THE EMBO JOURNAL

Manuscript EMBO-2011-77872

FOG-1 and GATA-1 act sequentially to specify definitive megakaryocytic and erythroid progenitors.

Elena Mancini, Alejandra Sanjuan-Pla, Luisa Luciani, Susan Moore, Amit Grover, Agnes Zay, Kasper D Rasmussen, Sidinh Luc, Daniel Bilbao, Donal OíCarroll, Sten Eirik Jacobsen and Claus Nerlov

Corresponding author: Claus Nerlov, University of Edinburgh

Review timeline:

Submission date:	13 April 2011
Editorial Decision:	01 June 2011
Revision received:	02 September 2011
Accepted:	05 October 2011

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 01 June 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. Please let me first apologise for the delay in getting back to you with a decision: unfortunately two of the referees were significantly late in returning their reports, and we are only now in a position to take a decision on your submission. The referees' reports are enclosed below. As you will see, both referees 1 and 3 are broadly in favour of publication, pending satisfactory revision, whereas referee 2 finds the conceptual advance here to be limited and does not recommend publication. He/she raises significant concerns as to appropriate citation and discussion of relevant literature. Moreover, he/she argues that your work does not provide new mechanistic insight into lineage commitment decisions in hematopoiesis.

Given the overall positive recommendations of referees 1 and 3 we are willing to over-rule the negative referee and to invite a revision on your manuscript, but it would be important to take his/her concerns into account, especially in terms of appropriate discussion of the literature. Moreover, and while we do recognise that this was not specifically requested by any of the referees, we would encourage you to consider extending the mechanistic insight of your paper by analysing further the potential cross-regulation between FOG-1/GATA and C/EBP: if, for example, you were able to show direct binding and regulation, that should significantly strengthen this important aspect of your study that is highlighted in your abstract.

In terms of additional analysis requested by the referees, we recognise that the final point of referee 1's report would require a very long time-scale experiment. If you have data addressing this point, it would clearly be useful, but we would not insist on this. The other specific concerns of referees 1 and 3, however, should be addressed.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. 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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension. Please don't hesitate to get in touch if you have any questions or comments about the revision.

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

This manuscript by Mancini et al. present data supportive of the hypothesis that factors mediating Mk/E lineage commitment act antagonistically to C/EBPs (GM lineage commitment). Conditional knockout approaches are used and the animals analysed by FACS and hematopoietic progenitor assays. The results are interesting and provide new data in the field of hematopoietic lineage specification and differentiation.

Specific comments
In CEBPs cKO, why is the percentage of CFU-E decreased? This seems inconsistent with the model. Can the authors please explain?

The lack of erythroid colony formation is shown in figure 3. Was the Meg potential of the sorted preMegEs tested at the same time? Please include this functional data.

The rationale of the transplantation (non-competitive) experiment is unclear. Why was the 1 month post-transplantation time point chosen? This would not allow testing of the stem cell or most immature progenitor population. What happens if the poly I-C treatment was performed 4 months post-transplantation? Is there a delay in lethality?

Referee #2

The manuscript by Mancini et al. describes an analysis of the function of GATA-1 and FOG-1 in the context of controlling hematopoiesis in the mouse. The investigators used a conditional knockout approach to assess factor requirements at specific stages of hematopoiesis, with a major focus on whether factors are required for commitment and specification of cell lineages. Overall, I found the work to be technically sound. However, major problems/limitations exist with the study. This is especially important, considering the goal of presenting work that illustrates important biological and/or mechanistic advances.

Specific Comments:
1) The authors argue that "transcriptional control of erythroid and megakaryocytic fates is less understood" relative to that of myeloid and lymphoid lineages, and set up the problem as if very

little work has been done on this issue. This could not be further more the truth, as extensive work has investigated the underlying mechanisms. Almost none of the mechanistic literature, a component of which is very important for understanding the in vivo observations, was considered in this manuscript.

2) While certain observations emerged from the analysis, which might be of interest to the specialist, one can argue that the study did not define new mechanisms nor did it significantly advance existing knowledge on GATA-1 and FOG-1 mechanisms. For example, a major conclusion was that FOG-1 and GATA-1 are required for formation of bipotent MK/E progenitors and their erythroid commitment. Again, while this conclusion might be of interest to the specialist, this finding would not particularly surprise experts in the field, which is not necessarily inconsistent with existing concepts in the field. Perhaps this would be better appreciated by the readership of a specialist hematology journal.

3) The authors argue that FOG-1 has an activity distinct from GATA-1, specifically to mediates transcriptional MK/E programming of HSCs. However, they largely disregard established knowledge that FOG-1 colocalizes with GATA-2 at many chromatin sites, FOG-1 can mediate certain GATA-2 actions in vivo, and FOG-1 is required for GATA-1 to repress the GATA-2 gene and a large cohort of other genes. Careful consideration of the respective extensive body of literature would almost certainly provide mechanistic insights to help explain the observational results presented in this manuscript.

Referee #3

Mancini et al. Review

Nerlov and colleagues have revisited the concept of lineage commitment, now analyzing the roles of transcription factors known to play a role in myeloerythroid fate decisions within the latest version of the hematopoietic tree. Updating the traditional CMP/MEP/GMP canon with more precise prospective isolation tools, this paper refines the nodes of lineage commitment and shows that FOG-1 is key for the generation of all Mk/E committed precursors, and GATA-1 key for the subsequent specification of E committed cell types. Overall, I found the data in the paper convincing, and conclusions well-supported by the experimental findings. I have no major critiques of the work.

Minor comments:

- Minor grammatical errors are present throughout the text. Revision by a native English speaker would thus be advised.

- Figure 1A: There exists a phenotypic shift in the CD150 x CD41 plot in the C/EBPcKO plot. Compared to WT controls, an overall shift to the right along the CD41 axis makes the apparent increase in MkPs unconvincing. If there is really a 4-fold increase in MkP activity, this should read out in increased Mk-forming ability in vitro if colony number is normalized to cell number input. This should be performed to confirm the phenotypic increase.

- This brings up another issue, which I think should be discussed in the context of the current work: The possibility that loss of transcription factor function may change expression of the cell surface receptors used to identify and isolate the precursor populations of interest. This is an important issue, and rarely discussed in these types of lineage commitment papers.

1st Revision - authors' response 02 September 2011

Referee #1

This manuscript by Mancini et al. present data supportive of the hypothesis that factors mediating Mk/E lineage commitment act antagonistically to C/EBPs (GM lineage commitment). Conditional knockout approaches are used and the animals analysed by FACS and hematopoietic progenitor

assays. The results are interesting and provide new data in the field of hematopoietic lineage specification and differentiation.

Specific comments
In CEBPs cKO, why is the percentage of CFU-E decreased? This seems inconsistent with the model. Can the authors please explain?

A valid point. One possibility is that C/EBPs play a role in regulating erythroid differentiation, and that progression of CFU-Es is accelerated, leading to an apparent decrease in progenitors. Alternatively, the deletion of C/EBPs, since it is systemic, affects a stromal component involved in progression of erythropoiesis. It is very difficult to distinguish between these possibilities based on the available evidence, which makes it hard to discuss the issue coherently. Addressing this in an accurate manner would be time-consuming, and given that lineage commitment (as measured by the earliest erythroid progenitors, preCFU-Es, which are the progenitors mentioned in the model Figure) is unaffected, the results of further investigation are unlikely to impinge in any significant way on the conclusions of the manuscript.

The lack of erythroid colony formation is shown in figure 3. Was the Meg potential of the sorted preMegEs tested at the same time? Please include this functional data.

Megakaryopoiesis in the absence of GATA-1 has previously been extensively characterized, including Mk colony forming assays (Guiterrez, 2008). Given that we see no loss of platelets after *Gata1* deletion it did not seem relevant to include this particular experiment, since it would essentially only confirm an already published observation. The above paper observes a mild decrease in platelet counts 3-4 weeks after *Gata1* deletion, consistent with a mild defect in platelet production in the absence of GATA-1; however, nothing suggest that Mk differentiation if fundamentally affected. We have now added a more explicit reference to these published observations to the text.

The rationale of the transplantation (non-competitive) experiment is unclear. Why was the 1 month post-transplantation time point chosen? This would not allow testing of the stem cell or most immature progenitor population. What happens if the poly I-C treatment was performed 4 months post-transplantation? Is there a delay in lethality?

Analysis (i.e. polyIC injection) at 1 month post-reconstitution is the standard procedure which allows full hematopoietic reconstitution, and there are no issues that we are aware of regarding stem cell/progenitor phenotyping using this time point for initiation of polyIC injection. We therefore have no reason to believe that waiting until 4 month post-transplantation before polyIC injection would affect the outcome of this experiment. In addition, given the time frame of the revision, it is difficult to experimentally test this.

Referee #2

The manuscript by Mancini et al. describes an analysis of the function of GATA-1 and FOG-1 in the context of controlling hematopoiesis in the mouse. The investigators used a conditional knockout approach to assess factor requirements at specific stages of hematopoiesis, with a major focus on whether factors are required for commitment and specification of cell lineages. Overall, I found the work to be technically sound. However, major problems/limitations exist with the study. This is especially important, considering the goal of presenting work that illustrates important biological and/or mechanistic advances.

Specific Comments:
1) The authors argue that "transcriptional control of erythroid and megakaryocytic fates is less understood" relative to that of myeloid and lymphoid lineages, and set up the problem as if very little work has been done on this issue. This could not be further more the truth, as extensive work has investigated the underlying mechanisms. Almost none of the mechanistic literature, a component of which is very important for understanding the in vivo observations, was considered in this manuscript.

We do not aim to ignore the considerable amount of work done regarding the megakaryocytic/erythroid bifurcation. Several aspects of this work was mentioned in the introduction, and further elaborated on in the Discussion. However, we believe it is fair to state that this work has not been conclusive to date. This is to a large extent due to the fact that the relevant early committed progenitors (MkP, preCFU-E) have only recently been identified. However, it is also fair to say that thus far a clear answer has not emerged as to which are the critical regulators. For example, Fli-1 has been proposed as a key promoter of megakaryopoiesis, including Mk lineage commitment, with its antagonistic relationship with the erythroid transcription factor Klf1/Eklf (Frontelo, 2007) as the proposed mechanism. The most recent analysis of Fli1 deficient hematopoiesis (Starck, 2010; not previously cited since just published, but added in revision) clearly demonstrates that Fli1-deficient megakaryopoiesis is impaired; however, there is no demonstration that this is a lineage commitment defect. In fact, the suggested principal mechanism is defective megakaryocyte maturation. Notably, the progenitor phenotyping scheme used does not identify the early commitment steps discussed above, which makes definitive conclusions difficult. Similar arguments can be made regarding the Myb knockdown, which increases the number of CD41 Mk lineage cell in the bone marrow, with a concomitant decrease in CFU-Es (Emambokus, 2003). Also here the observation is a quantitative shift, not a block in erythropoiesis, and the available evidence does not allow any conclusions regarding lineage commitment, since accurate phenotyping of relevant progenitors was not performed. Similar arguments may be made about studies involving Notch signaling (Mercher, 2008), and miR-150 (Lu, 2008) (cited).

All of this is discussed in the manuscript, and we therefore feel that is rather unfair to say that we have ignored the literature in this area. However, if there are specific papers that the reviewer feels should have been cited in addition to those above we would be happy to include these.

2) While certain observations emerged from the analysis, which might be of interest to the specialist, one can argue that the study did not define new mechanisms nor did it significantly advance existing knowledge on GATA-1 and FOG-1 mechanisms. For example, a major conclusion was that FOG-1 and GATA-1 are required for formation of bipotent MK/E progenitors and their erythroid commitment. Again, while this conclusion might be of interest to the specialist, this finding would not particularly surprise experts in the field, which is not necessarily inconsistent with existing concepts in the field. Perhaps this would be better appreciated by the readership of a specialist hematology journal.

This will always remain a matter of opinion. However, it should be borne in mind that hematopoiesis, and the myelo-erythoid bifurcation in particular, has for decades served as a paradigm for other differentiation hierarchies, and as one of the key systems for mathematical modeling of lineage decisions. This manuscript provides strong evidence that some long-held beliefs regarding these lineage decisions are in need of considerable modification, and would thus arguably have a significant impact well beyond its own field. It can also be mentioned that FOG and C/EBP proteins are very widely expressed in vertebrate tissues, in which they play important roles in lineage specification and differentiation, and regulatory circuits through which they interact may therefore well be relevant beyond the hematopoietic field.

3) The authors argue that FOG-1 has an activity distinct from GATA-1, specifically to mediates transcriptional MK/E programming of HSCs. However, they largely disregard established knowledge that FOG-1 colocalizes with GATA-2 at many chromatin sites, FOG-1 can mediate certain GATA-2 actions in vivo, and FOG-1 is required for GATA-1 to repress the GATA-2 gene and a large cohort of other genes. Careful consideration of the respective extensive body of literature would almost certainly provide mechanistic insights to help explain the observational results presented in this manuscript.

There is little doubt that GATA-2/FOG-1 complexes are highly relevant to the function of FOG-1, and we indeed state (p7) that to our knowledge FOG-1 has no GATA-independent functions. However, we fail to see how this excludes that GATA-1 and FOG-1 have distinct functions in lineage commitment (which we believe is clearly shown in the manuscript). Indeed, the fact that loss of FOG-1 and GATA-1 results in distinct phenotypes is entirely consistent with FOG-1/GATA-2 executing critical aspects of Mk/E lineage commitment, and we now identify Trib2 as a GATA-2/FOG-1 target gene, and a potential mediator of FOG-1–C/EBP antagonism. The ability of GATA-2/FOG-1 to repress *Gata1* is, in our opinion, not of direct relevance to the manuscript, as it is

important for erythroid maturation where GATA-1 levels are very high; in most other cell types in the hematopoietic system GATA-1 and GATA-2 seem to be co-expressed, both at more moderate levels.

Referee #3

Mancini et al. Review

Nerlov and colleagues have revisited the concept of lineage commitment, now analyzing the roles of transcription factors known to play a role in myeloerythroid fate decisions within the latest version of the hematopoietic tree. Updating the traditional CMP/MEP/GMP canon with more precise prospective isolation tools, this paper refines the nodes of lineage commitment and shows that FOG-1 is key for the generation of all Mk/E committed precursors, and GATA-1 key for the subsequent specification of E committed cell types. Overall, I found the data in the paper convincing, and conclusions well-supported by the experimental findings. I have no major critiques of the work.

Minor comments:

- *Minor grammatical errors are present throughout the text. Revision by a native English speaker would thus be advised.*

This has been done, hopefully with the desired effect.

- *Figure 1A: There exists a phenotypic shift in the CD150 x CD41 plot in the C/EBPcKO plot. Compared to WT controls, an overall shift to the right along the CD41 axis makes the apparent increase in MkPs unconvincing. If there is really a 4-fold increase in MkP activity, this should read out in increased Mk-forming ability in vitro if colony number is normalized to cell number input. This should be performed to confirm the phenotypic increase.*

We have performed CFU-Mk assays on total bone marrow from the C/EBPcKO mice, and indeed observe an increase in Mk colony formation (new Figure 1C)

- *This brings up another issue, which I think should be discussed in the context of the current work: The possibility that loss of transcription factor function may change expression of the cell surface receptors used to identify and isolate the precursor populations of interest. This is an important issue, and rarely discussed in these types of lineage commitment papers.*

This is a very valid issue, which is why we in the manuscript attempt to back up the progenitor phenotyping with colony forming assays whenever possible. Overall, we observe very good correlation between these readouts. In general, and in the case of FOG-1–deficient preMegEs in particular, this is further supported by analysis of gene expression in the relevant stem- and progenitor cells, which shows correlation between lineage potential and lineage priming.

For myeloid and lymphoid differentiation proposed progenitor defects can usually be backed up by demonstration that the downstream differentiated progeny is absent or reduced. A particular difficulty when dealing with the erythroid and megakaryocytic lineages is that this has so far been very difficult, due to the inability of the standard CD45 allotypic markers to distinguish donor, competitor and recipient erythrocytes and platelets. We have in the meantime devised a strategy for fluorescent identification of platelets and erythrocytes in peripheral blood, which relies on a von Willebrand factor (Vwf)-EGFP BAC transgene and a miR-144/451 EGFP knockin allele. These efficiently EGFP label platelets and erythrocytes, respectively. By using competitor bone marrow carrying these transgenes we could now show that deletion of FOG-1 led to the depletion of both platelets and erythrocytes derived from FOG-1^{cKO} HSCs in a competitive repopulation setting. It is, to our knowledge, the first time such a demonstration has been made technically possible, and these assays could potentially set a new standard for the field.

