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RUNX1 reshapes the epigenetic landscape at the onset of hematopoiesis

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1st Editorial Decision 30 April 2012

Thank you for submitting your research manuscript (EMBOJ-2012-81494) to our editorial office. It has now been seen by three referees and their comments are provided below.

As you can see, the referees appreciate that your genome-wide analysis of binding profiles of the three hematopoietic transcription factors SCL/TAL1, FLI1 and C/EBPbeta in early hemogenic endothelium in the absence or presence of RUNX1 is of significant interest and provides an important resource for researchers in the field. All three reviewers are overall supportive of publication here, and although they do raise a number of specific concerns, these should not require too much further work to address. However, the conclusion that Runx1 directly causes the relocalization of FLI1 and SCL/TAL1 does need more support. In particular referee #2 provides reasonable suggestions for how to extend this part of the analysis and this should be included in a revised version.

Given the comments provided, I would like to invite you to submit a suitably revised manuscript to The EMBO journal that addresses the raised concerns in full. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

In this paper, the authors have used an inducible RUNX1 ES cell line to analyze the dynamics of transcription factor binding during the Runx1-dependent transition from hemogenic endothelium to hematopoietic progenitors. ChIP-seq analysis of the binding of C/EBPb, SCL/TAL1 and FLI1 in the absence of RUNX1 showed widespread priming of hematopoietic genes with these TFs binding to many of the same genes but at different sites. They claim that this early priming is important for correct temporal expression of downstream genes although the only gene for which this importance is assessed is PU.1. A Fli1 binding site present in the -14kb URE was deleted and the kinetics of PU.1 expression were delayed during macrophage differentiation suggesting that although this site is not absolutely required for PU.1 expression, it is required for the correct temporal activation of PU.1. It would be interesting to know the behavior of these genes that are "primed" during development - does the priming associate with upregulation in Kit+ progenitors vs uninduced hemogenic endothelium? The authors address this by using GSEA analysis using a previously published hematopoietic gene expression set, although it would have been better to use a data set obtained from the same cell populations using RNAseq. The binding of FLI1 and SCL/TAL1 was then assessed upon induction of Runx1 overnight with dox and surprisingly although the location of SCL and FLI1 binding was significantly altered, the genes that were bound were similar suggesting that RUNX1 moves TFs from one location to another within a single gene locus. The gained peaks were significantly associated with Runx1 binding within 400bp and co-IP evidence supports the physical interaction between Runx1 and SCL/TAL1, FLI1 and C/EBPb, suggesting that Runx1 actually attracts the TFs to a new position by mediating long-range interactions between enhancers.

The data presented is technically and theoretically sound. In addition to the description of the global binding patterns of 4 major TFs during the onset of hematopoiesis in a relevant model system, the authors have provided evidence to support their priming hypothesis using genetically engineered Pu.1 ki ES cells as well, providing compelling evidence for a role for Runx1 in reshaping the epigenetic landscape during the endothelial to hematopoietic transition by means of an inducible Runx1 system in combination with ChIP-seq.

The idea that Runx1 actually creates new enhancers and may actually move TFs from other cis locations to these new enhancers in order to promote hematopoiesis is a novel concept and builds upon recent data suggesting that Runx1 mediates long range interactions between distant cis-regulatory elements. Therefore it will be of great interest to researchers in the blood field but may also provide insight into mechanisms of action of Runx genes in general and moreover, may provide insight into the mechanism of action of various Runx1 fusion genes that are associated with several leukemias.

In addition, the priming hypothesis has long been touted as a potential mechanism for initiating cell fate decisions and by cataloguing these priming events associated with the hematopoietic program, this paper may enable and encourage researchers to delve deeper into the mechanism of priming at different genes beyond PU.1. Priming may represent a general mechanism by which cell fates are specified during development and investigating the process may aid in the understanding of diverse events such as carcinogenesis and reprogramming.

We strongly recommend this paper for publication in EMBOJ.

Referee #2

The authors report the genome-wide analysis of Tal1, Fli1, Cebpb and Runx1 in the earliest steps of hematopoietic specification. These factors represent essential hematopoietic regulators. Using a Runx1 inducible ES cell line they show that Tal1, Fli1 and Cebpb are able to bind hematopoietic genes in the absence of Runx1 and that induction of Runx1 expression drives a shift in Tal1 and Fli1 binding patterns.

The data presented here represent an important contribution to the field of developmental hematopoiesis and are of broad biological significance.

The major claim is that Runx1 drives the shift of Tal1 and Fli1 binding patterns.
The authors provide circumstantial evidence that Runx1 plays a key role in this process. They show that a significant fraction of the newly acquired Tal1 and Fli1 peaks are in the vicinity of Runx1 peaks. They also show that Runx1 co-immunoprecipitates with both factors in HE cells indicating that it may physically drive re-localization of its protein partners.
This may be an attractive hypothesis, but the authors cannot rule out the possibility that this effect is indirect. Runx1 may activate specific targets, which in turn could drive the process of Tal1/Fli1 re-localization (possibly in combination with Runx1). It remains possible that Runx1 may not have the ability to relocate Tal1/Fli1 alone, but may require additional factors to do so. Therefore the statement in the discussion "...highlight a direct role of RUNX1 in orchestrating the formation of a hematopoiesis-specific transcription factor binding pattern..." remains unproven. there is simply too much emphasis on Runx1 in the absence of functional experiments. In particular, thye authors should showwhat would happen if Runx1 expression is suppressed or knocked-down after the induction period? Would the newly acquired Tal1 and Fli1 peaks (that relocate near Runx1 binding sites after induction) disappear? It is very well possible that once relocated next to Runx1 binding sites, Tal1 and Fli1 binding would remain even in the absence of Runx1, although one might expect to see some changes. This experiment should be added. Alternatively, showing in vitro that Runx1 displaces Tal1 or Fli1 from their binding sites would at least go some way towards a validation of the exciting findings reported in this paper.

If the authors address this point, the paper would make an important contribution to the field of hematopoiesis and to the general mechanisms of gene regulation during development.

Referee #3

In this manuscript, the authors study genome-wide binding of key hematopoietic regulators (SCL/TAL1, FLI1 and CEBPβ) in the absence or after induction of RUNX1 expression in hematopoietic progenitors. Using an ES cell differentiation system, they describe that binding of SCL/TAL1, FLI1 and CEBPβ in the early stage of the hemogenic endothelium (HE) primes hematopoietic genes. Subsequent binding of RUNX1, critical for the endothelial to hematopoietic transition (EHT), seems to redistribute SCL and FLI1 in gene loci; this establishes a hematopoietic pattern of transcription factor binding that presents some similarities with that found in hematopoietic cells.

This study is conceptually very interesting and provides a wealth of information that will be useful to dissect some of the molecular mechanisms that underlie important steps in hematopoietic development. The authors have taken advantage of the fact that hematopoietic development is blocked in Runx1-/- cells to have access to cells representing the early and transient HE stage in numbers sufficient for ChIP-seq analyses. Induction of Runx1 expression then releases the block to allow the study of the next stages of hematopoietic development. The data provide an important resource for investigators in the field. More generally, the concept will also be of interest to scientists involved in cell fate specification.

The whole study is based on an ES cell differentiation assay. The protocol used needs however to be clarified. Indeed, the differentiation system seems different from what has been published so far.

After purifying day 3.25 Flk1+ cells, the authors used a 2-step protocol instead of the well-described blast colony assay, Flk1+ cells are first plated in blast media for 2-2.5 days and, (after re-sorting?), Flk1+ cells are then replated in the HE media for another 2 days. What is the rationale behind this replating at day 2? The cells will have grown for a total of 4-4.5 days and will certainly contain haematopoietic cells. So the HE is not a population of cells that has grown for 2 or 3 days as shown in Figure 1A. This is confusing and needs to be clarified. It is mentioned that the cells are checked for purity; but it is important to show the FACS plots (see below).

Although it is interesting to analyse binding of CEBPβ (as an example of a lineage-specific regulator), comparing and incorporating these analyses with the rest of the data is slightly confusing. The authors do show that this factor binds genes involved in hematopoiesis and in the morphological alterations that may occur during the EHT, but (i) CEBPβ redistribution by Runx1 is not fully investigated, (ii) its is essentially presented as binding to myeloid genes that will be required later for macrophage differentiation, and (iii) it is not clear as to whether it participates in EHT or even in early hematopoietic development at a functional level. Could the authors maybe present these data in a slightly different way and mainly focus on the fact that myeloid genes are marked very early on by CEBPβ?

Other major points

1. As mentioned above, the protocols and overview of the differentiation for each cell line and each hematopoietic population analysed need to be more detailed. Importantly, the exact day at which the cells are harvested for analysis should be included in a schematic at the top of each Figure (such as 2A, 5A, 6A, 7A)

The FACS plots corresponding to day2, day3 and day4 of differentiation and to production of macrophages (as shown in Figure 1A) need to be included in the manuscript (perhaps as supplementary material) to be able to judge the efficiency of the differentiation system.

2. All the PCR analyses are performed using Sybr green primers. It is critical to explain how the primers were tested. Indeed, Sybr green primers can be difficult to use if their specificity has not been fully validated. Therefore it is important to confirm that the authors have analysed melting curves and standard dilution curves, and have loaded the PCR reactions on an agarose gel to confirm the presence of one unique PCR product and no primer dimers. The authors also need to mention how normalization is performed.

3. Figure 1B. Tal1 seems to be expressed in Macrophages. Is this expected? Or does this reflect the immaturity of the macrophages obtained in the differentiation system? FACS data and cellular staining would be useful.

4. There is no indication of the controls (such as no antibody or input) used in the ChIP experiments. Unspecific binding can be detected in ChIP experiments that will vary depending on the quality of the antibodies used. In that respect, have all the antibodies been used in previous ChIP-seq analyses; if not, how was their specificity validated?

5. If they haven't already done so, could the authors deposit the ChIP-seq data in a public database and include accession numbers in the manuscript.

6. Figure 2 and Supplementary Figure 2. Differentiation from iRunx1 (-DOX) and Runx1-/- cells to generate Tie2+ckit+CD41- cells; the FACS plots showing the purity of the populations need to be shown.
Also, it is difficult to be sure of when exactly the cells are harvested. At day 2 of differentiation? Or did the authors leave the cells slightly longer in culture to obtain more material? If the latter is true, then the observations made could be an artifact of the prolonged culture in absence of Runx1. In that case, it would be crucial to confirm some of the ChIP-seq data on chosen loci from day 2 Tie2+ckit+CD41- cells differentiated from wild-type ES cells.

7. Figure 2E and all de novo motif discovery figures. It might be useful to present the occurrence of each motif by measuring their frequency against a pool of random sequences of a size similar to the ChIP peaks. Especially useful to show an increase in the occurrence of GATA motifs in the

sequences underlying the TAL1 peaks after DOX induction.

Figures 2F, S5D, S6B. The authors show the % of peaks bound by a given transcription factor and containing its specific consensus recognition motif, but they never comment on the data. This could be included in a table showing the occurrence of all identified motifs for each ChIP-seq analysis and any interesting data discussed.

How was the motif AGGAAA/GCAG (bottom of Figure 2E) defined as an ETS/Ebox motif?

8. Figure S2C. To validate some of the ChIP-seq data, ChIP assays from Runx1-/- cells were analysed by PCR. This is supposed to show absence of leakiness of the inducible system. In addition to this, measurement of Runx1 levels before and after DOX induction should be compared to Runx1-/- and wild-type cells. Showing expression of Runx1 after DOX induction is also important for Figure 6A. Together with FACS data, it will confirm that the differentiation block is released; at the moment, there is no direct indication that induction has worked.

9. Figure 3A. It would have been informative to add a set of microarray expression data from HE populations to the GSEA analysis. That would allow comparison to directly relevant sets of genes.

10. Figure 4. Could the authors precise the type of mutation that was knocked into the PU.1 cis-element and why this leads to a 100bp difference between the PCR products derived from the wt and mutated alleles.

FLI1 ChIP must be performed on the mutated allele to confirm absence of FLI1 binding. ChIP with other transcription factors (such as SCL/TAL1, CEBPβ, Runx1, PU.1) must also be shown to exclude additional perturbation in the cis-element. WT cells must be used as a positive control in the expression analyses as Pu.1 expression could also be affected in heterozygous cells. This would show the full extent of how Pu.1 expression is affected by the mutation.

11. Figure 5. Could you make clear that the cells used in this figure derive from wild-type ES cells and have been differentiated for 4 days in the blast conditions. Perhaps as a drawing in 5A. Please show the FACS plots showing their immunophenotype (ckit+CD41+Tie2-).

12. Figure 5D. The fact that all the SCL/TAL1 datasets cluster together does not seem in favour of a redistribution of the peaks after RUNX1 induction, although Figure 5C and S5E do suggest it is. Could you comment on this discrepancy and perhaps show more representative examples?

13. Following this up, the examples shown in Figure 7B and S7A do not fully support the redistribution either. There is no change in SCL/TAL1 binding in the un-induced versus induced cells on Sox7. The binding is then lost in ckit+ cells. Regarding FLI1, binding is detected in un-induced cells and increased in induced cells. In the Erg locus, contrary to what is written on page 15, SCL binding is not lost in induced cells; in fact there is an additional peak. The same peak appears in the FLI1 track. The only peak bound by SCL in ckit+ cells was bound in un-induced and induced cells.
Although there are definitely differences in the binding patterns, it might be difficult to make this a general mechanism upon RUNX1 binding. Please comment.

14. Could you please explain how "the gene set bound by RUNX1 was basically identical to that bound by SCL/TAL1, FLI1 and CEBPβ"? Figure 6C shows that 2067 genes are uniquely bound by RUNX1. Separately, it may be useful to compare the list of genes bound by the 4 factors and those only bound by the EHT proteins (RUNX1, SCL/TAL1 and FLI1).

15. The coIP presented Figure 8B do not show that "the HE contains complexes containing SCL/TAL1, CEBPβ and FLI1 which are associated with RUNX1". This is not strictly right. They show that SCL/TAL1, CEBPβ and FLI1 individually coIP with RUNX1.

16. Some of the supplementary data (see below in Minor points) is not referred to nor analysed in the manuscript. If they are not necessary, these figures should be removed. This would help focus on the critical data.

Minor points

1. Two references may be added in the Introduction. Kissa and Herbomel, Nature 2010 (the most "visual" study showing the EHT) and Visvader et al, Genes Dev 1998 (the demonstration that SCL/TAL1 is required for vessel development).

2. Page 7, last paragraph. The first two sentences need to be rephrased. It is not clear whether both figures (S2C and S2D; text and legend) show data from Runx1-/- cells. And for how long the cells were differentiated. Probably same as in Figure 2A, but this needs to be described.

In the same paragraph, the 3rd sentence refers to Figure S2C, but mentions "all 3 factors" when only 2 are shown. RUNX1 is not shown. Also, there is some repetition in the sentence; this needs to be corrected.
Supplementary Figure 1B is mentioned but there is no such Figure in the paper. Generally speaking, there are a few incorrectly referenced Figures and Tables. Could the authors check the whole manuscript?
Finally, it is said that "none of the transcription factors bound to the Csfr promoter and its intronic enhancer (FIRE)". Figure S2C does show binding. Could you please comment?

Could you also analyse the data presented in Figure S2D.

3. Figure 2D and all other ChIP-seq tracks. Can the genes presented be drawn under the tracks. It is impossible to see where the genes lie and where their promoters are. Also, for PU,1, it is important to annotate the locus to show the cis-elements that will be validated in Figure S2C. More generally, the ChIP-seq peaks that are validated manually need to be identified on the tracks for easy comparison with PCR data.
What is the track at the bottom of the ChIP-seq figures?

4. ChIP-PCR experiments: How are the PCR signals normalized? Have no antibody or IgG controls been used in the ChIP assay?

5. Page 8, last paragraph. "the gene set that was bound by all 3 factors showed the highest enrichment score for hematopoietic genes". Should we read "for hematopoietic stem cell genes"? Please correct.

6. Page 9, last paragraph. Referring to Figure 1B, the authors write "The onset of Pu.1 gene expression was absolutely dependent on the presence of RUNX1". This is not strictly true as Figure 1B does not show a requirement for Runx1 but shows that the onset of Pu1 expression is concomitant with Runx1 up-regulation. Evidence for this requirement is presented in the next sentence. Please rephrase.

7. Figure S3C. What do the red stars show?

8. Where is Supplementary Figure 6E presented?

1st Revision - authors' response 11 July 2012

Referee 1:

His/her only slightly critical remark refers to a lack of gene expression data in the iRUNX1 system which was also picked up by the other referees. Part of the reason for not including such data was that our consortium has a paper in preparation that will carefully characterize the nature and biological function of RUNX1 target genes, a task that goes far beyond the scope of this study. However, we now show microarray expression data for the un-induced and induced iRUNX1 hemogenic endothelium stage, and will make these data available to the community via submission to the NCBI/EBI databases. These data very nicely show that (i) the majority of genes binding FLI1, SCL/TAL1 and C/EBPbeta in the HE are expressed, (ii) genes responding to RUNX1 induction are

direct targets of RUNX1 and (iii) that RUNX1 binding correlates with both the up- as well with the down-regulation of expression.

Referee 2:

This referee is in favour of publication, but is more critical regarding some interpretations of our data. He/she states:

"The major claim is that Runx1 drives the shift of Tal1 and Fli1 binding patterns.
The authors provide circumstantial evidence that Runx1 plays a key role in this process. They show that a significant fraction of the newly acquired Tal1 and Fli1 peaks are in the vicinity of Runx1 peaks. They also show that Runx1 co-immunoprecipitates with both factors in HE cells indicating that it may physically drive re-localization of its protein partners.
This may be an attractive hypothesis, but the authors cannot rule out the possibility that this effect is indirect. Runx1 may activate specific targets, which in turn could drive the process of Tal1/Fli1 re-localization (possibly in combination with Runx1). It remains possible that Runx1 may not have the ability to relocate Tal1/Fli1 alone, but may require additional factors to do so. Therefore the statement in the discussion "...highlight a direct role of RUNX1 in orchestrating the formation of a hematopoiesis-specific transcription factor binding pattern..." remains unproven. There is simply too much emphasis on Runx1 in the absence of functional experiments. "

The referee is of course correct in stating that some of the effects of RUNX1 with respect to redirecting factor binding may be indirect and is it certainly the case that RUNX1 needs to collaborate with other factors to form stable complexes. We therefore have toned down that statement in the discussion. "Direct" was meant in the sense of crucially important to set things in motion, and the fact that SCL/TAL1 and FLI-1 peaks move closer towards an actual RUNX1 binding event shows that RUNX1 may indeed directly organize factor complexes at these sites, but probably not everywhere. We actually discussed different scenarios in the previous version of the paper where we highlighted the fact that RUNX1 induces other factors, such as PU.1 which may directly displace FLI-1 from its binding sites. We have now rewritten the discussion to make this even clearer. However, we also performed another crucial line of experiments highlighting the roles of RUNX1 (see below)

In particular, the authors should show what would happen if Runx1 expression is suppressed or knocked-down after the induction period? Would the newly acquired Tal1 and Fli1 peaks (that relocate near Runx1 binding sites after induction) disappear? It is very well possible that once relocated next to Runx1 binding sites Tal1 and Fli1 binding would remain even in the absence of Runx1, although one might expect to see some changes. This experiment should be added. Alternatively, showing in vitro that Runx1 displaces Tal1 or Fli1 from their binding sites would at least go some way towards a validation of the exciting findings reported in this paper.
If the authors address this point, the paper would make an important contribution to the field of hematopoiesis and to the general mechanisms of gene regulation during development.

This was an excellent suggestion as it added another dimension to our analysis and in fact has opened up an entirely new line of research on the function of RUNX1. It is well known from studies with knock-out mice that a pulse of RUNX1 is enough to drive stem cell development (Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E, Speck NA. Nature. 2009 Feb 12;457(7231):887-91). In addition, my lab has previously used the iRUNX1 system to show that it is possible to withdraw RUNX1 without an effect on the maintenance of expression of PU.1 and genes regulated by this factor (Hoogenkamp et al., 2009, Blood) in precursor cells, although their induction is crucially dependent on RUNX1.
As suggested by the referee, in the revised version of this paper we have now measured FLI-1 and SCL/TAL1 binding after a short pulse of RUNX1 at a number of genes where we have shown that these factors relocate after induction, using quantitative ChIP assays. We did this experiment three times, and reproducibly show that transcription factor binding responds to RUNX1 withdrawal at the majority of these sites, demonstrating that RUNX1 is required to maintain the binding pattern and that at early stages after induction transcription factor assembly is still not completely "locked-in". However, as demonstrated by *Pu.*1 (and also *Sox17)* this does not appear to be a black-and-white scenario. Genes respond with different kinetics.

We therefore thank the referee for this suggestion. We are going to study at which point after RUNX1 induction factor assembly is stabilized and how.

Referee 3:

This referee has read the paper very carefully in meticulous and extensive detail and has made a multitude of helpful suggestions which we have followed as much as possible.

"The whole study is based on an ES cell differentiation assay. The protocol used needs however to be clarified. Indeed, the differentiation system seems different from what has been published so far. After purifying day 3.25 Flk1+ cells, the authors used a 2-step protocol instead of the well-described blast colony assay, Flk1+ cells are first plated in blast media for 2-2.5 days and, (after re-sorting?), Flk1+ cells are then replated in the HE media for another 2 days. What is the rationale behind this replating at day 2? The cells will have grown for a total of 4-4.5 days and will certainly contain haematopoietic cells. So the HE is not a population of cells that has grown for 2 or 3 days as shown in Figure 1A. This is confusing and needs to be clarified. It is mentioned that the cells are checked for purity; but it is important to show the FACS plots (see below)."

The referee is right in saying that we did not explain this properly. The step-wise culture system we used was first published by the Lacaud/Kouskoff lab in their 2009 Nature paper. Their studies demonstrate that this culture system is a traceable system that faithfully recapitulates the different steps in blood cell development, and it allows tracing and "trapping" cells at specific developmental stages by placing them in culture conditions optimized for forming blast cells and the hemogenic endothelium, respectively. The details of this methodology can be found in:
1. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. Lancrin C, Sroczynska P, Stephenson C, Allen T, Kouskoff V, Lacaud G. Nature. 2009 Feb 12;457(7231):892-5.;
2. In vitro differentiation of mouse embryonic stem cells as a model of early hematopoietic development. Sroczynska P, Lancrin C, Pearson S, Kouskoff V, Lacaud G. Methods Mol Biol. 2009;538:317-34.
These papers have now been explicitly cited. The referee is also correct in stating that the timing of these cultures is different than in wild-type cells and we apologize for the confusion. However, the order of expression of hematopoietic genes and their relative expression level is preserved in this system (as shown in Supplementary Figure 6 G), and by sorting FLK1 positive cells twice (once before and – indeed - once after blast culture) we ensure that the resulting cell population only contains HE cells, and not hematopoietic cells. In the revised version we have now clarified this issue and also have changed the schematic depiction, for example in in Figure 2 A (and others) to highlight this fact. However, to accommodate the large number of requested additional experiments, we had to move the detailed description of this culture system into Supplementary methods.

Although it is interesting to analyse binding of CEBPβ (as an example of a lineage-specific regulator), comparing and incorporating these analyses with the rest of the data is slightly confusing. The authors do show that this factor binds genes involved in hematopoiesis and in the morphological alterations that may occur during the EHT, but (i) CEBPβ redistribution by Runx1 is not fully investigated, (ii) its is essentially presented as binding to myeloid genes that will be required later for macrophage differentiation, and (iii) it is not clear as to whether it participates in EHT or even in early hematopoietic development at a functional level. Could the authors maybe present these data in a slightly different way and mainly focus on the fact that myeloid genes are marked very early on by CEBPβ?

In the revised version we have followed the referee's suggestion and removed this part of the discussion.

Other major points

As mentioned above, the protocols and overview of the differentiation for each cell line and each hematopoietic population analysed need to be more detailed. Importantly, the exact day at which the cells are harvested for analysis should be included in a schematic at the top of each Figure (such as 2A, 5A, 6A, 7A)

This has now been done.

The FACS plots corresponding to day2, day3 and day4 of differentiation and to production of macrophages (as shown in Figure 1A) need to be included in the manuscript (perhaps as supplementary material) to be able to judge the efficiency of the differentiation system.

We felt that it was not feasible to show FACS plots for every single experiment as this would have meant to produce a very large number of additional figures. The manuscript already has a vast number of supplementary figures, and the additional crucial data we included have expanded this even more. We have added FACS plots where we think it makes sense in Suppl. Figure 1, 6 and 8. We are following a published protocol, and the referee needs to trust us that our data are produced from the correct cell populations. Very detailed FACS profiles of the entire time course are shown in Figure 1 of Lancrin et al., 2009, Nature, and our profiles look exactly the same.

All the PCR analyses are performed using Sybr green primers. It is critical to explain how the primers were tested. Indeed, Sybr green primers can be difficult to use if their specificity has not been fully validated. Therefore it is important to confirm that the authors have analysed melting curves and standard dilution curves, and have loaded the PCR reactions on an agarose gel to confirm the presence of one unique PCR product and no primer dimers. The authors also need to mention how normalization is performed.

We follow standard procedures that have been established in our previous publications. The specificity of all of our Real-Time PCR reactions are of course controlled by producing a melting curve plot at the end of the reaction and they always only show one product. The amplification efficiency is further normalized by running a standard curve for each primer in parallel. Also, the newly generated microarray data confirm gene expression changes assayed by SYBR-Green based Q-RT-PCR, and thus provide validation using totally different technology.

Figure 1B. Tal1 seems to be expressed in Macrophages. Is this expected? Or does this reflect the immaturity of the macrophages obtained in the differentiation system? FACS data and cellular staining would be useful.

TAL1 is indeed expressed in myeloid cells. See: The TAL1/SCL transcription factor regulates cell cycle progression and proliferation in differentiating murine bone marrow monocyte precursors. Dey S, Curtis DJ, Jane SM, Brandt SJ. Mol Cell Biol. 2010 May;30(9):2181-92.

There is no indication of the controls (such as no antibody or input) used in the ChIP experiments. Unspecific binding can be detected in ChIP experiments that will vary depending on the quality of the antibodies used. In that respect, have all the antibodies been used in previous ChIP-seq analyses; if not, how was their specificity validated?

We apologize for the oversight. All of our ChIP-seq experiments are of course normalized against input. This is now mentioned in Materials and Methods.

If they haven't already done so, could the authors deposit the ChIP-seq data in a public database and include accession numbers in the manuscript.

We will do so upon acceptance of the manuscript.

Figure 2E and all de novo motif discovery figures. It might be useful to present the occurrence of each motif by measuring their frequency against a pool of random sequences of a size similar to the ChIP peaks. Especially useful to show an increase in the occurrence of GATA motifs in the sequences underlying the TAL1 peaks after DOX induction.

The motifs were identified in an unbiased fashion using the HOMER software suite and the p-Values are indeed calculated compared to random sequence. The software does that automatically.

Figures 2F, S5D, S6B. The authors show the % of peaks bound by a given transcription factor and containing its specific consensus recognition motif, but they never comment on the data. This could

be included in a table showing the occurrence of all identified motifs for each ChIP-seq analysis and any interesting data discussed.

The only time the percentage of peaks is shown is in Figure 8, all the other numbers are p-values that highlight the significance of motif occurrence. We have now added a sentence commenting on the data.

8. Figure S2C. To validate some of the ChIP-seq data, ChIP assays from Runx1-/- cells were analysed by PCR. This is supposed to show absence of leakiness of the inducible system. In addition to this, measurement of Runx1 levels before and after DOX induction should be compared to Runx1-/- and wild-type cells. Showing expression of Runx1 after DOX induction is also important for Figure 6A. Together with FACS data, it will confirm that the differentiation block is released; at the moment, there is no direct indication that induction has worked.

We control induction every time by analysing for the expression of CD41 on the surface. As shown by Lancrin et al. 2009, this is a very sensitive marker for RUNX1 activity. To illustrate this point, we have included a FACS plot showing an example of such an analysis (Supplementary Figure 6).

Figure 3A. It would have been informative to add a set of microarray expression data from HE populations to the GSEA analysis. That would allow comparison to directly relevant sets of genes.

This has now been done.

Figure 4. Could the authors precise the type of mutation that was knocked into the PU.1 cis-element…..

This information has now been added in Supplementary Figure 4 A.

FLI1 ChIP must be performed on the mutated allele to confirm absence of FLI1 binding. ChIP with other transcription factors (such as SCL/TAL1, CEBPβ, Runx1, PU.1) must also be shown to exclude additional perturbation in the cis-element. WT cells must be used as a positive control in the expression analyses as Pu.1 expression could also be affected in heterozygous cells. This would show the full extent of how Pu.1 expression is affected by the mutation.

A detailed analysis of mice carrying such a mutation by the Tenen lab (including ChIP assays to confirm lack of binding) is currently under revision at Molecular Cell. Our experiments were aimed at showing that the intactness of this binding site is necessary for the correct timing of expression of Pu.1 as compared to other genes. The importance of the priming aspect of this manuscript has been emphasized by Referee 2. In light of the fact that this experiment is not an absolutely essential part of the manuscript, but illustrates the importance of priming in a very nice way, we would be grateful if the referee would await the publication of the above study.

Figure 5. Could you make clear that the cells used in this figure derive from wild-type ES cells and have been differentiated for 4 days in the blast conditions. Perhaps as a drawing in 5A. Please show the FACS plots showing their immunophenotype (ckit+CD41+Tie2-).

This has been clarified. Regarding the FACS plots: see my comment above.

Figure 5D. The fact that all the SCL/TAL1 datasets cluster together does not seem in favour of a redistribution of the peaks after RUNX1 induction, although Figure 5C and S5E do suggest it is. Could you comment on this discrepancy and perhaps show more representative examples?

The clustering looks at common sequence features whereas the Venn diagram shows peaks that are directly overlapping. Clustering is therefore less stringent as it is dominated by occurrences with large differences and is optimized to show tissue-specific global binding patterns. However, note that in the Venn-diagram there are less peak overlaps with FLI-1 than for SCL/TAL1.

Following this up, the examples shown in Figure 7B and S7A do not fully support the redistribution either. There is no change in SCL/TAL1 binding in the un-induced versus induced cells on Sox7. The binding is then lost in ckit+ cells. Regarding FLI1, binding is detected in un-induced cells and

increased in induced cells. In the Erg locus, contrary to what is written on page 15, SCL binding is not lost in induced cells; in fact there is an additional peak. The same peak appears in the FLI1 track. The only peak bound by SCL in ckit+ cells was bound in un-induced and induced cells.
Although there are definitely differences in the binding patterns, it might be difficult to make this a general mechanism upon RUNX1 binding. Please comment.

The examples shown have been chosen to exemplify a global trend, i.e. a change in binding pattern (loss and appearance of peaks, as exemplified by *Erg*). At *Sox17*, a new FLI-1 peak is detected after RUNX1 induction which is not seen before (note that we show non-manipulated data, the small increase in reads in un-induced cells is not significant and not called as peak). It should also be noted that our clustering analysis is not the only evidence for a role of RUNX1. Figure 8 shows that newly acquired FLI1 and SCL/TAL1 binding sites now associate with RUNX1 motifs which they did not before. Moreover, they are now more closely localized with an actual RUNX1 binding site in a highly statistically significant fashion. This result is very difficult to explain in a scenario that does not involve RUNX1.

Could you please explain how "the gene set bound by RUNX1 was basically identical to that bound by SCL/TAL1, FLI1 and CEBPβ? Figure 6C shows that 2067 genes are uniquely bound by RUNX1. Separately, it may be useful to compare the list of genes bound by the 4 factors and those only bound by the EHT proteins (RUNX1, SCL/TAL1 and FLI1).

A study like ours has global data that we cannot all describe in one paper. We are already at the word-limit. Our study will provide a wealth of information for other scientists to analyse, since all data-sets and gene sets will be publicly available. Also note my comment above to referee 1: a publication containing more detailed analyses of the biological function of RUNX1 target genes is currently in preparation. Such an analysis is outside the scope of this paper.

The coIP presented Figure 8B do not show that "the HE contains complexes containing SCL/TAL1, CEBPβ and FLI1 which are associated with RUNX1". This is not strictly right. They show that SCL/TAL1, CEBPβ and FLI1 individually coIP with RUNX1.

The referee is correct. The statement has been altered accordingly

Some of the supplementary data (see below in Minor points) is not referred to nor analysed in the manuscript. If they are not necessary, these figures should be removed. This would help focus on the critical data.

We have now tried and commented on each experiment as much as possible.

Minor points

Two references may be added in the Introduction. Kissa and Herbomel, Nature 2010 (the most "visual" study showing the EHT) and Visvader et al, Genes Dev 1998 (the demonstration that SCL/TAL1 is required for vessel development).

We apologize for the oversight. These references have now been included.

Page 7, last paragraph. The first two sentences need to be rephrased. It is not clear whether both figures (S2C and S2D; text and legend) show data from Runx1-/- cells. And for how long the cells were differentiated. Probably same as in Figure 2A, but this needs to be described.

This has now been clarified

In the same paragraph, the 3rd sentence refers to Figure S2C, but mentions "all 3 factors" when only 2 are shown. RUNX1 is not shown. Also, there is some repetition in the sentence; this needs to be corrected.
Supplementary Figure 1B is mentioned but there is no such Figure in the paper. Generally speaking, there are a few incorrectly referenced Figures and Tables. Could the authors check the whole manuscript?

Finally, it is said that "none of the transcription factors bound to the Csfr promoter and its intronic enhancer (FIRE)". Figure S2C does show binding. Could you please comment?

These inaccuracies have been corrected.

Figure 2D and all other ChIP-seq tracks. Can the genes presented be drawn under the tracks. It is impossible to see where the genes lie and where their promoters are. Also, for PU,1, it is important to annotate the locus to show the cis-elements that will be validated in Figure S2C. More generally, the ChIP-seq peaks that are validated manually need to be identified on the tracks for easy comparison with PCR data.

The cis-elements have now been annotated

ChIP-PCR experiments: How are the PCR signals normalized? Have no antibody or IgG controls been used in the ChIP assay?

All ChIP-PCR experiments were normalized to an internal negative control ("Chr2"). We find that this controls against differences in background caused by different antibodies and we prefer this to the IgG control which gives very low signals that are highly variable, thus causing normalization artifacts.

Page 8, last paragraph. "the gene set that was bound by all 3 factors showed the highest enrichment score for hematopoietic genes". Should we read "for hematopoietic stem cell genes"? Please correct.

This has been done

6. Page 9, last paragraph. Referring to Figure 1B, the authors write "The onset of Pu.1 gene expression was absolutely dependent on the presence of RUNX1". This is not strictly true as Figure 1B does not show a requirement for Runx1 but shows that the onset of Pu1 expression is concomitant with Runx1 up-regulation. Evidence for this requirement is presented in the next sentence. Please rephrase.

This has been done

7. Figure S3C. What do the red stars show?
They show genes within the focal adhesion pathway that are bound by SCL/TAL1, FLI1 and C/EBPb. This explanation has now been added.

2nd Editorial Decision 20 August 2012

Thank you for submitting your revised manuscript for our consideration. I am pleased to inform you that in light of the re-review comments from one of the original referees (provided below), we are happy to accept the paper, pending modification of a few minor points as follows:

- the referee maintains that FACS blots for Fig. 1A and 2A should be included. Although I take your point that it might not be feasible to include FACS blots for every single experiment in the manuscript, this distinct request does seem reasonable.

- her/his additional points, which only require text changes and some re-formatting should be taken into consideration as well.

- according to the figure legend of Fig.1B, the data is based on biological duplicates. As it is not statistically sound to derive error bars from only two data points, I would like to ask you to either repeat the experiment one more time or to remove the error bars.

- please remember to add the data accession numbers for all high-throughput data sets.

I am returning the manuscript to you for one last round of minor amendments, hoping that you will be able to upload and re-submit the final version at your earliest convenience. After that, we should be able to swiftly proceed with formal acceptance and production of the manuscript!

If you have any questions in this regard, please do not hesitate to contact me directly.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT

Referee 3:

"The whole study is based on an ES cell differentiation assay. The protocol used needs however to be clarified. Indeed, the differentiation system seems different from what has been published so far. After purifying day 3.25 Flk1+ cells, the authors used a 2-step protocol instead of the well-described blast colony assay, Flk1+ cells are first plated in blast media for 2-2.5 days and, (after re-sorting?), Flk1+ cells are then replated in the HE media for another 2 days. What is the rationale behind this replating at day 2? The cells will have grown for a total of 4-4.5 days and will certainly contain haematopoietic cells. So the HE is not a population of cells that has grown for 2 or 3 days as shown in Figure 1A. This is confusing and needs to be clarified. It is mentioned that the cells are checked for purity; but it is important to show the FACS plots (see below)."

The referee is right in saying that we did not explain this properly. The step-wise culture system we used was first published by the Lacaud/Kouskoff lab in their 2009 Nature paper. Their studies demonstrate that this culture system is a traceable system that faithfully recapitulates the different steps in blood cell development, and it allows tracing and "trapping" cells at specific developmental stages by placing them in culture conditions optimized for forming blast cells and the hemogenic endothelium, respectively. The details of this methodology can be found in:
1. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. Lancrin C, Sroczynska P, Stephenson C, Allen T, Kouskoff V, Lacaud G. Nature. 2009 Feb 12;457(7231):892-5.;
2. In vitro differentiation of mouse embryonic stem cells as a model of early hematopoietic development. Sroczynska P, Lancrin C, Pearson S, Kouskoff V, Lacaud G. Methods Mol Biol. 2009;538:317-34.
These papers have now been explicitly cited. The referee is also correct in stating that the timing of these cultures is different than in wild-type cells and we apologize for the confusion. However, the order of expression of hematopoietic genes and their relative expression level is preserved in this system (as shown in Supplementary Figure 6 G), and by sorting FLK1 positive cells twice (once before and - indeed - once after blast culture) we ensure that the resulting cell population only contains HE cells, and not hematopoietic cells. In the revised version we have now clarified this issue and also have changed the schematic depiction, for example in in Figure 2 A (and others) to highlight this fact. However, to accommodate the large number of requested additional experiments, we had to move the detailed description of this culture system into Supplementary methods.

In the Lancrin paper, the cells sorted at day 2-3 of BL-CFC cultures are Tie2+cKit+CD41-, not Flk1+. This is done to analyse the hematopoietic potential of the cells. So this is different from what is done in this manuscript, where the authors replate a heterogeneous population of Flk1+ cells to generate the HE. It is still not clear to me why this is done. Moreover, the FACS data in Fig.1 in Lancrin et al clearly shows that, at day 2, there are already CD41+ cells whilst in Fig1. of Lichtinger et al, the schematic shows CD41- cells only at day2. All I am asking for is for the authors to incorporate the FACS plots underneath day2, day3 and day4 in Fig1A to confirm the immunophenotype described at the top of the figure. And to do the same in Fig 2A to show that the dark blue cells are indeed the same as in Fig. 1A (as the differentiation protocols are different). In fact, the FACS plots presented in Fig. S6A show 20% CD41+ cells before Runx1 induction, as in the Lancrin paper. This is certainly a reflection of the leakiness of the inducible system.

Other major points

The FACS plots corresponding to day2, day3 and day4 of differentiation and to production of macrophages (as shown in Figure 1A) need to be included in the manuscript (perhaps as supplementary material) to be able to judge the efficiency of the differentiation system.

We felt that it was not feasible to show FACS plots for every single experiment as this would have meant to produce a very large number of additional figures. The manuscript already has a vast number of supplementary figures, and the additional crucial data we included have expanded this even more. We have added FACS plots where we think it makes sense in Suppl. Figure 1, 6 and 8. We are following a published protocol, and the referee needs to trust us that our data are produced from the correct cell populations. Very detailed FACS profiles of the entire time course are shown in Figure 1 of Lancrin et al., 2009, Nature, and our profiles look exactly the same.

See above comment.

All the PCR analyses are performed using Sybr green primers. It is critical to explain how the primers were tested. Indeed, Sybr green primers can be difficult to use if their specificity has not been fully validated. Therefore it is important to confirm that the authors have analysed melting curves and standard dilution curves, and have loaded the PCR reactions on an agarose gel to confirm the presence of one unique PCR product and no primer dimers. The authors also need to mention how normalization is performed.

We follow standard procedures that have been established in our previous publications. The specificity of all of our Real-Time PCR reactions are of course controlled by producing a melting curve plot at the end of the reaction and they always only show one product. The amplification efficiency is further normalized by running a standard curve for each primer in parallel. Also, the newly generated microarray data confirm gene expression changes assayed by SYBR-Green based Q-RT-PCR, and thus provide validation using totally different technology.

Could you then just add a sentence below Table S2 saying that specificity of all the primers has been checked by standard procedures. It is always useful to know this is the case when searching for published sequences.

The following point has not been answered:
Figure 2 and Supplementary Figure 2.
Also, it is difficult to be sure of when exactly the cells are harvested. At day 2 of differentiation? Or did the authors leave the cells slightly longer in culture to obtain more material? If the latter is true, then the observations made could be an artifact of the prolonged culture in absence of Runx1 (especially now that we know that the system is leaky). In that case, it would be crucial to confirm some of the ChIP-seq data on chosen loci from day 2 Tie2+ckit+CD41- cells differentiated from wild-type ES cells or from Runx1-/- (as opposed to iRunx1).
This question really comes from the fact that the experimental procedures lack details.

Minor points

Figure 2D and all other ChIP-seq tracks. Can the genes presented be drawn under the tracks. It is impossible to see where the genes of interest lie and where their promoters are.
This has not been done. We still cannot read the names of the genes. They may have to be added manually.

Also, for PU,1, it is important to annotate the locus to show the cis-elements that will be validated in Figure S2C. More generally, the ChIP-seq peaks that are validated manually need to be identified on the tracks for easy comparison with PCR data.

The cis-elements have now been annotated.
I cannot see the annotations.

2nd Revision - authors' response 27 August 2012

Response to instructions from the editor:

- the referee maintains that FACS blots for Fig. 1A and 2A should be included. Although I take your point that it might not be feasible to include FACS blots for every single experiment in the manuscript, this distinct request does seem reasonable.
Such profiles have now been included, and further explanations have been added in Materials and Methods. Further details are outlined in our comments addressing the referee's remarks below.

- her/his additional points, which only require text changes and some re-formatting should be taken into consideration as well.
This has been done as outlined below.

- according to the figure legend of Fig.1B, the data is based on biological duplicates. As it is not statistically sound to derive error bars from only two data points, I would like to ask you to either repeat the experiment one more time or to remove the error bars.
This is of course correct, as two of our 6 measurements have only been performed twice instead of 3 – 4 times. We therefore now show the mean value of these two measurements and indicate the individual values for each measurement. Error bars for measurements independently performed 3 times and more have been retained. This fact is highlighted in the revised Figure Legend.

- please remember to add the data accession numbers for all high-throughput data sets.

The data have been uploaded on GEO and the accession number is indicated. Data will be released immediately upon publication.

Detailed responses to further comments by Referee 3:

Referee 3: *In the Lancrin paper, the cells sorted at day 2-3 of BL-CFC cultures are Tie2+cKit+CD41-, not Flk1+. This is done to analyse the hematopoietic potential of the cells. So this is different from what is done in this manuscript, where the authors replate a heterogeneous population of Flk1+ cells to generate the HE. It is still not clear to me why this is done. Moreover, the FACS data in Fig.1 in Lancrin et al clearly shows that, at day 2, there are already CD41+ cells whilst in Fig1. of Lichtinger et al, the schematic shows CD41- cells only at day2.*
All I am asking for is for the authors to incorporate the FACS plots underneath day2, day3 and day4 in Fig1A to confirm the immunophenotype described at the top of the figure. And to do the same in Fig 2A to show that the dark blue cells are indeed the same as in Fig. 1A (as the differentiation protocols are different).

Our response:

We apologize for misunderstanding the real purpose of referee's query and thus creating more confusion. The Figures 1 and 2 depict two different schemes of ES cell differentiation and analyses of cell populations which, however, generate the same cell types.

In the first Figure depicting experiments with wild-type cells, hemangioblast cells are purified by Flk1 selection and are re-plated into blast culture. In the original Figure, the main populations that are sequentially generated upon hematopoietic and macrophage development were indicated at the days at which they are the most prominent. This scheme of differentiation has been extensively described in the literature and the differentiation potential of the cells displaying the different surface marker phenotypes has been described (Lancrin 2009, Hoogenkamp 2009, Lancrin 2012). To avoid confusion, we now have removed the annotation by days in all figures and indicate the sorting and blast culture step.
As requested, we now show additional FACS profiles to illustrate the composition of our blast cultures at different days (new Figure 1, left panel) from which we purify the cells representing the different developmental stages. Since our differentiation system is not completely synchronized, we find cells of all surface marker combinations at day 3 of blast culture (this is the profile shown in the new Supplementary Figure 1A, top right panel). We therefore do not sort cells at different days, but we prospectively sort cells with defined surface marker characteristics and developmental potential at day 3 of blast culture and test their purity by additional flow cytometry (as shown in

Supplementary Figure 1 bottom right panel). These pure cell populations are then used for our gene expression analyses. Additional explanations have been added in the Figure legend and Materials and Methods and we hope that this is clearer now.

We also added more FACS profiles and explanations to Supplementary Figure 2. In the experiments described in the second Figure, iRunx1 ES cells are differentiated in the same way as outlined in Figure 1 up to the blast culture stage. Blast culture day 2 Flk1-sorted cells are then re-plated in hemogenic endothelium medium in order to generate a pure hemogenic endothelium cell population whereby we only use MACS columns, instead of sorting for the phenotype Tie2CkitCD41 as this requires longer sorting times and allows recovery of more cells. This can be done since all hemogenic endothelial cells deficient for Runx1 do not progress further than the first stage of hemogenic endothelium (Tie2+, ckit+, CD41-) and still carry the marker Flk1. The cells are then cultured as adherent cells for a short time, thus removing any floating cells. This procedure has now been described in more detail. We now also show the FACS profiles for such purified cells assaying their Tie2, c-kit and CD41 expression (Supplementary Figure 2A). This unequivocally demonstrates that these cells correspond to the cell types described in Figure 1.

We have now re-written Materials and Methods as well as the Figure legends to make this absolutely clear.

Referee 3: …the FACS plots presented in Fig. S6A show 20% CD41+ cells before Runx1 induction, as in the Lancrin paper.
This is certainly a reflection of the leakiness of the inducible system.

Our response: There are two arguments against a leakiness of our inducible system.
(i) The referee is right to remark that a small proportion of these iRunx1-/- HE cells show CD41 expression even in the absence of RUNX1. This has indeed already been documented in Lancrin et al 2009 and we have shown that these cells do not display any definitive hematopoietic potential upon re-plating, in contrast to the CD41+ cells generated upon re-expression of RUNX1. In addition, the number of these cells does not change during culture which would be the case if the system was leaky. We included additional FACS data emphasizing this point (Supplementary Figure 2A). The cells with low CD41 expression generated in the absence of RUNX1 are therefore not the result of the leakiness of the system but they are most likely primitive erythroid cells.
(ii) The second argument against extensive leakiness comes from our ChIP-seq data. In Figure 6 E/F we show a ChIP-seq experiment in the absence of RUNX1 induction and while we see a signal in the absence of RUNX1 across all genes (pink curve), the fraction of genes showing a RUNX1 signal is tiny, demonstrating tight regulation of our inducible system where it matters: at the level of RUNX1 interaction with the genome.

Could you then just add a sentence below Table S2 saying that specificity of all the primers has been checked by standard procedures. It is always useful to know this is the case when searching for published sequences.
This has now been included.

The following point has not been answered:
Figure 2 and Supplementary Figure 2. Also, it is difficult to be sure of when exactly the cells are harvested. At day 2 of differentiation? Or did the authors leave the cells slightly longer in culture to obtain more material? If the latter is true, then the observations made could be an artifact of the prolonged culture in absence of Runx1 (especially now that we know that the system is leaky). In that case, it would be crucial to confirm some of the ChIP-seq data on chosen loci from day 2 Tie2+ckit+CD41- cells differentiated from wild-type ES cells or from Runx1-/- (as opposed to iRunx1).
This question really comes from the fact that the experimental procedures lack details.

Our response: The questions about when we purified the cells have been answered above. Control ChIP experiments with RUNX1-/- cells which are blocked in differentiation at the Tie2+ckit+CD41- stage are shown in Supplementary Figure 2 C.

Minor points

We still cannot read the names of the genes. They may have to be added manually.
I cannot see the annotations.

Our response: Cis-elements have now been further annotated and the names of the genes have been depicted even bigger. We also indicated the position of primers used for RT-PCR.

