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ERG promotes the maintenance of hematopoietic stem cells by restricting their differentiation

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The balance between self-renewal and differentiation is crucial for the maintenance of hematopoietic stem cells (HSCs). Whereas numerous gene regulatory factors have been shown to control HSC self-renewal or drive their differentiation, we have relatively few insights into transcription factors that serve to restrict HSC differentiation. In the present work, we identify ETS (E-twenty-six)-related gene (ERG) as a critical factor protecting HSCs from differentiation. Specifically, loss of Erg accelerates HSC differentiation by >20-fold, thus leading to rapid depletion of immunophenotypic and functional HSCs. Molecularly, we could demonstrate that ERG, in addition to promoting the expression of HSC self-renewal genes, also represses a group of MYC targets, thereby explaining why Erg loss closely mimics Myc overexpression. Consistently, the BET domain inhibitor CPI-203, known to repress Myc expression, confers a partial phenotypic rescue. In summary, ERG plays a critical role in coordinating the balance between self-renewal and differentiation of HSCs.

[Keywords: hematopoietic stem cell; ERG; MYC; differentiation; self-renewal; CPI-203]

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Hematopoietic stem cells (HSCs) are essential for the lifelong production of blood cells, and careful regulation of their numbers is required for the daily replenishment of aged and defective cells. HSCs have therefore developed intricate molecular networks in order to balance precisely their cellular fate choices, including their propensities to maintain quiescence, proliferate, die, and differentiate, all with the purpose of maintaining hematopoietic homeostasis. Importantly, disturbances in any of these fate options may lead to alterations in HSC numbers that may ultimately result in a decline in hematopoietic output or set the stage for malignant transformation.

HSC fate options are controlled by numerous molecular pathways that in turn are affected by signals from the bone marrow (BM) microenvironment (i.e., the HSC niche) as well as intrinsic regulators, including transcription factors (Rossi et al. 2012). Numerous studies in mice have identified factors that support self-renewal, including C/EBPa, c-MYB, and SATb1 (Lieu and Reddy 2009; Will et al. 2013; Hasemann et al. 2014). Other factors, such as DNMT3a, shown to restrict self-renewal, are equally prevalent (Challen et al. 2012). Similarly, several studies have identified factors that appear to promote differentiation, including the well-studied oncogene MYC. Here, the conditional loss of Myc leads to a block in differentiation and accumulation of HSCs, whereas overexpression of Myc promotes HSC differentiation at the expense of self-renewal (Wilson et al. 2004). Finally, epigenetic regulators such as BMI-1 and TET2 have been reported to prevent HSC differentiation. (Iwama et al. 2004; Ko et al. 2011; Moran-Crusio et al. 2011; Quivoron et al. 2011).

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The E-twenty-six (ETS)-related gene (ERG) is a member of the ETS family of transcription factors, of which several, including PU.1, have been shown to play a role in HSC maintenance [Loughran et al. 2008; Ng et al. 2011]. Erg was identified in a sensitized genetic screen as a gene involved in HSC function, and the molecular defect was assigned to a point mutation in the DNA-binding domain of ERG [Loughran et al. 2008]. Erg<sup>Mld2/Mld2</sup> mice i.e., heterozygous carriers of the mutant Erg allele] had slightly reduced numbers of immunophenotypical HSCs that appeared functionally compromised in transplantation experiments. In addition, follow-up work demonstrated that although Erg haploinsufficiency was compatible with lifelong HSC self-renewal, it strongly impaired stress hematopoiesis following exposure to myelotoxic treatment [Ng et al. 2011]. Collectively, these data suggest that ERG could be particularly important during the expansion of HSCs. This notion gained further credibility from studies of the role of ERG during fetal hematopoiesis [Taoudi et al. 2011].

Using Erg<sup>Mld2/Mld2</sup> embryos as a model, ERG was shown to be dispensable for primitive hematopoiesis as well as HSC emergence. In contrast, ERG was found to be essential during the early phases of definitive hematopoiesis, which entail the expansion and maintenance of HSCs. Finally, evidence was provided for the direct ERG-dependent control of Gata2 and Runx1 expression, two well-known players of various aspects of HSC biology [Ichikawa et al. 2004, 2008; Rodrigues et al. 2005].

Although these studies provide ample evidence for a functional role of ERG in HSC biology, they also raise a number of additional questions, especially pertaining to the molecular consequences of the ERG variant. The Mld2 mutation mapped to the DNA-binding domain of ERG; however, it did not interfere with DNA binding per se but instead interfered with the transcriptional activity of the protein. As ETS family members share similar, albeit not identical, DNA recognition motifs, the Mld2 variant could in principle act as dominant-negative by interference with the activity of other ETS family members of known importance in hematopoiesis [Wei et al. 2010]. These issues could be resolved by the development of a conditional Erg knockout allele, which would allow the assessment of ERG function without any confounding impact on the function of other ETS factors.

Several factors known to be important in HSC biology have also been reported to play roles in the maintenance or development of leukemic stem cells. Indeed, we demonstrated recently that the myeloid tumor suppressor C/EBPa is essential for HSC maintenance as well as the initiation of mixed-lineage leukemia (MLL)-rearranged acute myeloid leukemia (AML) [Hasemann et al. 2014; Ohlsson et al. 2014]. Similarly, ERG has also been demonstrated to have an impact on the characteristics of leukemia development/maintenance in a number of settings. ERG is frequently overexpressed in human AML and T-cell acute lymphoblastic leukemia (T-ALL) and is associated with poor outcome in these leukemias [Marcucci et al. 2005, 2007; Baldus et al. 2006]. In mice, ectopic expression of Erg can lead to the development of either T-ALL through the acquisition of Notch1 mutations or a condition resembling acute megakaryocytic leukemia associated with Down syndrome [DS-AML] [Salek-Ardakani et al. 2009; Tsuzuki et al. 2011; Carmichael et al. 2012]. The latter is of particular interest, as ERG is located on chromosome 21 and thus is amplified in individuals with Down syndrome.

Finally, ectopic expression of ERG has been shown to promote the execution of a transcriptional program resembling that of human AML stem cells and progenitors [Goldberg et al. 2013]. However, despite the strong evidence that ERG can promote the onset of several types of human leukemia, no reports have addressed whether ERG is actually required for this process.

Here we set out to characterize the requirement of ERG for HSC function and for the development of leukemia using a newly generated mouse line that allows for the conditional ablation of the DNA-binding domain of ERG. Using this model, we could show that ablation of ERG function promotes the rapid loss of self-renewing HSCs. Moreover, analysis of HSC population dynamics following Erg ablation combined with mathematical modeling demonstrates that ERG is required to prevent HSCs from differentiating prematurely. Interestingly, we could show that this is associated with the up-regulation of an MYC-dependent transcriptional program and that ERG binds to MYC target genes, suggesting that ERG restricts HSC differentiation through repression of MYC activity. Consistently, the phenotype could be partially rescued by pharmacological down-regulation of MYC levels in vivo. In contrast to the pronounced importance of ERG in HSCs, the protein is largely dispensable for both myeloid and lymphoid transformation, pointing to a unique role for ERG in normal stem cell biology.

**Results**

**Conditional deletion of Erg results in a massive loss of BM cells**

In order to investigate the importance of ERG for adult hematopoiesis, we flanked exon 11, encoding the DNA-binding Ets domain, with LoxP sites, thereby generating a mouse line allowing for the functional ablation of ERG [Fig. 1A]. The resultant Erg<sup>fl/fl</sup> animals were subsequently crossed into the Mx1Cre strain, resulting in the generation of Erg<sup>Δ/Δ</sup>; Mx1Cre animals in which ERG function can be efficiently abolished in hematopoietic cells following polyinosinic:polycytidylic acid (pIpC)-mediated activation of the Cre recombinase. Specifically, pIpC injections led to the essentially complete deletion of Erg, as assessed by PCR-based genotyping of BM cells [Fig. 1B] and quantitative RT–PCR (qRT–PCR) analysis of Erg expression in FACS-sorted multipotent progenitor (MPP) cells (LSK CD150<sup>+</sup>CD48<sup>−</sup>) [Fig. 1C].

We first assessed the functional consequences of Erg deletion 2 wk [15 d] and 4 wk following pIpC injection and found a substantial reduction of BM cells in Erg-deleted [Erg<sup>Δ/Δ</sup>] mice versus Erg<sup>fl/fl</sup> littermate controls [Fig. 1D]. Erg is predominantly expressed in immature hematopoietic cells [Supplemental Fig. S1A], and we consistently found the numbers of megakaryocytic, erythroid, and
monocytic/granulocytic progenitors to be severely reduced 2 wk following deletion of Erg (Fig. 1E,F). This translated into a specific depletion of monocytes and granulocytes in both the BM and peripheral blood (PB) (Fig. 1G; Table 1). The reduction of cell numbers was still present at the 4-wk time point, after which partially deleted escaper cells started to repopulate the BM (data not shown).

Erg is required for HSC activity

A hypomorphic variant of ERG has previously been shown to impact negatively on key HSC properties. However, the role of ERG within the adult HSC compartment has not been assessed in the context of a complete functional ablation of ERG. We therefore set out to functionally characterize the stem and progenitor (HSC/hematopoietic progenitor cell [HPC]) compartment in our newly developed conditional Erg knockout model.

BM analysis 2 wk after complete Erg deletion revealed a severe reduction of both immunophenotypic HSCs (Lineage− Sca-1+ c-Kit+ [LSK] CD150+ CD48−) and MPP cells (LSK CD150− CD48+) in ErgΔ/Δ BM, thus demonstrating an essential role for ERG in HSC maintenance (Fig. 2A, B). This effect was intrinsic to the hematopoietic compartment, as Ergfl/fl; Mx1Cre recipients transplanted with wild-type BM cells followed by plpC injection showed no profound hematopoietic defects (Supplemental Fig.

Figure 1. Conditional deletion of Erg results in loss of myeloid cells. (A) Schematic drawing of the conditional Erg knockout mouse model. (B) PCR-based genotyping of BM cells derived from plpC-injected Ergfl/fl;Mx1Cre animals illustrating the floxed [fl] and deleted [Δ] Erg alleles 2 wk after initiating plpC injections. We estimate deletion frequencies of >99%. (C) The expression of Erg mRNA in MPPs derived from ErgΔ/Δ [n = 3] and Ergfl/fl [n = 3] BM 2 wk after initiating plpC injections. The expression level of the full-length Erg transcript is reduced by >100-fold in ErgΔ/Δ MPPs. (D) BM (2× femur, tibia, and ilium) cell numbers in Ergfl/fl [n = 6] and ErgΔ/Δ [n = 6] mice 2 and 4 wk after plpC treatment. (E) The distribution of myeloid progenitors in Ergfl/fl and ErgΔ/Δ mice was analyzed by FACS. (F) Quantification of the data in E. Ergfl/fl, n = 6; ErgΔ/Δ, n = 7. (G) The distribution of mature myeloid cells in Ergfl/fl [n = 6] and ErgΔ/Δ [n = 7] mice was analyzed by FACS. Data are represented as mean ± SD. (** P < 0.05; (*** P < 0.01; (**** P < 0.001; ns not significant. [GMP] Granulocyte–macrophage progenitor; [preGMP] pregranulocyte–macrophage; [CFU-E] erythroid colony-forming unit; [pro-E] proerythroblast; [preCFU-E] pre-erythroid colony-forming unit; [preMeg-E] premegakaryoblast–erythroid progenitor; [MkP] megakaryocyte progenitor. See also Supplemental Figure S1.
ERG is mainly important for truly reconstituting HSCs and MPPs (Supplemental Fig. S3C,D) as well as immunophenotypic HSCs and MPPs (Supplemental Fig. S3E,F). Importantly, we also found that these animals were almost completely reconstituted by wild-type BM, whereas wild-type BM failed to engraft in ErgΔfl/fl recipients under the same conditions [Fig. 2I; Supplemental Fig. S2B]. These data suggest that the ErgΔfl/fl; Mx1Cre model might serve as a useful tool in BM transplantation assays when conditioning by irradiation or drugs is not appropriate. In summary, loss of Erg has dramatic effects on both the numbers and the ability of HSCs to reconstitute the hematopoietic system, and, collectively, our functional analyses demonstrate that ERG is indispensable for HSC maintenance and self-renewal.

HSC migration, homing, and adhesion are not dependent on ERG activity

Having demonstrated the unique importance of ERG for HSC function, we next set out to identify which key properties were affected by its loss. The rapid and massive reduction in ErgΔfl/fl HSCs could in principle be explained by HSC egress from the BM to the PB and/or other hematopoietic organs. To test this, we quantified the numbers of HSCs (CD45+ EPCR+ CD150+ CD48Δ) from ErgΔfl/fl; Mx1Cre model might serve as a useful tool in BM transplantation assays when conditioning by irradiation or drugs is not appropriate. In summary, loss of Erg has dramatic effects on both the numbers and the ability of HSCs to reconstitute the hematopoietic system, and, collectively, our functional analyses demonstrate that ERG is indispensable for HSC maintenance and self-renewal.

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ERG restricts HSC differentiation

ERG maintains quiescence and prevents differentiation of HSCs

Our phenotypic analyses of the HSC compartment demonstrate that ERG is indispensable for HSC maintenance. This was corroborated further by in vitro long-term culture-initiating cell (LTC-IC) assays (a surrogate self-renewal assay) in which the ability of \( \text{Erg}^{Δ/Δ} \) HSCs to form colonies was reduced by 80% (Fig. 4A). To determine whether this reduction was caused by alterations in proliferation, we single-sorted \( \text{Erg}^{fl/fl}; \text{R26-CreER} \) HSCs and assessed their potential for proliferation in vitro following the deletion of \( \text{Erg} \) by 4-hydroxytamoxifen (4-OHT). However, no differences were observed between control and knockout HSCs in terms of either plating efficiency or their ability to divide (Fig. 4B). Consistent with the in vitro data, cell cycle analyses of freshly isolated \( \text{Erg}^{+/−} \) and \( \text{Erg}^{+/+} \) HSCs demonstrated similar amounts of cells in the S/G2/M phases, suggesting that ERG does not affect the proliferative capacity of HSCs per se (Fig. 4D,E).
However, we did observe a marked decrease in the amount of quiescent (G0) HSCs and a compensatory increase in the amount of cells in G1, suggesting that ERG is important for maintaining HSCs in quiescence. As this increase is comparable with what is observed in other mouse lines exhibiting far less dramatic HSC phenotypes (e.g., Cebpa-deficient HSCs), we consider it unlikely that this is responsible for the complete loss of HSCs observed upon ERG ablation [Hasemann et al. 2014]. Significantly, other cellular properties associated with reduced HSC maintenance, such as accumulation of reactive oxygen species (ROS) and cell death, were also not different between Erg+/− and Erg+/+ HSCs [Fig. 4C,D,F].

Exit from quiescence is presumably one of the first steps when HSCs commit to differentiation, and we reasoned that loss of Erg could potentially increase the propensity of HSCs to differentiate, which in turn could explain the dramatic drop in HSC numbers that we observed. As a first step to test this hypothesis, we assessed the myeloid differentiation potential of FACS-sorted HSCs, MPPs, pre-granulocyte/macrophages [preGMs] and granulocyte/macrophage progenitors [GMPs] and found that loss of Erg was entirely compatible with normal myeloid differentiation [Fig. 4G,H]. Next, we rationalized that if Erg−/− HSCs were lost through increased differentiation, this should initially be discernable in the HSC compartment before becoming apparent in the downstream MPP populations. Interestingly, we saw a dramatic decrease in the HSC/MPP ratio 2 wk following deletion of Erg, which was normalized 2 wk later [Fig. 4I]. We next subjected these data to mathematical modeling (see the Supplemental Material). Significantly, the model that best fits the experimental data displays a 25-fold increase in the relative differentiation rate of Erg−/− HSCs [Fig. 4J, Supplemental Fig. S4]. Importantly, the increased differentiation rate in Erg−/− HSCs is very robust and only varies between 17-fold and 34-fold when the relative proliferation rate of Erg−/− to wild-type HSCs is changed from 0.5 to 2.0, thereby demonstrating a remarkable stability of the model over a range of relative proliferation rates [Fig. 4K]. In conclusion, both the experimental data and our mathematical modeling are consistent with a role for ERG in restricting HSC differentiation.

ERG regulates stem cell genes directly

To identify the molecular mechanisms underlying the function of ERG in HSCs, we performed gene expression analysis in Erg−/− and Erg+/+ HSCs [LSK CD150+ CD48−]. We identified 179 and 174 genes that were down-regulated and up-regulated, respectively, by at least 1.5-fold in Erg−/− HSCs compared with controls [Supplemental Table S1], and several of the genes, such as Cxcl12, are known to play a role in HSC biology. Supporting an important role of ERG in HSC maintenance, gene set enrichment analysis (GSEA) demonstrated down-regulation of stem cell signatures [NG_STEM and NG_S_MPP] in Erg−/− HSCs. Similarly, a signature associated with lymphoid–myeloid priming of HSCs [NG_S_MYLY] is also down-regulated in Erg−/− HSCs, whereas a signature associated with erythroid differentiation is up-regulated [MANSION_E], in line with the relative mild erythroid phenotypes in Erg−/− animals [Fig. 5A, Supplemental Fig. S3]. Consistent with the analysis of Erg−/− versus Erg+/+ HSCs, further gene expression analyses in Erg−/− versus Erg+/+ MPPs demonstrated that loss of Erg in the MPP compartment also correlated negatively with HSC gene signatures and positively with an erythroid gene signature [Fig. 5B, Supplemental Table S2]. Finally, we note that a signature representing MYC downstream targets is selectively up-regulated in Erg−/− HSCs [Fig. 5C].

In order to identify direct ERG target genes, we next compared the Erg−/− and Erg+/+ HSC gene expression data set with published ERG ChIP-seq [chromatin immunoprecipitation [ChIP] combined with deep sequencing] data from the HPC-7 progenitor cell line [Wilson et al. 2010]. Sixty-two percent of the down-regulated genes [Supplemental Table S3] and 56% of the up-regulated
Figure 4. Loss of Erg results in differentiation of the HSCs. (A) LTC-IC assay of sorted Erg<sup>fl/fl</sup> [n = 220 HSCs from four mice] and Erg<sup>Δ/Δ</sup> [n = 168 HSCs from six mice] HSCs (Lineage<sup>−</sup> EPCR<sup>−</sup> CD45<sup>−</sup> CD150<sup>−</sup> CD48<sup>−</sup>). Sorted HSCs were cultured on FBMD-1 stromal cells for 5 wk, and LTC-IC capacity was evaluated by counting colonies arising after an additional week of culture in semisolid M3434 medium. (B) Colony-forming ability (seedling efficiency [left panel] and colony size [right panel]) of single-sorted, in vitro excised Erg<sup>fl/fl</sup> [n = 330 HSCs from five mice] and Erg<sup>Δ/Δ</sup> [n = 330 HSCs from six mice] HSCs (Lineage<sup>−</sup> EPCR<sup>−</sup> CD45<sup>−</sup> CD150<sup>−</sup> CD48<sup>−</sup>) cultured in SFEM supplemented with cytokines for 8 d. (C) ROS levels in Erg<sup>−/−</sup> [n = 4] and Erg<sup>−/+</sup> [n = 4] HSCs were measured by CM-H2DCFDA staining followed by FACS analysis. (MFI) Mean fluorescence intensity. (D) FACS analysis of cells stained with Ki67 and DAPI to analyze cell cycle distribution and cell death in Erg<sup>−/−</sup> [n = 10] and Erg<sup>−/+</sup> [n = 13] HSCs. (E) Quantification of the cell cycle data in D. (F) Quantification of the cell death data in D. (G) CFU assays (in M3434 semisolid medium) of sorted stem and progenitor cells from Erg<sup>fl/fl</sup> [HSCs, n = 6; MPPs, n = 2; preGMs, n = 6; GMPs, n = 2], and Erg<sup>Δ/Δ</sup> [HSCs, n = 8; MPPs, n = 2; preGMs, n = 8; GMPs, n = 2]. (H) Genotyping of colonies from CFU assays in G. (Top gel) Erg<sup>fl/fl</sup> (Bottom gel) Erg<sup>Δ/Δ</sup> (I) Numbers of HSCs and MPPs at different time points after pIpC injection were determined by FACS analysis. The graph illustrates HSC/MPP ratios at different time points after pIpC injections in Erg<sup>fl/fl</sup> [No pIpC, n = 4, 2 wk, n = 6, 4 wk, n = 7] and Erg<sup>Δ/Δ</sup> [No pIpC, n = 3, 2 wk, n = 7, 4 wk, n = 5] BM. (J) Mathematical modeling of the data in I showing the changes in HSC and MPP numbers in Erg<sup>Δ/Δ</sup> BM. Please note that the model takes into account that the deletion is <100%, leading to the emergence of escaper cells [HSC Erg<sup>Δ/Δ</sup> and MPP Erg<sup>Δ/Δ</sup>]. The experimental data are depicted by circles. (K) a of the relative differentiation rates of knockout/wild-type HSCs of the best-fitted model, plotted as a function of the relative proliferation rates of knockout/wild-type HSCs. See the Supplemental Material for further details of the analyses. Data are represented as mean ± SD. (“) P < 0.01; (**) P < 0.001; (ns) not significant. See also Supplemental Figure S4.
sets (Supplemental Table S5), suggesting that ERG activates the expression of a group of genes important for HSC function by direct binding to their promoters. Moreover, in line with the GSEA data above, gene ontology analysis of the ERG-bound up-regulated genes identified several MYC target gene sets (Supplemental Table S6), suggesting that ERG might functionally repress the expression of MYC downstream targets in HSCs. This is further supported by motif analysis that, besides identifying the ERG consensus motif and motifs bound by already known coregulators such as RUNX and GATA factors, also identifies the MYC consensus motif in ERG-bound regions (Fig. 5E; Supplemental Table S7). Further analysis of the ERG-bound genes revealed that genes down-regulated upon Erg deletion were enriched for GATA/RUNX motifs in their promoters and frequently constitute genes involved in stem cell function (Supplemental Tables S8, S9). In contrast, ERG-bound genes that were up-regulated upon Erg deletion had increased occurrences of GATA/MYC motifs in their promoters and preferentially constituted MYC target genes. The latter finding is consistent with a model in which ERG represses the expression of MYC target genes by direct binding to their promoters.

Collectively, our bioinformatics analyses suggest that ERG functions as both a transcriptional activator and a repressor in HSCs and coordinately regulates the expression of genes associated with self-renewal and differentiation.
ERG controls MYC-dependent differentiation in HSCs

Our data are compatible with a model in which the main function of ERG is to restrict HSC differentiation, and this potentially could be mediated through repression of a MYC-dependent transcriptional program in HSCs. In other words, loss of ERG would phenotypically mimic overexpression of Myc, which is associated with increased HSC differentiation [Wilson et al. 2004]. Chemicals targeting the bromodomain and extraterminal (BET) family of proteins such as CPI-203 and JQ-1 are known inhibitors of Myc mRNA expression and act through interfering with a superenhancer that controls Myc expression [King et al. 2013; Loven et al. 2013]. Thus, to substantiate our model, we tested whether CPI-203 could rescue the Erg-deficient HSC phenotype.

We first confirmed the activity of this compound in HSCs by culturing sorted LSK CD150+CD48- cells from ErgΔ/Δ and ErgΔ/+ mice in the presence of 0.5 μM CPI-203 for 24 h. As expected, Myc mRNA was reduced by twofold to threefold compared with mock-treated HSCs in both groups, while there was no difference in Myc expression between the ErgΔ/Δ and ErgΔ/+ groups [Fig. 6A]. We next treated ErgΔ/+ and ErgΔ/+; Mx1Cre mice with CPI-203 simultaneously with Erg deletion through plpC injection [Fig. 6B]. Strikingly, CPI-203 restored the numbers of immunophenotypic HSCs (defined as c-Kit+EPCR+CD48−; since Sca-1 expression is not reliable simultaneously with Erg deletion) compared with controls (Fig. 6C). Similarly, CPI-203 rescued the numbers of immunophenotypic ErgΔ/Δ HSCs [LSK CD150+CD48-] to normal levels in ErgΔ/Δ BM while increasing HSC numbers in ErgΔ/+ controls [Fig. 6C]. Interestingly, CPI-203 restored the numbers of immunophenotypic HSCs (defined as c-kit+EPCR+CD150+CD48-) since Sca-1 expression is not reliable shortly after plpC administration) to normal levels in ErgΔ/Δ BM while increasing HSC numbers in ErgΔ/+ controls (Fig. 6C). Similarly, CPI-203 rescued the numbers of immunophenotypic ErgΔ/Δ HSCs [LSK CD150+CD48-] to normal levels and again increased HSC numbers in control animals [Fig. 6D,E]. These experiments clearly demonstrate that a reduction of MYC activity can indeed rescue the effect of Erg deletion on HSC numbers, likely through an anti-differentiation mode of action. Although HSCs are increased above normal levels upon Myc down-regulation in ErgΔ/+ mice, it is interesting to note that the extent of this increase is larger in ErgΔ/Δ mice [2.3-fold] than in the ErgΔ/+ controls [1.5-fold] [Fig. 6E]. In addition, there seems to be a particularly enhancing effect of CPI-203 on the proportion of CD150+CD48- cells within the LSK population in ErgΔ/Δ mice compared with controls [Fig. 6F], pointing toward an exclusively restoring effect of Myc down-regulation in the differentiation-prone Erg-deficient HSCs.

To test whether Myc inhibition would have a similar rescuing effect on HSC function as well, we transplanted BM cells from CPI-203-treated ErgΔ/+ and ErgΔ/Δ mice into lethally irradiated mice along with unmanipulated BM competitor cells [Fig. 6D]. CPI-203 slightly but significantly improves the multilineage engraftment capacity of ErgΔ/+ HSCs compared with mock treatment, demonstrating that Myc down-regulation partly restores HSC function [Fig. 6G,H]. Consistent with its effect on the numbers of immunophenotypic HSCs, Myc down-regulation also increases the engraftment of ErgΔ/+ cells. Chimerism within the HSC compartment 20 wk after transplantation did not vary significantly between the CPI-203-treated and mock-treated groups irrespective of genotypic genotype [Fig. 6I]. This is an anticipated result given that CPI-203 is not administered to the recipients and further verifies the anti-differentiating effect of this drug rather than having effects on other HSC-promoting properties like self-renewal or survival. Such effects would be expected to read out as increased long-term repopulation capacity of the HSC pool. The transplantation experiments therefore confirm that a short period of Myc inhibition in Erg-deficient donor mice is able to prevent differentiation of HSCs and thereby partly rescue the numbers of multilineage reconstituting HSCs.

Finally, we wanted to test whether Myc down-regulation leads to normalization of genes otherwise up-regulated in the Erg-deficient setting. For this, we selected top-scoring genes from the MYC target gene set that were found to be up-regulated upon Erg loss [Supplemental Table S6] and performed qRT–PCR analysis on ErgΔ/Δ and ErgΔ/+ c-Kit+ cells treated with CPI-203 for 24 h in vitro. We verified the up-regulated expression of several MYC target genes in ErgΔ/+ cells [Dkc1, Ccnb1, Cks2, Tfcc, and Pigp], while Myc mRNA itself was unchanged, consistent with our gene expression analysis [Fig. 6J]. Moreover, a subset of the tested MYC target genes was down-regulated in both ErgΔ/Δ and ErgΔ/+ c-Kit+ cells following CPI-203 treatment in vitro [Dkc1, Ccnb1, and Pol2], indicating that these genes are indeed direct targets of MYC and that Erg status can influence their expression levels. Other tested genes were increased in ErgΔ/Δ cells compared with control but were not obviously sensitive to CPI-203 treatment [Cks2, Tfcc, and Pigp] and may therefore not be coregulated by ERG/MYC in this setting [Fig. 6J]. Collectively, these data support a model in which ERG regulates the balance between HSC self-renewal and differentiation through repression of MYC target genes and activation of self-renewal genes [Fig. 6K].

ERG is dispensable for leukemic transformation

It is well established that ERG overexpression or ERG-containing fusion proteins are drivers of malignant transformation in leukemia and solid tumors and that in vitro depletion of ERG in these cases can attenuate cell growth [Marcucci et al. 2005, 2007; Baldus et al. 2006; Salek-Ardakani et al. 2009; Tsuzuki et al. 2011; Carmichael et al. 2012; Rosen et al. 2012]. However, the extent to which the presence of ERG is a general requirement for tumor initiation and maintenance has not been addressed. To address this important issue, we crossed the Erg+ allele into well-established models of murine T-ALL and AML. Initially, we generated ErgΔ/+; CD2iCre mice in which the expression of CRE initiates in common lymphoid progenitors (CLPs), resulting in a pan-leukemic deletion of Erg. BM cells from these animals and ErgΔ/+ controls were then transduced with retrovirus expressing either NOTCH-ICD or NrasG12D and subsequently transplanted into lethally irradiated recipients. These mice developed short latency T-ALLs with accumulation of mainly CD4+CD8+ or CD4lowCD8+ leukemic cells in hematopoietic organs [Fig. 7A–C]. Although the Erg-
deficient leukemias displayed slightly longer latencies compared with controls, the differences were not dramatic—a fact paralleled by the normal T-cell development in Erg
\textsuperscript{fl/fl}; CD2iCre animals (Fig. 7D–F).

To test whether ERG is equally dispensable for myeloid transformation, we transduced BM progenitors from Erg
\textsuperscript{fl/fl}; R26Cre-ER mice with the potent myeloid oncogene MLL-ENL and subjected them to Erg deletion.
with 4-OHT followed by serial replating in colony assays [Fig. 7G,H]. Strikingly, but in line with the results from the T-ALL experiments, this surrogate assay of leukemic stem cell maintenance demonstrated no requirement for the presence of ERG. We therefore conclude that ERG is dispensable for both lymphoid and myeloid transformation. This excludes a general role for ERG in the maintenance of hematopoietic cells with stem-like properties such as leukemic stem cells, thus further highlighting its unique role within the normal HSC compartment.

Discussion

HSCs are endowed with the ability to sustain the lifelong production of hematopoietic cells. Crucial to this process is their ability to regulate the balance between self-renewal and differentiation such that HSC numbers are maintained while at the same time ensuring the sufficient production of mature blood cells.

Here we explored the function of the ETS factor ERG in HSCs. ERG has previously been found to be essential for fetal hematopoiesis and adult stress hematopoiesis in a
manner that appeared to converge at the inability of Erg mutant HSCs to expand in a variety of settings (Loughran et al. 2008; Ng et al. 2011; Taoudi et al. 2011). These earlier studies all used the Erg<sup>Mld2</sup> allele that encodes an ERG variant with a mutation in the DNA-binding ETS domain. Although this mutant is inactive in reporter assays, it still binds DNA, thus raising the concern that it potentially could interfere with the function of other ETS proteins through binding to ETS consensus binding sites. To circumvent this problem, we generated an Erg mutant allele that facilitates the conditional deletion of the entire ETS domain, thus generating an ERG variant with no DNA-binding activity, which in turn allows us to study the specific role of ERG without any potential confounding impact on other ETS factors. Importantly, this model makes it possible to assess the impact of complete functional loss of ERG in an adult setting.

Using this newly developed conditional Erg knockout model, we found that ablation of ERG in adult mice leads to a complete loss of HSCs without any apparent changes in their proliferative or apoptotic behavior. Instead, kinetic analysis of HSCs and their immediate descendants following ERG ablation demonstrated that HSCs were depleted prior to the loss of MPPs. Moreover, mathematical modeling of the data is consistent with a model in which the loss of Erg leads to a >20-fold increase in the differentiation rate of HSCs, implying that ERG serves to restrict the differentiation of HSCs. Thus, our data add considerably to the earlier proposed function of ERG in HSCs, and, to our knowledge, this is the first example of a transcription factor that serves to restrict premature differentiation of HSCs.

Numerous studies have implicated the proto-oncogene Myc in the regulation of self-renewal and differentiation of HSCs. Thus, whereas conditional deletion of Myc in the hematopoietic system leads to a block in differentiation and accumulation of HSCs, overexpression of Myc is associated with HSC depletion and increased differentiation [Wilson et al. 2004]. Importantly, none of these phenotypes was associated with any changes in HSC proliferation and apoptosis, and the Myc overexpression HSC phenotype therefore perfectly mirrors the loss of Erg in HSCs. Furthermore, we found that several previously reported MYC target gene signatures are up-regulated in Erg-deficient HSCs and that these are enriched for genes directly targeted by ERG in the HPC-7 HSC/HPC line. We therefore propose a model in which ERG represses the activity of some MYC target genes, thus restricting the MYC-driven differentiation of HSCs. According to this model, modulation of MYC and/or ERG function or levels would consequently affect the delicate balance of HSC self-renewal versus differentiation [Fig. 6K]. Consistent with this model, we found that inhibition of MYC activity through the BET inhibitor CPI-203 in Erg-deficient mice is able to improve the HSC defect significantly, with stabilization of HSC levels in the BM of both Erg<sup>S/A</sup> and Erg<sup>Y-</sup> mice after only 1 wk of treatment. Similarly, HSCs from CPI-203-treated mice display improved functional properties with increased long-term multilineage engraftment of PB hematopoiesis compared with control, arguing that the balance again has been shifted back in favor of self-renewal. Of note, we do realize that Myc is not the only target of CPI-203 but is clearly one of those responding most efficiently to the drug, and previous studies have indicated that BET inhibitors appear to phenocopy genetic ablation of Myc [King et al. 2013; Loven et al. 2013]. We therefore conclude that most of CPI-203’s effects on HSC properties are due to modulation of MYC activity.

It is interesting to note that Myc repression also has a clear impact on wild-type HSCs where ERG levels are normal. These results indicate that lowering of MYC activity has an HSC-promoting effect independent of ERG status. This is compatible with the observations reported previously for Myc-null HSCs, which exhibit a dramatic block in differentiation. Nevertheless, these observations do not undermine the proposed role of ERG as a major HSC maintenance factor and do not exclude that ERG and MYC coregulate differentiation genes. Instead, we show that MYC targets that were up-regulated in Erg-deficient HSCs could be lowered upon Myc down-regulation, clearly arguing for a situation in which the combination of ERG and MYC controls the expression levels of certain differentiation-promoting genes.

Finally, in addition to its role as a modulator of MYC-induced gene expression, our gene expression analysis also demonstrated that ERG induces the expression of several genes previously identified as HSC self-renewal factors. ERG therefore emerges as a key HSC regulator, as it is involved in governing several aspects of HSC homeostasis. Our work also offers novel insights into differences in the wiring of self-renewal/differentiation pathways between HSCs and leukemic cells. Thus, in contrast to the crucial role of ERG in the maintenance of normal HSCs, we found that ERG is essentially dispensable for the development of lymphoid leukemia as well as the transformation of myeloid cells into myeloid leukemia. These findings suggest that the leukemic stem cells responsible for the maintenance of both lymphoid and myeloid leukemias are not always dependent on normal self-renewal/survival pathways. High expression of Erg in both T-ALL and AML patients has been associated with an adverse prognosis, and ERG overexpression has been shown to drive the formation of both lymphoid and myeloid leukemias in mice [Marcucci et al. 2005, 2007; Baldus et al. 2006; Salek-Ardakani et al. 2009; Tsuzuki et al. 2011; Carmichael et al. 2012]. The fact that ERG is implicated in tumors of both myeloid and lymphoid origin as well as the data described in the present work suggest a model in which the main role of ERG overexpression in patients is to expand the numbers of HSCs, presumably by restricting their differentiation. This may in turn render HSCs amenable to the acquisition of secondary mutations that ultimately drives leukemic transformation.

In summary, our work demonstrates that ERG plays different roles in normal HSCs and their leukemic counterparts. We propose a model in which ERG serves to restrict HSC differentiation by activating stem cell genes and repressing the expression of MYC target genes, thereby placing ERG at center stage in the regulation of the delicate balance between HSC self-renewal and differentiation.
Material and methods

Flow cytometry

BM (2× femur, tibia, and ilium) and spleens were collected and crushed in PBS and 3% fetal calf serum [FCS] (GE Healthcare). Cell suspensions were stained for mature cells using antibodies against Ter119, Mac-1, Gr-1, B220, CD4, and CD8α (eBioscience); myelo-erythroid progenitor cells using antibodies against lineage (CD3e, B220, Mac-1, and Gr-1), Sca-1, c-Kit, CD105, CD41, FcgRII/III, Ter119 (eBioscience), and CD150 (Biolegend); and HSCs/HPCs using antibodies against either B220, CD3, CD11b, Gr1, Ter119, Sca-1, c-Kit (eBioscience), CD150, and CD48 (Biolegend) [2- and 4-wk time points] or B220, CD3, CD11b, Gr1, Ter119, Sca-1, c-Kit (eBioscience), CD150, and CD48 (Biolegend) [2- and 4-wk time points] or B220, CD3, CD11b, Gr1, Ter119, Sca-1, c-Kit (eBioscience), CD150, and CD48 (Biolegend) [1-wk time point]. For the analysis of transplanted animals, antibody cocktails were supplemented with CD45.1 (eBioscience) and CD45.2 (BD) antibodies. After washing, stained cells were resuspended in PBS and 3% FCS containing 0.2 μg/mL DAPI (Invitrogen) or 1 μg/mL 7AAD (Invitrogen). For stem and progenitor cell purification, c-Kit+ cells were enriched with anti-c-Kit antibodies. After washing, stained cells were resuspended in PBS and 3% FCS containing 0.2 μg/mL DAPI (Invitrogen). For stem and progenitor cell purification, c-Kit+ cells were enriched with anti-c-Kit antibodies. After washing, stained cells were resuspended in PBS and 3% FCS containing 0.2 μg/mL DAPI (Invitrogen) or 1 μg/mL 7AAD (Invitrogen). For stem and progenitor cell purification, c-Kit+ cells were enriched with anti-c-Kit antibodies. After washing, stained cells were resuspended in PBS and 3% FCS containing 0.2 μg/mL DAPI (Invitrogen) or 1 μg/mL 7AAD (Invitrogen).

Transplantation assays

All transplantation assays were performed using the Ly-5 congenic mouse system.

For reciprocal transplantation, 3 × 10^6 Ly5.1 BM cells (CD45.1) were transplanted by tail vein injection into either irradiated (900 cGy) or nonirradiated ErgΔ/Δ or ErgΔ/Δ Ly-5.2 (CD45.2) mice 10 d after initiating pIpC treatment (five injections in total).

For transplantation with nonedited donor cells, 4.5 × 10^6 BM cells from ErgΔ/Δ or ErgΔ/Δ; Mx1Cre (CD45.2) mice were intravenously injected into irradiated (900 cGy) Ly-5.1 [CD45.1] recipients along with 5 × 10^4 CD45.1 competitor cells. Four months after transplantation, the blood chimerism was analyzed, and, 2 wk later, excision of Erg was induced with pIpC injections.

For whole BM competitive repopulation assays, 3 × 10^6 or 3 × 10^6 BM cells from ErgΔ/Δ or ErgΔ/Δ Ly-5.2 (CD45.2) mice [2 wk after excision of Erg] were mixed with 3 × 10^4 competitor BM cells Ly-5.1 [CD45.1] and transplanted by tail vein injection into lethally irradiated (900 cGy) Ly-5.1 [CD45.1] mice. For competitive transplantations of ErgΔ/Δ or ErgΔ/Δ BM, 2.5 × 10^6 + 2.5 × 10^6 and 4.5 × 10^6 + 0.5 × 10^6 cells were injected [1:1 and 10:1 donor:competitor ratio, respectively].

For HSC/MPP competitive repopulation assays, 30 freshly isolated HSCs [LSK CD150^+ or 150 MPPs (LSK CD150^-) from ErgΔ/Δ or ErgΔ/Δ Ly-5.2 (CD45.2)] mice 2 wk after excision of Erg were mixed with 2 × 10^6 Ly-5.1 (CD45.1) competitor BM cells and transplanted by tail vein injection into irradiated [900 cGy] Ly-5.1 (CD45.1) mice.

Gene expression profiling

RNA was purified using the RNeasy microkit (Qiagen) from sorted ErgΔ/Δ and ErgΔ/Δ HSCs [LSK CD150^+ or ErgΔ/Δ and ErgΔ/Δ MPPs (LSK CD150^-)]. The ERG ChIP-seq data were obtained from Wilson et al. (2007). The curated signatures were kindly provided by Susan Moore and Claus Nerlov.

Gene expression profiling

RNA was purified using the RNeasy microkit (Qiagen) from sorted ErgΔ/Δ and ErgΔ/Δ HSCs [LSK CD150^+ or ErgΔ/Δ and ErgΔ/Δ MPPs (LSK CD150^-)] and subjected to microarray [Mouse Gene 1.0 ST GeneChip array, Affymetrix] as described in Hasemann et al. (2014). Microarray data were normalized by RMA [robust multiarray analysis] followed by mean one-step probe set summarization using the Partek genomics suite 6.5 followed by analysis of differential expression between experimental groups (expression fold change >1.5; P < 0.05; q < 0.22, ANOVA F-test). The raw data can be accessed at the Gene Expression Omnibus under accession number GSE69873.

GSEA

GSEA was performed using GSEA version 4.0 (http://www.broadinstitute.org/gsea). Gene sets originated from the MSigDB [http://www.broadinstitute.org/gsea/msigdb] and a list of curated signatures extracted from Venezia et al. (2004), Mansson et al. (2007), and Pronk et al. (2007). The curated signatures were kindly provided by Susan Moore and Claus Nerlov.

ERG ChIP-seq data analysis

The ERG ChIP-seq data were obtained from Wilson et al. (2010), where summits from the union of called peaks between three peak callers were used for further analysis. We defined ERG-binding sites in a window of ±70 bp with respect to the summit positions. Genes from microarray analysis were deemed significantly differentially expressed using a fold change threshold of >1.5 (P < 0.05). Promoter regions defined by TSS ± 800 bp, with sequences acquired from RefSeq mm9 assembly using the longest isoform of differentially expressed genes were overlaid with ERG peaks. Using this union, the significance of overlap was calculated using a permutation test drawing
a similar sample size 10,000 times from random genes on the microarray.

De novo motif discovery among ERG peaks was performed using MEME-chip [Machanick and Bailey 2011]. The frequency tables from DREME [matching against the JASPAR database] [Sandelin et al. 2004; Bailey 2011] was used as input to FIMO [Grant et al. 2011] to find significant occurrences of selected ERG CHIP-seq motifs within sequences of the differentially expressed genes \( P < 0.01 \). Based on counts of these significant occurrences, the upper quartile of differentially expressed genes was overlapped with gene signatures from the MSigDB (C2, C5, and C6; hypergeometric test, \( P < 0.05 \)).

AML and T-ALL analysis

To generate AML on an Erg-deficient background, c-Kit-enriched BM from \( \text{Erg}^{fl/fl} \) and \( \text{Erg}^{G12D} \); R26-CreER mice were transduced with \( \text{MLL-ENL-IRES-GFP} \) [Ohlsson et al. 2014] and transformed through three passages in colony assays [Stem Cell Technologies, M3231] supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 20 ng/mL murine SCF (mSCF) [PeproTech], 10 ng/mL human IL-6 (hIL-6) [PeproTech], 10 ng/mL human GM-CSF, and 10 ng/mL murine IL-3 [PeproTech]. \( \text{Erg}^{fl/fl} \) and \( \text{Erg}^{G12D} \); R26-CreER transformed colonies were subsequently dissociated and passedaged in RPMI 1640 [In-vitrogen] supplemented with 20% FCS, 20% WEHI-conditioned medium, 20 ng/mL mSCF, and 10 ng/mL hIL-6 ± 0.5 μM 4-OHT to allow for excision of Erg. Finally, serial replating assays were performed as described in Ohlsson et al. [2014]. For T-ALL analysis, c-Kit-enriched BM from \( \text{Erg}^{fl/fl} \) and \( \text{Erg}^{G12D} \); Cd2iCre mice was transduced with \( \text{NOTCH-ICD} \) [Chiang et al. 2008] or \( \text{Nras}^{G12D} \) in which luciferase was replaced with Venus [Zuber et al. 2009] and transplanted to irradiated (900 cGy) Ly5.1 recipients (CD45.1). Log rank test was used to analyze differences in survival.

Pharmacological targeting of Myc expression

CPI-203 [DC Chemicals] was dissolved in 0.9% saline solution, 5% DMSO [Sigma-Aldrich], and 10% hydroxypropyl-β-cyclodextrin [Sigma-Aldrich] and administered by intraperitoneal injection twice daily at 5 mg/kg. Mock-treated mice were injected with the corresponding volume of saline/DMSO/hydroxypropyl-β-cyclodextrin solution. Sorted LSK CD150+ CD48 cells were cultured in StemSpan SFEM [Stem Cell Technologies] with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 20 ng/mL mSCF, and 0.5 μM CPI-203 or the corresponding volume of DMSO. C-Kit-enriched cells were cultured in IMDM with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 ng/mL mSCF, and 0.5 μM CPI-203 or the corresponding volume of DMSO. Cells were harvested after 24 h of culture, immediately dissolved in RLT lysis buffer from the RNeasy microkit, and stored at −80°C until further purification.

Statistical analyses

Student two-tailed \( t \)-test was used to test for significance throughout, unless specifically stated.

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