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Induction of hematopoietic and endothelial cell program orchestrated by ETS transcription factor ER71/ETV2

Fang Liu, Daofeng Li, Yik Yeung Lawrence Yu, Inyoung Kang, Min-Ji Cha, Ju Young Kim, Changwon Park, Dennis K. Watson, Ting Wang and Kyunghee Choi

Corresponding author: Kyunghee Choi and Ting Wang, Washington University School of Medicine

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Esther Schnapp

1st Editorial Decision 07 January 2015

Thank you for your patience while your manuscript was peer-reviewed for EMBO reports. We have now received the enclosed set of referee reports and also comments from an advisor regarding the main concern by referee 2 that the ChIP-Seq experiments should have been performed with an antibody against endogenous ER71/Etv2.

Given that the advisor and both referees 1 and 3 do not agree with this concern (as per their referee cross-comments not included here), we have decided that ChIP-Seq experiments based on endogenous ER71/Etv2 expression are not required for publication of the manuscript here. However, please make it clear in the manuscript text that you have followed an overexpression approach. All other referee comments are relatively minor and should be addressed. It is important that the study is placed better/more accurately into its context, that related, previous findings are acknowledged, that it is made clear what exactly is novel here, and that statistical analyses are performed.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the issue further.

In your case, we can either publish the manuscript as a short report, or as a normal article, as we have decided recently that we will also publish longer articles from now on. For a short report, the revised manuscript text should not exceed 35,000 characters (including spaces and references) and 5 main plus 5 supplementary figures. Shortening of the manuscript text may be made easier by combining the results and discussion section which may help to eliminate some redundancy that is inevitable when discussing the same experiments twice. Commonly used materials and methods can further be moved to the supplementary information, however, please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file.
For a normal article, 7 main figures are fine, but please also reduce the character count as much as possible as suggested above. The reference style further needs to be changed into the numbered EMBO reports style, which will also help to reduce the character count.

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

REFEREE REPORTS:

Referee #1:

The manuscript is focused on the role of the ER71 protein during hematopoiesis and endothelial cell commitment. ER71 is and ets factor that is only transiently expressed during embryogenesis, yet deficient embryos lack blood and blood vessel formation, and do not survive. This has left the question of how it exerts its non-redundant role in this process; as such, the authors have used conditional KO and ChIP-seq strategies. The latter studies were performed with V5 tagged-ER71 ES cells but utilized both anti-V5 and anti ER71 antibodies, with the focus on their overlap. The underlying motif was present in 85% of the peaks and represented an apparent ETS binding site, and was present in both promoters and distal enhancers including introns. This data was overlapped with expression data, enriching for targets important for hematopoietic and endothelial cell development and differentiation. These sites were found to be methylated in ES cells, but significantly hypomethylated in bone marrow. VEGF and notch signaling pathways were enriched, as well as co-occurrence of ETS, GATA, and E-box binding. Multiple components of the VEGF pathway that show binding by ER71 were verified and also checked for responsiveness to ER71 by luciferease reporter assays. IN addition, a reciprocal relationship was found between gain- and loss- of function for these targets. Importantly, the overlap with Flk1 suggests its signaling is required. The relationship to the hemagioblast took advantage of the surface property of Flk1+Pdgfra-; this population is missing in the ER71 embryo. Conditional deletion of ER71 after Flk1 expression shows it is still required, as hematopoiesis and vascular defects remain. Among validated ER71 target genes are multiple ets factors, including ER71 itself. A careful analysis of the kinetics of their onset shows that ER71 precedes their expression, and altering Fli1 (the downstream target) had no effect. Interestingly, Fli1-null cells still generate Flk1+Pdgfra- cells, although its overexpression can skew the results, unlike with Ets1 or Ets2, but less so that ER71, results further supported by experiments with overexpressing combinations of ets factors with Gata2 and Scl. A very nice final study shows that even though ER71 expression is transient its binding site switches to an interaction with Fli1 at later stages.

This is a very complete, carefully done, well-analyzed and clearly presented study. I have no concerns accepting the manuscript as it is. I would only suggest that the abstract include an explicit sum of the important VEGF signaling that is dependent on ER71, and the connection to Flk1+Pdgfra- cells; my point is simply that I don't feel the abstract quite conveys the range in the paper.

Referee #2:

Liu et al report analysis of Etv2 binding patterns in transgenic ES cells overexpressing Etv2 protein. These new datasets are combined with some analysis of Etv2 function during early haemangioblast development, much of which confirms previous studies. Etv2 is a major regulator of early mesoderm diversification, and as such identification of its target genes is of significant interest to the field. The current paper however falls short in terms of providing definitive new answers, because too much of the data depends on ectopic overexpression systems.
Specific comments

1) The authors use Er71 throughout their paper, when the accepted and official gene name is Etv2. EMBO Reports should not permit the use of inofficial gene nomenclature. It just perpetuates confusion within the field.

2) All the CHIP-Seq data come from inducible overexpression systems. It is well established that expression levels are critical determinants of binding profiles. We therefore cannot be certain at all to what extent the binding profiles described correspond to endogenous Etv2 binding (with the exception of the few regions validated by qRT-PCR of endogenous Etv2 ChIP in Figure S3A). For a journal like EMBO Reports, I would expect the authors to sequence this sample, and present ChIP-Seq of the actual endogenous Etv2. I understand that some optimisation may be required because the levels of enrichment are lower, but they seem close to what is needed for CHIP-Seq. According to the legend of Figure S3A, the ChIP was done without enriching for appropriate cell types. That alone should be enough to get enrichment levels good enough for sequencing the sample.

3) Page 5: The authors refer to the Ismailoglu 2008 paper as "we previously described", when there doesn't seem to be any authors shared between this paper and the current manuscript. Also, the 2008 paper is about Scl/Tal1, not Etv2?

4) ChIP-Seq peaks: I feel it is not satisfactory in terms of advancing the field to just throw away more than half of the peaks for each of the V5 and polyAB samples. Some detailed analysis of this needs to be provided before we can just accept that the intersect peak set is indeed a useful peak set. In any case, this all reinforces my view that the authors should really focus on reporting the ChIP-Seq for the endogenous Etv2 (see my point 2).

5) The authors seem to really like using the word "significant", but rarely use it in the context of an actual statistical test (p-values commonly missing in figures). This is done throughout the text, and also description of figures.

6) The authors need to be much clearer that the hierarchy of Etv2 upstream of Fli1 has been reported before (by the Nishikawa and Kouskoff groups). This weakens the novelty of this current submission.

7) Figure 7: Control experiments with just Gata2 and Scl/Tal1 are missing

8) Discussion: Etv2 has already been used successfully in reprogramming studies. It should therefore not be discussed as a hypothetical option, but instead credit should be given to the groups who have already published on this.

9) Figure 2D: The authors should clarify if these peaks occur on previously identified regulatory regions, and cite relevant papers where appropriate. If so, it would strengthen their case that Etv2 binding contributes to the activity of validated elements.

10) Figure 3B: There are 17 dots in the figure, but apparently only 15 regions in Figure 3A???

11) Figure S3C: The authors make the point in the text that the main target population for Etv2 function is Flk1+, but this figure also shows big effects in the Flk1+/Pdgfra+ population? Although these do not appear to have been quantified by the authors (why not??).

Minor Comments:
1) A recent BLOOD paper suggested distinct developmental origins for the 1st wave of blood cells and endothelium (e.g. not derived from a bipotential haemangioblast). The authors should mention in their introduction that there is an emerging opinion in the field which suggests that the primitive red blood cells in the yolk sac are not developmentally related to yolk sac endothelium.

2) Figure 2C is hard to read

3) Figure 6B: What does the Y-axis show (relative to what???)

4) Figure S1: Axes need to be explained better

5) Figure S3A: explain in figure legend what numbers 1-15 mean

Referee #3:

This manuscript by Liu et al., describes the comprehensive identification of ER71-binding regions in Flk1-positive mesoderm from differentiating embryoid bodies. They demonstrate that Flk1, Flt1, Nrp1 and other VEGF signaling pathway genes are regulated by ER71, based on microarray and transient reporter analysis. The authors find ER71-binding motifs are localized in close relationship to GATA2 and SCL-binding motifs. The authors also describe that ER71 is the earliest Ets factor and regulates directly other Ets genes including Fli1, Ets1, Erg and Ets2, and that there is a Ets switching, based on ChIP assay, qRT-PCR and transient reporter assay. Although overall, this paper is well written and bring new insights into hematopoietic and endothelial cell biology field, the reviewer has a comment as below.

Minor comment.

1) Figure 4; CD31 staining of Flk1-Cre;ER71 CKO at E8.5 and E9.5 should be presented to show the early phenotype. Ideally, Tie2-Cre or VE-cadherin-Cre would be better to determine if ER71 is still required once hematopoietic and endothelial progenitors are formed.

1st Revision - authors' response 09 February 2015

We report three major findings in this paper. One, we mapped ETV2 enhancer regions genome-widely, which revealed that ETV2 induces key genes regulating hematopoietic and endothelial cell lineage specification. Two, we established that FLK1^{high}PDGFRa- cell population, enriched for hemangiogenic progenitors, is exclusively sensitive to ETV2. As such, ETV2 dependent VEGFR2 signaling is required for optimal levels of the hematopoietic and endothelial cell progenitor formation. Three, ETV2 induces other Ets genes, thereby ETV2 establishes an ETS hierarchy. Importantly, ETV2 is replaced by other ETS factors, which maintain hematopoietic and endothelial cell gene expression. These findings collectively provide a comprehensive understanding of how hematopoietic and endothelial cell program is established. Sections containing changes are indicated in blue.

Reviewer 1
This is a very complete, carefully done, well-analyzed and clearly presented study. I have no concerns accepting the manuscript as it is.

We would like to thank this reviewer for recognizing the completeness of the work. We believe this work will be valuable for researchers in many different areas including hematopoietic, vascular and cardiovascular development, stem cell field and regenerative medicine.

I would only suggest that the abstract include an explicit sum of the important VEGF signaling that is dependent on ER71, and the connection to Flk1+Pdgfra- cells; my point is simply that I don't feel the abstract quite conveys the range in the paper.

The abstract is modified to reflect the scope of the findings. Especially, we now emphasize the importance of the VEGF signaling in the context of ETV2.

Reviewer 3
This paper is well written and bring new insights into hematopoietic and endothelial cell biology field.

We would like to thank this reviewer for recognizing the importance of the work. As mentioned, we hope this work will be instrumental to the folks in hematopoietic and cardiovascular research, stem and regenerative medicine.

Minor comment.
1) Figure 4; CD31 staining of Flk1-Cre;ER71 CKO at E8.5 and E9.5 should be presented to show the early phenotype. Ideally, Tie2-Cre or VE-cadherin-Cre would be better to determine if ER71 is still required once hematopoietic and endothelial progenitors are formed.

As discussed in the original submission, Kataoka et al. (2013) [1] have reported that Etv2 deletion using Tie2-Cre leads to normal development. They also showed in that same paper that Tamoxifen injection from E9.5 in ROSACreER;*Er71*$^{f/f}$ mice also leads to normal development. We have independent studies showing that Tie2-Cre;Etv2$^{f/f}$ or VEcadherin-Cre; Etv2$^{f/f}$ mice are born alive (unpublished data). These studies would argue that Etv2 is no longer required once hematopoietic and endothelial cell progenitors are formed.

As suggested, we subjected E9.5 yolk sac Flk1Cre;Etv2 CKO embryos to CD31 analysis (Figure S3E). Consistent with CD31 wholemount staining data of the yolk sac shown in Figure 4D, we observed CD31 expressing cells in the CKO yolk sacs at E9.5. However, the frequency of CD31+ cells seems reduced from the CKO animals compared to that of the controls (controls, 9.3 and 10% vs CKOs, 7 and 8.5%). However, we cannot make an argument that it is statistically significant as the number of animals analyzed is only two for each genotype.

Reviewer 2
Etv2 is a major regulator of early mesoderm diversification, and as such identification of its target genes is of significant interest to the field. The current paper however falls short in terms of providing definitive new answers, because too much of the data depends on ectopic overexpression systems.

We would like to thank this reviewer for acknowledging the significance of ETV2 target gene identification and characterization. We do indeed agree with this reviewer that our approach using overexpression system might have some limitations. However, previous studies have demonstrated that ectopic Etv2 expression in irrelevant cell types using transgenic system did not lead to aberrant phenotype, suggesting that ETV2 would not activate irrelevant genes even when overexpressed [2]. Moreover, in microarray studies there is a great overlap in global gene expression changes between genes upregulated or downregulated by *Etv2* overexpression vs. *Etv2* knockout system [3]. Importantly, ChIP-Seq validation using control wild type EB cells (no *Etv2* overexpression) and ETV2 polyclonal antibodies confirmed the overall validity of the ChIP-Seq peaks identified from the overexpression system (Figure 7E, Figure S3A). To warrant that the target genes that we identified represent authentic targets, we strictly focused on the gene list that also showed greatest changes in gene expression pattern in overexpression as well as knockout system. From these studies, ETV2 targets identified from these studies will most likely to be real endogenous targets.

1) The authors use Er71 throughout their paper, when the accepted and official gene name is Etv2. EMBO Reports should not permit the use of inofficial gene nomenclature. It just perpetuates confusion within the field.

ETV2 is used to indicate human gene, etsrp for zebrafish and ER71 or ETV2 is interchangeably

used to indicate mouse gene. It is not clear whether the official nomenclature of the mouse gene is ETV2. However, to make it simple, we changed the nomenclature from ER71 to ETV2.

2) All the CHIP-Seq data come from inducible overexpression systems. It is well established that expression levels are critical determinants of binding profiles. We therefore cannot be certain at all to what extent the binding profiles described correspond to endogenous Etv2 binding (with the exception of the few regions validated by qRT-PCR of endogenous Etv2 ChIP in Figure S3A). For a journal like EMBO Reports, I would expect the authors to sequence this sample, and present ChIP-Seq of the actual endogenous Etv2. I understand that some optimisation may be required because the levels of enrichment are lower, but they seem close to what is needed for CHIP-Seq. According to the legend of Figure S3A, the ChIP was done without enriching for appropriate cell types. That alone should be enough to get enrichment levels good enough for sequencing the sample.

Naturally, we attempted to perform ChIP-Seq using non-overexpression system. However, using the whole EB cells for ChIP-Seq studies have proven to be rather challenging, as we found that Etv2 expression was low and the Etv2 expressing cell population represented a minor cell population. Thus, the quality of our ChIP-Seq data was not impressive, as the signal to the noise ratio was not that distinct. Therefore, as discussed earlier, validations were performed using control EB cells and endogenous ETV2 antibody (Figure 7E and Figure 3SA). These include Lmo2, Gata2, Scl/Tal1, Tek/Tie2 and Cdh5/Vecadherin, Flk1/Kdr, Flt1, Nrp1 and Nrp2. Hopefully in the future we will be able to isolate ETV2 expressing cell population and perform genome-wide target gene identification to further advance the field of ETV2 biology in relationship to hematopoietic and vascular development.

3) Page 5: The authors refer to the Ismailoglu 2008 paper as "we previously described", when there doesn't seem to be any authors shared between this paper and the current manuscript. Also, the 2008 paper is about Scl/Tal1, not Etv2?

Thanks for catching our mistake. The Ismailoglu (2008) [4] paper is a reference for the A2 ES cells. We now have Lee et al. (2008)[5] and Liu et al., (2012)[3] papers as correct references of the inducible Etv2 ES system.

4) ChIP-Seq peaks: I feel it is not satisfactory in terms of advancing the field to just throw away more than half of the peaks for each of the V5 and polyAB samples. Some detailed analysis of this needs to be provided before we can just accept that the intersect peak set is indeed a useful peak set. In any case, this all reinforces my view that the authors should really focus on reporting the ChIP-Seq for the endogenous Etv2 (see my point 2).

We thank the reviewer for keeping a high standard for reporting ChIP-seq peaks. As we discussed in response to point 2, technical challenges still exist to profile endogenous Etv2 binding. An over-expression system has the advantage of increasing the sensitivity of our ChIP-seq experiment, but has the disadvantage of higher likelihood of false positives. An effective strategy to reduce false positives without compromising the high sensitivity of our assay is to use well designed biological replicates – which is precisely what our experimental strategy was designed to do. The intersection between V5 and polyAB represents a list of binding peaks with higher confidence. Indeed, peaks that belong to this intersection are 40% more likely to associate with genes for which we also have gene expression support, than peaks that are either unique to V5 or to polyAB, see attached table. This data suggests that the intersection represents a collection of peaks of higher confidence. However, we recognize the reviewer's insight that peaks unique to V5 or polyAB may also contain useful biological information. Thus, we have provided genomic locations of these peaks in Supplementary Table S1.

	Number of peaks	Number of associated genes	Number of genes with expression support	Fold increase
Peaks unique to polyAb and V5	12045	9941	670	
Peaks shared by polyAb and V5	3933	4580	425	**1.38**

5) The authors seem to really like using the word "significant", but rarely use it in the context of an actual statistical test (p-values commonly missing in figures). This is done throughout the text, and also description of figures.

Taking to heart what the reviewer has suggested, we have now used “significantly” when the statement is statistically supported (page 6, line 4; page 7, line 8; page 9, line 10). Otherwise, we have changed “statistically” to various synonyms to avoid the confusion.

6) The authors need to be much clearer that the hierarchy of Etv2 upstream of Fli1 has been reported before (by the Nishikawa and Kouskoff groups). This weakens the novelty of this current submission.

We have already acknowledged and cited these two papers In the original submission, recognizing that Fli1 was a previously identified ETV2 target. However, these two studies are limited to only showing that ETV2 can activate Fli1 expression. Thus, the scope of the studies showing the relationship between ETV2 and Fli1 was rather narrow. In comparison, the novel finding of our work is that many Ets genes are direct ETV2 targets. Fli1 is one of the Ets genes that are activated by ETV2. Additional novel finding of our study is that while ETV2 is necessary and sufficient, Fli1 is sufficient but not necessary, as we show that Fli1 deficient embryos still express similar levels of Etv2 as wild type embryos (Figure 6E) and still generate FLK1^{high}PDGFRa- hemangiogenic progenitors (Figure 7A). These studies demonstrate a functional hierarchy between ETV2 and FLI1. Another novel finding is that ETV2 is replaced by FLI1 on the target sites once *Etv2* expression is no longer detected, thereby maintain the continuous target gene expression in blood and endothelial cells. Collectively, these studies demonstrate how ETV2 coordinates ETS expression and function to establish and maintain hematopoietic and endothelial cell development.

7) Figure 7: Control experiments with just Gata2 and Scl/Tal1 are missing

The hemangiogenic progenitors, i.e hematopoietic and endothelial cell progenitors, are sensitive only to ETV2. We previously reported the cell phenotype of Etv2-/-, Gata2-/-, Scl-/-, inducible Etv2, Gata2 and Scl ES cells (Liu et al., 2013)[6], which showed that ETV2 is a determining factor for the generation of the FLK1^{+}PDGFRa- cell population. Specifically, *Etv2* alone, not Gata2 or Scl, could skew mesoderm into hemangiogenic progenitors. When coexpressed with *Gata2* and *Scl*, Etv2 skewing potential was even more strenghtened [6]. The goal of the experiment shown in Figure 7 was to assess different ETS factors for their ability to skew mesoderm to hemagniogenic progenitors compared to that of Etv2 in the context of Gata2 and Scl coexpression. Thus, comparison of the skewing effect compared to Gata2 and Scl may not provide relevant information.

8) Discussion: Etv2 has already been used successfully in reprogramming studies. It should therefore not be discussed as a hypothetical option, but instead credit should be given to the groups who have already published on this.

We modified the discussion as suggested.

9) Figure 2D: The authors should clarify if these peaks occur on previously identified regulatory regions, and cite relevant papers where appropriate. If so, it would strengthen their case that Etv2 binding contributes to the activity of validated elements.

While some of the previously published ETV2 regulatory regions overlap with the ETV2 ChIP-Seq data, some don’t. As for the overlapping sites, our current study confirmed ETV2 binding to the regulatory region of Cdh5 that was published in Blood (2012)[3]. On the other hand, Kataoka H. et al reported ETV2 binding on the 3’ enhancer of Scl (+197534) (2011, Blood)[8]. Wareing S. et al have reported that ETV2 was enriched at the -4.11 and -3.78kb from the exon 1 of Scl (2012, Stem Cells)[9]. Our current study revealed novel and more precise regulatory regions on Scl, which include the 5’ enhancer of -12.6 and -9.6kb from TSS site. Moreover, Koyano-Nakagawa N. et al have published that the +1kb enhancer of Lmo2 was bound by ETV2 [10]. Here, we have identified a different region (-178b from TSS site) of Lmo2 and confirmed the binding the ETV2 on this site.

As these previous studies have relied on sequence conservation analysis, it is possible that some of the sites identified may not be occupied by ETV2 in the chromatin context. However, one important

consideration is that ETV2 core consensus binding motif is similar to that of FLI1 and other ETS factors (Figure 1C, [7]). Potentially, although ETV2 can bind to these non-overlapping sites in vitro, these non-overlapping sites could be recognized by different ETS factors in the genome, such as FLI1.

10) Figure 3B: There are 17 dots in the figure, but apparently only 15 regions in Figure 3A???

We corrected the Figure 3B.

11) Figure S3C: The authors make the point in the text that the main target population for Etv2 function is Flk1+, but this figure also shows big effects in the Flk1+/Pdgfra+ population? Although these do not appear to have been quantified by the authors (why not??).

We previously reported that FLK1+PDGFRa+ cells, enriched for cardiac potential, were generated at a higher level from the ETV2-/- ES cells, compared to wild type controls [3].
While FLK1+PDGFRa+ cells also appear to be increased in the Etv2 KO embryos, similar to Etv2-/- ES cells, we did not make a strong case of this, as we did not assess whether cardiac potential of these cells also increased. Thus, we simply focused on the fact that FLK1+PDGFa- cell population was directly relevant to ETV2 and FLK1 signaling. Future studies are warranted to further characterize FLK1+PDGFa+ cells from the embryo in the context of ETV2 expression.

Minor Comments:
1) A recent BLOOD paper suggested distinct developmental origins for the 1st wave of blood cells and endothelium (e.g. not derived from a bipotential haemangioblast). The authors should mention in their introduction that there is an emerging opinion in the field, which suggests that the primitive red blood cells in the yolk sac are not developmentally related to yolk sac endothelium.

We included this new paper in the revised manuscript.

2) Figure 2C is hard to read

We modified the figure and hopefully the figure is easy to read.

3) Figure 6B: What does the Y-axis show (relative to what???)

Genes were normalized against *Gapdh*, and then the ration of the gene quantity (+DOX) to gene quantity (-DOX) was determined to yield fold change shown on the Y-axis.

4) Figure S1: Axes need to be explained better.

We modified the Figure S1 to be clear.

5) Figure S3A: explain in figure legend what numbers 1-15 mean.

We modified the figure legend to include the description of the numbers 1-15.

Reference:

1. Kataoka, H., et al., *Region-specific Etv2 ablation revealed the critical origin of hemogenic capacity from Hox6-positive caudal-lateral primitive mesoderm.* Exp Hematol, 2013. **41**(6): p. 567-581 e9.
2. Hayashi, M., et al., *Endothelialization and altered hematopoiesis by persistent Etv2 expression in mice.* Exp Hematol, 2012. **40**(9): p. 738-750 e11.
3. Liu, F., et al., *ER71 specifies Flk-1+ hemangiogenic mesoderm by inhibiting cardiac mesoderm and Wnt signaling.* Blood, 2012. **119**(14): p. 3295-305.
4. Ismailoglu, I., et al., *Mesodermal patterning activity of SCL.* Exp Hematol, 2008. **36**(12): p. 1593-603.

5. Lee, D., et al., *ER71 acts downstream of BMP, Notch, and Wnt signaling in blood and vessel progenitor specification.* Cell Stem Cell, 2008. **2**(5): p. 497-507.
6. Liu, F., et al., *Enhanced hemangioblast generation and improved vascular repair and regeneration from embryonic stem cells by defined transcription factors.* Stem Cell Reports, 2013. **1**(2): p. 166-82.
7. Wilson, N.K., et al., *Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators.* Cell Stem Cell, 2010. **7**(4): p. 532-44.
8. Kataoka, H., et al., *Etv2/ER71 induces vascular mesoderm from Flk1+PDGFRalpha+ primitive mesoderm.* Blood, 2011. **118**(26): p. 6975-86.
9. Wareing, S., et al., *The Flk1-Cre-mediated deletion of ETV2 defines its narrow temporal requirement during embryonic hematopoietic development.* Stem Cells, 2012. **30**(7): p. 1521-31.
10. Koyano-Nakagawa, N., et al., *Etv2 is expressed in the yolk sac hematopoietic and endothelial progenitors and regulates Lmo2 gene expression.* Stem Cells, 2012. **30**(8): p. 1611-23.

2nd Editorial Decision 23 February 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed comments from referee 1 who was asked to assess it, and s/he supports publication of the manuscript now. Only a few changes need to be made before we can proceed with the official acceptance of your study.

I noticed that not all figure legends define the number of independent experiments performed (n) and the error bars. Can you please add this information to figure legends 3H, 4E and G, 5B and D, 6A, S1E, S2B and S3A? Please also note that n should be used for the number of independent experiments, and not the number of mice per group, which should be given too. In the latter case, it is unclear whether only one or several experiments were performed. Please clearly state in all figure legends whether only one or several experiments were performed.

I would like to suggest a few minor changes to the title and abstract. I think it would be better to mention that ETV2 is a transcription factor in the title, and to include both names ER71 and ETV2 in the title. Is the first sentence of the abstract referring to mouse data only? In this case it would be good to add mouse to the first sentence. The abstract also needs to be written in present tense.

Title: Induction of hematopoietic and endothelial cell program orchestrated by ETS transcription factor ER71/ETV2

Abstract:
The ETS factor ETV2 is essential for the generation of the blood and vascular system, as ETV2 deficiency leads to a complete block in blood and endothelial cell formation and embryonic lethality. However, the ETV2-mediated gene regulatory network and signaling governing hematopoietic and endothelial cell development are poorly understood. Here, we map ETV2 global binding sites and carry out in vitro differentiation of embryonic stem cells, and germ-line and conditional knockout mouse studies to uncover mechanisms involved in the hemangiogenic fate commitment from mesoderm. We show that ETV2 binds to enhancers that specify hematopoietic and endothelial cell lineages. We find that the hemangiogenic progenitor population in the developing embryo can be identified as FLK1highPDGFRα-. Notably, these hemangiogenic progenitors are exclusively sensitive to ETV2-dependent FLK1 signaling. Importantly, ETV2 turns on other Ets genes, thereby establishing an ETS hierarchy. Consequently, the hematopoietic and endothelial cell program initiated by ETV2 is maintained partly by other ETS factors through an ETS switching mechanism. These findings highlight the molecular and cellular basis established by ETV2 in the regulation of hematopoietic and endothelial cell lineage specification and stability following [OK?] transient ETV2 expression.

Please read the abstract carefully and let me know whether you agree with the changes.

I look forward to seeing a final revised version of your manuscript as soon as possible.

REFEREE REPORT:

Referee #1:

I had enjoyed the first version of the manuscript, and felt its three major findings were a novel and significant contribution, suitable for publication in EMBO Reports. In this revision, the authors have addressed all the comments (as specified by the editor) and have more clearly laid out the results and their significance in the text and the abstract (as I requested). The minor comment of reviewer #3 was also addressed. More critically, all the comments from reviewer #2 have been addressed by clarifications in the text and/or figures, additional references, and supplemental data. The response also offers a direct explanation for the experimental decisions taken by the authors in this study. All of the other (what I consider minor) suggestions have been addressed, and some indeed indicate that the reviewer had missed some points already brought up in the first version of the paper or that were in the references therein.
The bottom line is that I feel the authors have addressed everything requested from the reviewers, and I see no further issues with this very complete, carefully done, well-analyzed and clearly presented study.

2nd Revision - authors' response 24 February 2015

I would like to thank you for the positive evaluation of our manuscript. It has been constructive and productive experience working with you. We modified the title and the abstract per your request. We also modified the figure legends to add more statistics details. Changes in the second revision are marked in red. I hope the manuscript is now acceptable for publication. Thanks again for your support.

Accepted 27 February 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

