (as the nearest contacts do not – by chance – yield PCR products in this size range).

'*3. I am confused about the numbers shown for contacts in Fig. 2B. The library data, if I understand the approach correctly, is based on sequencing just ~80 clones and produces something like 30 "contacts". The high throughput sequencing approach in contrast has millions of reads, yet produces only a few fold more "contacts".*' As stated, we do not analyze all contacts, but focus on a few that are seen most. '*Why?*' As stated, we wished to establish the principle using the contacts seen most frequently (and had no wish to perform a genome-wide analysis which would inevitably require us to analyze many other unrelated issues). '*Does this mean that the number of true contacts is relatively small?*' No. '*Does it mean that the vast majority of reads in the 4C-Seq readout are just hits on the same small number of contacts?*' There are many lower frequency hits that were not analyzed – for exactly the reason hinted here (that because they are of lower frequency, they might be considered as 'background' events!). '*Does it mean that the library contacts are primarily the high frequency contacts while the additional contacts revealed by the Seq approach are primarily much lower probability events?*' No, but the sensitivity of the 'seq approach' reveals a broader range of probabilities. '*What is the expected fraction of contacts that can be expected to be detected by the Seq approach at the given read rate-ie what is the estimated coverage of these contacts or how many are found in two different Seq runs?*' To repeat what is clearly stated: we use approaches with different biases, concentrate on contacts seen most frequently, want to reveal the key principles driving the formation of the interactome (without aiming to present a full interactome), and find that the different approaches nevertheless yield the same general results.

'*4. On a related note, exactly how many TNF-p65 regulated genes are there that show significant upregulation? How do these numbers compare to the number of contacts? How do they compare to the number of phospho-p65 foci?*' We have a huge microarray data-set (http://157.82.78.238/huvecdb/main_search.jsp), and – depending on the threshold used – can define 100-400 early-response genes (those becoming active within 1 h). As we look in detail at just two responsive genes by 4C, and at the top 69 by ChIA-PET, the number of contacts seen using both approaches appears reasonable. There are a comparable number (i.e., ~150) of bright p65^{P} foci in the nuclei shown in Suppl. Fig. S5.

'*5. It would be nice to separate the idea of contacts because of co-regulation by the same tx factor versus contacts by chance dictated primarily by the level of tx activity. The current scheme of looking at either TNF responsive OR p65 binding OR pol 2 binding doesn't do that.*' This was done – Fig. 5B and Suppl. Fig. S6 show that clustering does not correlate with high activity.

'*For that matter, I am very curious about the significant difference in fraction of non-responsive contacts in the library versus Seq approaches. There is a big jump in nonresponsive genes in the Seq approach which makes me think there is a big selection in the library approach that may represent it's contacts corresponding to the highest probability contact events, which may then also represent contacts with high tx activity.*' As discussed above, we clearly state that the two approaches have different biases, and so it is not surprising that they give different results; however,

both uncover the same trends. Again the issue of '*tx activity*' is raised, and this time we offer yet another control: Fig. S3A clearly shows that our contacts are not biased towards highly-active genes (compared to a random set, and *GAPDH*).

'*6. Can the authors come up with some scheme that compares level of tx activity with probability of contact? I assume that TNF induction leads to high tx activity of target genes after induction. How do their activities (not sure how you measure this-ideally by run on tx or RNA FISH) compare to typical house-keeping genes? What about a better control list of the most active house-keeping genes in this cell type?*' Again '*tx activity*': see Fig. S6C(iv) or Fig. S3A. '*How would the top 100 most highly expressed house-keeping genes compare in terms of representation in the contact list versus those with lower activities?*' This is a variation on '*tx activity*', and I think any sensible reader would accept Fig. S6C(iv) as a relevant alternative (also, the random set in Fig. 2 and Table S1 contains a number of 'low activity' genes, which have been used in such comparisons).

'*7. Similarly, what about a control for Fig. 4 that involves one of the most highly active but non-responsive housekeeping genes?*' Both control genes used in Fig. 4 are highly active (*EDN1* is as active as *SAMD4A*, while *RCOR1* has an activity comparable to *GAPDH*; Wada *et al*., 2009; Papantonis *et al*., 2010). We now state this in the manuscript.

'*8. What fraction of phospho-p65 foci overlap with RNA pol 2 foci (ie tx factories) or Br-UTP foci?* As stated above, we have shown that the p65 foci colocalize with sites rich in nascent Br-RNA (i.e., transcription factories) – we have now included these results, where phospho-p65 is non-randomly associated with factories marked by BrRNA. *How many p65 binding sites are there in the Chip-Seq data? Again how many significantly (by some fold threshold) upregulated genes are there after TNF stimulation?*' There are >12,000 p65 binding sites across the genome (Antonaki *et al*., 2012; Kasowski *et al*., 2009; our ChIP-seq data), and 100-400 up-regulated genes (depending on the threshold; above).

'9. What fraction of RNA FISH signals to intron probes actually colocalize with tx factories?' It depends on how you define a factory. We defined one as a site containing at least two active RNA polymerases, and – if you accept this definition and that a nascent transcript is found with an active polymerase (above) – then the percentage colocalization is given in the relevant Figures. '*What about RNA-DNA FISH to examine the distance of these intron signals to the genes? Short of that what about two color RNA FISH where they also use exon-intron junction probes or exon probes.'* Is this relevant here (again, I think most people are happy using the nascent transcript copied from a gene as a marker for that gene!)?

'*10. Is there strong selection for the most highly expressed genes by the ChIA-PET approach? Numbers of contacts seem to scale disproportionally to levels of tx measured by FISH for the micro-RNAs.*' The ChIA-PET dataset involved a pull-down of phosphorylated polymerase II, and so (as we clearly state) involved a strong selection for highly-expressed genes. And the contacts that are seen reflect this – and correlate with the RNA FISH (which shows, for instance, that >30% *SAMD4A* alleles are active compared to ~15% for *MIR155HG*).

'*11. My major cognitive dissonance in reading this paper involves the 60% colocalization frequency of the SAMD4A gene with the 7 multiplex contact list. This is really high and seems to point to a relatively small number of foci. If each contact has equal probability, this works out to about 10% rate per contact. Assuming that these contacts are random with regard to the number of NF-KB "factories" doesn't this imply a low number then of about 10 factories per nucleus?*' This repeats the point above; see the 'speculative scenario' that involves ~250 such factories per nucleus. And our frequencies are within the range seen by others (we now state this in the main text, and cite Brown *et al*., 2008, and Schoenfelder *et al*., 2010).

'*What am I missing? Is it possible that this low number of estimated factories suggests that the colocalization occurs at a nuclear body of lower frequency than tx factories or p65 foci?*' Again, see the 'speculative scenario'.

'*I would really like to see costaining of the RNA FISH data with nuclear bodies such as speckles, Cajal bodies, PML bodies of such low frequency.*' I suggest this is beyond the scope of this manuscript, especially as these bodies are known to be transcriptionally inactive, and the associations seen (by Brown *et al*., 2008, and Schoenfelder *et al*., 2010) are – in any case – low.

'*Alternatively, the choice of this 7 multiplex mixture might be yielding unusually high*

fractions, and biased by one or two of these contacts having an exceptionally large colocalization frequency.' This is probably so, as the genes were chosen because they had a high interaction frequency. '*So on that note, where are these 7 contacts relative to the chromosome location of the target SAM4DA start site? Are any in cis and close by, resulting in a very high fraction of colocalization? This is For instance a contact within 10s of Mbp of the SAM4DA site might show a very high percentage of colocalization because the probability of colocalizing to the same nuclear body is much higher than a contact on a different chromosome.*' 3 of 7 are in *cis* (all >0.5 Mbp away) and the rest are on 4 different chromosomes (as stated). We have added the required experiment without probes targeting the 3 *cis* contacts; colocalization now drops to ~30% (from ~60%). We also note that none of the 7 contacts were expressed more than *SAMD4A* (assessed by RNA FISH).

'*Very crudely, if you divide the genome size by the number of NF-KB factories, then this would give the genomic distance expected for colocalization to the same factory.*' This repeats the point above; the 'speculative scenario' involving ~8 factories per ~100 Mbp chromosome is a plausible number.

Editorial Correspondence 28 June 2012

Thank you for your email requesting us to reconsider our decision not to invite a revised version of your manuscript. I have rediscussed your case with my colleagues in the editorial team in light of the comments made in your point-by-point response, and we find that we could be willing to change our decision, if the referees find that your manuscript would be acceptable in a revised version without the originally requested additional experimental data.

I have therefore contacted one of the original referees, and this person has agreed to look at your point-by-point response; with your permission, I will pass this on for consideration. If the referee finds that the concerns have been sufficiently addressed to preclude the addition of further experimental work, we would consider a revised version. However, in that case we would for technical reasons encourage you to submit the revised paper as a new manuscript to our system. We would then attempt to have it reviewed as fast as possible by the original referees to avoid further delays.

I will inform you of our final decision for your current manuscript, when we have considered the evaluation of your point-by-point response from the original referee.

Thank you again for offering The EMBO Journal the opportunity to consider your manuscript. Please feel free to contact me with any additional questions.

Yours sincerely,

Editor
The EMBO Journal

Editorial Correspondence 09 July 2012

Sorry for the delay in getting back to you with our decision.

I have now been in touch with one of the original referees regarding the additional experiments discussed in your point-by-point response, and the referee still has a number of concerns which crystallize to the following: 1) The need for direct visualization of DNA interactions in the proposed transcription factories and 2) the high co-localization rate seen for the few genes investigated in detail relative to the high number of transcription factories observed in total.

Based on this outcome, I have to insist that you perform additional DNA-FISH experiments to

provide visual proof that co-localization occurs for DNA and not just for nascent transcript before we can proceed towards publication of your manuscript. My suggestion would be that you also include the Br-RNA data you mention in the point-by-point response to strengthen the evidence for the observed foci as sites of active transcription. I understand that the most likely explanation would be that the observed foci indeed reflect DNA interactions, but nevertheless this needs to be formally proven to exclude that the observed localization is caused by nascent RNA accumulating at foci at a distance from the transcribed locus. The referee quotes recent work from Jeanne Lawrence's lab, which has shown that transcripts frequently accumulate with some offset from the actual gene location.

The referee also brings up the high co-localization frequency observed for single genes and the possible co-localization of transcription factories with existing nuclear bodies, and s/he argues that even if speckles are transcriptionally inactive, they can still be associated with active genes whose nascent transcripts go inside the speckle. However, I would not ask you to provide additional experimental data to address this option, but instead ask you to carefully rephrase the text to clearly highlight and discuss the basis for the co-localization frequencies observed, the possibility of co-localization with known nuclear bodies, and the caveats of the different measuring techniques and the impact of this on the observed interaction rates.

If you would be able to provide these additional experiments to demonstrate DNA-DNA interactions and to implement an extended rephrasing and discussion in the manuscript I would be willing to consider a revised version of the manuscript.

Yours sincerely,

Editor
The EMBO Journal

Author Correspondence 10 July 2012

Thanks for your consideration. We will:
1. Do the DNA FISH expt.
2. Include the Br-RNA data as you suggest (in the supplementary material).
3. Rephrase the text as suggested.
Re the last two points, I can't resist pointing out that nascent RNA cannot (by definition) accumulate at sites distant from the transcription site, that Jeanne Lawrence's lab did look at sites of accumulation (and not at nascent transcripts), and that nascent transcripts are not found in speckles (note we did use super-resolution localization!).

1st Revision - Authors' Response 11 August 2012

We now submit a revised version of our manuscript; we have done everything you requested, and we detail below how we have responded to each point made by the referees. I do hope that you now find this revised version acceptable.

Your points

You said *'I have to insist that you perform additional DNA-FISH experiments to provide visual proof that co-localization occurs for DNA and not just for nascent transcript before we can proceed towards publication of your manuscript'*, and *'my suggestion would be that you also include the Br-RNA data you mention in the point-by-point response to strengthen the evidence for the observed foci as sites of active transcription'*. We have done both (in Supplementary Fig S5A, and E, and the results are as expected). You also asked us to '*to carefully rephrase the text to clearly highlight and discuss the basis for the colocalization frequencies observed, the possibility of co-*

localization with known nuclear bodies, and the caveats of the different measuring techniques and the impact of this on the observed interaction rates' as the referee *'argues that even if speckles are transcriptionally inactive, they can still be associated with active genes whose nascent transcripts go inside the speckle'*. We now include more RNA FISH data, and speculate at length (in Supplementary information in a section entitled '*Estimating numbers of "NFκB" factories'*) on the colocalization frequencies. These frequencies are no different from those obtained by others (we now state this in the main text, and cite Brown *et al*., 2008, and Schoenfelder *et al*., 2010), and they are expected to be high simply because we selected genes that interacted at a high frequency. In this discussion (which includes new ChIA-PET data and an approach in Suppl. Fig. S6D that is additional to everything requested by referees), we have tried to highlight the major assumptions that were made, and whether the different calculations give upper or lower estimates. We also include in the main text a statement on association with nuclear bodies, such as speckles, and why our data and that of others would not lead us to expect such an association.

Editorial Correspondence 13 August 2012

Thank you for submitting a revised version of your manuscript. I will pass the revised version on to the original referees along with your point-by-point response, and I will let you know as soon as possible whether the referees now recommend the manuscript for publication in The EMBO Journal.
With regards to your difficulties with uploading the revised manuscript, this was probably caused by the manuscript status in our system; however we will now reset the status and upload the files you sent to me by email in house.
Please do not hesitate to contact me if you have any further questions.

Yours sincerely,

Editor

The EMBO Journal

2nd Editorial Decision 18 September 2012

Thank you very much for submitting a revised version of your manuscript and my apologies for the delay in getting back to you with our decision.

Your revised manuscript has now been seen by one of the original referees whose comments are shown below. As you will see, the referee finds that the new FISH data addresses the main concern raised by referees #1 and #2, and s/he now supports publication provided that certain adjustments are made to the manuscript text.
These include statistics for the FISH data, the rationale behind selecting the investigated gene sets, and the transcriptional activity of nuclear speckles. Another important issue is the extended discussion of gene interaction frequencies and expression levels that was added to the revised version. Since this was a major concern for both referee #1 and #2 in the original manuscript, I would ask you to address it more directly in the main discussion. This would then present the message of the manuscript in a clear and comprehensive manner as requested by the referee.

In addition, we now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you for giving us the opportunity to consider your manuscript. I look forward to your revision.

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORT:

Referee #2:

With regard to previous concerns:

1. DNA FISH is now included. A 5% experimental versus 0% control localization is seen. However, numbers relative to these percentages are somewhat low and a calculation of statistical significance should be made and stated. (By my rapid calculation which might be off the predicted standard deviation of the mean would be 0.0165, so I would assume it is significant but the authors should do this calculation to verify or if need be collect larger numbers of cells.)

2. The paper remains confusing to at least this reviewer because the authors don't state what they do in the appeal to provide appropriate information to the reader.

This includes:

a. The number of TNFalpha responsive genes seen by microarray
b. How the random gene control set was chosen- are these active genes versus randomly selected irregardless of gene activity. If they are selected as active, a couple of sentences about how they were selected and how the level of activity compares with the TNFalpha responsive genes are in order.

THIS IS IMPORTANT! The authors state that there are significantly fewer contacts with other highly active, non-responsive genes as compared to the TNFalpha 3C related genes, but it appeared to me that the actual difference was several fold. Therefore exactly how this gene control set is chosen could make a huge difference in the actual "significance" of these differences.

c. The gene set for the pooled RNA FISH colocalization apparently includes 3/7 probes that are in cis on the same chromosome. This should be stated, as well as the statistics for colocalization when these cis probes are excluded.

3. I disagree with the statements that speckles for instance are tx inactive. This is technically correct but for the purpose of this paper conceptually misleading. Speckles are surrounded by active genes whose nascent transcripts would obviously localize on the surface of the speckle. The Lawrence lab has shown that a subset of these transcripts will actually enter the speckle. Therefore when the authors use nascent transcript RNA FISH as their localization method, some of these transcripts could be localizing on the surface of nuclear speckles. As it has been shown that some incompletely spliced transcripts can enter nuclear speckles, then some of these transcripts could even be localized within speckles.

Minor point- on pg. 6

"Where next-generation sequencing is used we additionally require >10 identical reads per contact..."

This was very confusing to me. From the previous statements I got the impression that identical reads were likely due to PCR artifacts and come from a single molecule prior to PCR amplification. So if multiple reads are counted as one, why is there a threshold of >10?

3rd Revision - Authors' Response 22 September 2012

Thank you very much for your response to our revised submission. We have now re-revised the manuscript as requested to include: (i) the missing statistics in Suppl. Fig. 5A, (ii) more details on the gene sets used for comparisons, (iii) a lengthier discussion on speckles and interaction frequencies/ expression levels (all within the 55,000 character limit). We also provide point-to-point answers to the referee's latest comments (see below).

Lastly, as regards 'Source data files': the only electrophoretic gel in the main manuscript is in Fig. 3B and concerns 3C-PCR; a 'source file' showing scans of whole gels plus annotation of the parts relevant to Fig. 3B is now provided.

Answers to remarks by Referee #2:

1. DNA FISH is now included. A 5% experimental versus 0% control localization is seen. However, numbers relative to these percentages are somewhat low and a calculation of statistical significance should be made and stated. The *P* value (=0.0249) for the DNA FISH experiment was calculated and stated in the legend of Suppl. Fig. 5A; thus, the 5% versus 0% difference is significant.

2a. The number of TNFalpha responsive genes seen by microarray. This may vary according to the criteria used and the time-point concerned. In this case, 60 min post-induction, there are ~500 genes with >1.5-fold increase in expression level and a detectable activity; for clarity this is now included in the manuscript (see Methods, p. 18: 'Assessment of responsiveness to TNFa and SMAD binding').

2b. How the random gene control set was chosen- are these active genes versus randomly selected irregardless of gene activity. If they are selected as active, a couple of sentences about how they were selected and how the level of activity compares with the TNFalpha responsive genes are in order. The 'random gene control' set (Fig. 2 and Suppl. Table 1) was generated by randomly shuffling the microarray gene list, irrespective of gene activity. Responsiveness of these 75 genes is shown (Suppl. Table 1) and their activity varies greatly, from very lowly- to highly-expressed. This is now stated (p. 6); moreover, activities of a subset of these randomly-selected and responsive genes are compared at different time-points and <1/4 of them are expressed at levels lower than *GAPDH* (Suppl. Fig. S3A). The randomly-selected gene set used in Suppl. Fig. S6F is described in the legend and (because of the RNA polymerase pull-down in the ChIA-PET protocol) contains active genes of various expression levels (biased towards the most active). *The authors state that there are significantly fewer contacts with other highly active, non-responsive genes as compared to the TNFalpha 3C related genes, but it appeared to me that the actual difference was several fold. Therefore exactly how this gene control set is chosen could make a huge difference in the actual "significance" of these differences.* Highly-active, non-responsive genes are the top 69 genes from the microarray dataset; their levels 60 min post-induction do not change ±1.5-fold compared to 0-min levels—this is now clearly stated (Fig. 5, legend).

2c. The gene set for the pooled RNA FISH colocalization apparently includes 3/7 probes that are in cis on the same chromosome. This should be stated, as well as the statistics for colocalization when these cis probes are excluded. Statistics for this experiment ($P<0.0001$) are now added in the figure legend (Suppl. Fig. S5). The details of target chromosomal locations for the multiplexed probe set were already included in the Methods section, but are now also added to the text (p. 8).

3. I disagree with the statements that speckles for instance are tx inactive. This is technically correct but for the purpose of this paper conceptually misleading. Speckles are surrounded by active genes whose nascent transcripts would obviously localize on the surface of the

speckle. The Lawrence lab has shown that a subset of these transcripts will actually enter the speckle. Therefore when the authors use nascent transcript RNA FISH as their localization method, some of these transcripts could be localizing on the surface of nuclear speckles. As it has been shown that some incompletely spliced transcripts can enter nuclear speckles, then some of these transcripts could even be localized within speckles. We agree it is 'technically correct' to characterize speckles as 'transcriptionally inactive'—but why should this be 'misleading'? We also agree that it is formally possible that some of our (incompletely-spliced) transcripts go to speckles, but (as we've stated before) our reference genes were chosen partly because they were spliced co-transcriptionally (Wada *et al*., 2009; Papantonis and Cook, unpublished data)—and so we are talking about a minor fraction. Even so, let's assume this (hypothetical) minor fraction exists: it would still have to go with other transcripts with the same (hypothetical) properties to colocalize within a 160-nm region within the known dimensions of a speckle (diameter 0.5-3 mm). Moreover, as stated in Methods (RNA FISH, p. 18), each of our intronic probes yields at most two foci in our diploid G0 HUVECs. If our (hypothetical) incompletely-spliced transcripts were going to speckles we should detect >2 foci; we never do. Nonetheless, we have included the following in the Discussion (p. 12):

"RNA FISH (applied with intronic probes and coupled to high-resolution microscopy) confirms that nascent transcripts encoded by these responsive genes often lie together on the surface of 90-nm factories (**Figure 4**). Could these genes/transcripts associate with some structure other than a factory—for example, a nuclear "speckle" which is known to lie near nascent RNA (Brown *et al*, 2008; Spector and Lamond, 2011)? It seems unlikely, as speckles are themselves transcriptionally-inactive (Pombo and Cook, 1996) and have larger diameters (i.e., 0.5-3 mm; Hall *et al*, 2006) inconsistent with the profile seen in **Figure 4B**. While incompletely-spliced transcripts can associate with speckles (Hall *et al*, 2006; Spector and Lamond, 2011), *SAMD4A* transcripts are spliced co-transcriptionally (Wada *et al*, 2009) so FISH signals in **Figure 4A** should mark transcription sites."

Minor point- on pg. 6 "Where next-generation sequencing is used we additionally require >10 identical reads per contact..." This was very confusing to me. From the previous statements I got the impression that identical reads were likely due to PCR artifacts and come from a single molecule prior to PCR amplification. So if multiple reads are counted as one, why is there a threshold of >10? This was used as a minimum cutoff to exclude rare, randomly-amplified DNA fragments from being considered 'significant' hits, but it applied to negligibly few sequences. To avoid confusion the sentence has now been moved to the Methods section (p. 16).