Megakaryocytes and erythroid cells are thought to derive from a common progenitor during hematopoietic differentiation. Although a number of transcriptional regulators are important for this process, they do not explain the bipotential result. We now show by gain- and loss-of-function studies that erythroid Krüppel-like factor (EKLF), a transcription factor whose role in erythroid gene regulation is well established, plays an unexpected directive role in the megakaryocyte lineage. EKLF inhibits the formation of megakaryocytes while at the same time stimulating erythroid differentiation. Quantitative examination of expression during hematopoiesis shows that, unlike genes whose presence is required for establishment of both lineages, EKLF is uniquely down-regulated in megakaryocytes after formation of the megakaryocyte-erythroid progenitor. Expression profiling and molecular analyses support these observations and suggest that megakaryocytic inhibition is achieved, at least in part, by EKLF repression of Fli-1 message levels. (Blood. 2007;110:3871-3880) © 2007 by The American Society of Hematology

Introduction

Hematopoiesis is the process by which a self-renewing population of stem cells provide a continuous replenishment of differentiated blood cells by generating progeny with sequentially altered gene expression patterns. Identification of these cells has relied on selective enrichment by cell-surface markers combined with culture and in vivo cellular assays that enable detection of cells at specific stages of differentiation. Although stem cells are multipotent, individual steps of subsequent differentiative decisions are performed by a series of simpler, even bipotential, decisions whereby one cell type gives rise to 2 or 3 descendants of differing character. This has led to a commonly accepted pattern of parent and progeny relations, although variations of it have recently been suggested (but see Forsberg et al)

A large number of genetic, cellular, and gene expression studies point to the critical importance of cytokine pathways and expression patterns of transcription factors for establishing and maintaining steady state numbers of lymphoid, myeloid, and erythroid cells that, at the same time, can respond quickly to changes in the organismal environment and increase or decrease the cellularity of specific blood cell types.

The megakaryocyte and erythocyte lineages are proposed to derive from a common precursor, the megakaryocyte-erythroid progenitor (MEP) (reviewed in Pang et al). Strikingly, these 2 lineages share a number of commonalities with respect to transcription factors that are absolutely required (eg, GATA1, FOG1, SCL, Gfi-1b). At the same time, the protein partners that form with these factors as differentiation proceeds can be significantly different between lineages. However, because these factors are all positively required for both lineages, we are still left with an incomplete picture of how these lineages are differentially established during hematopoiesis.

Erythroid Krüppel-like factor (EKLF; KLF21) is a zinc finger transcription factor that plays a critical role in erythroid gene expression, with adult β-globin being a particularly well-studied target for activation. EKLF is highly restricted in its expression pattern to hematopoietic organs such as the yolk sac, fetal liver, adult bone marrow, and the red pulp of the spleen. Recent studies have expanded its activation target repertoire to protein-stabilizing, heme biosynthetic pathway, and red cell membrane proteins. However, along with other cellular and molecular studies, they also suggest that there are genes that are repressed by EKLF.

EKLF’s role is absolutely critical for the erythroid lineage, as supported by gene ablation studies, of which the most obvious effect is a profound β-thalassemia that leads to lethality in murine embryos at the time of the switch to adult β-globin expression. Enigmatically, however, EKLF is also expressed in multipotential hematopoietic cell lines and in cultured primary hematopoietic cells. As a result, we sought to determine by both gain- and loss-of-function approaches whether EKLF might be playing a heretofore undiscovered role in hematopoietic lineage decisions. Most unexpectedly, we find that, unlike its cohorts within the erythroid lineage that are also required for megakaryocyte development, EKLF plays a negative role and points to its expression level playing a part in megakaryocyte lineage commitment while remaining critical for erythroid maturation.
Materials and methods

Embryonic stem-cell and embryoid body differentiation

Full-length Flag-tagged EKLF cDNA was inserted into a modified plox vector35 to generate ploxEKLF/IRES GFP. Ainv18 embryonic stem (ES) cells were targeted with ploxEKLF/IRES GFP by coelectroporation of ploxEKLF/IRES GFP and a Cre recombinase expression plasmid followed by selection in G418 to generate the inducible cell line, tetO-EKLF-GFP. Culture of tetO-EKLF-GFP after removal from feeder cells and embryoid body (EB) differentiation precisely followed established protocols.36

Megakaryocyte cultures

The tetO-EKLF-GFP ES cell line was differentiated to day 6 as EBs before disaggregation and plating on irradiated OP9 feeder cells as described.37 OP9 cultures also included 10 to 20 ng/mL thrombopoietin. Doxycycline (0.25-1 μg/mL) was added as required when indicated. Fresh media was added every 3 days for extended cultures (up to 17 days). Liquid cultures from E13.5 fetal livers from wild-type or EKLF-null mice was performed as described.38 Megakaryocyte colony assays used the Megacult system as described.39 Megakaryocyte expansion and differentiation.14-19 These data suggest that EKLF expression negatively affects the megakaryocyte lineage.

Results

EKLF is normally down-regulated during megakaryocyte differentiation

As hematopoietic EKLF expression had been previously monitored only in tissue culture35 and a limited population of primary34 cells, we wanted to obtain a more precise idea of EKLF expression patterns during hematopoiesis. We used well-established criteria for isolation of specific subpopulations of cells emanating from long-term hematopoietic stem cells (defined as the c-Kit+, Thy1.1lo, Lin−, Sca1+, Flt3− population)41. Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis performed on cell-surface marker–sorted samples (Table S1) shows that EKLF is expressed at barely detectable levels in hematopoietic stem cells and multipotent progenitors (Figure 1A). A clear difference in expression subsequently becomes established, with EKLF absent in common lymphoid progenitors and their B- and T-cell progeny, yet increased in the common myeloid progenitor (CMP). At this point there is another clear demarcation in expression within the CMP progeny, because EKLF levels become 5-fold higher in the megakaryocyte/erythroid progenitor (MEP) but decline further in the GMP. EKLF expression within the GMP does not develop any further, because neither monocytes, macrophage, nor dendritic cells express it (Figure 1B). EKLF expression is also much higher in the Flt3− than in the Flt3+ population. These data show that there is a gradual restriction in expression of EKLF as hematopoiesis proceeds, ending with high levels within the MEP population (Figure 1C).

Of particular interest, however, the bipotential differentiation of MEPs leads to a dramatic difference in EKLF expression, with erythroid progenitors exhibiting an 80-fold greater level of expression compared with megakaryocyte progenitors (Figure 1B). This demarcates EKLF as having significantly different properties from GATA1, FOG, Gfi1b, and SCL, transcription factors whose presence are required for both erythropoiesis megakaryocytic expansion and differentiation.14-19 These data show that EKLF is normally down-regulated as MEPs differentiate down the megakaryocytic lineage.

EKLF expression negatively affects the megakaryocyte lineage but stimulates erythroid differentiation

Given the expression pattern of EKLF in hematopoietic cells, we investigated the effects of its misregulation on the normal hematopoietic progression, beginning with gain-of-function studies. To do this we took advantage of the embryoid body (EB) differentiation system, in particular the doxycycline-inducible plasmids and embryonic stem cell lines we had previously used to induce chimeric proteins as desired during differentiation.35,42 For the present experiments, we cloned the complete EKLF vector35 to generate ploxEKLF/IRES GFP. Ainv18 embryonic stem (ES) cells were targeted with ploxEKLF/IRES GFP by coelectroporation of the EKLF expression patterns during hematopoiesis. We used well-established criteria for isolation of specific subpopulations of cells emanating from long-term hematopoietic stem cells (defined as the c-Kit+, Thy1.1lo, Lin−, Sca1+, Flt3− population). Quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis performed on cell-surface marker–sorted samples (Table S1) shows that EKLF is expressed at barely detectable levels in hematopoietic stem cells and multipotent progenitors (Figure 1A). A clear difference in expression subsequently becomes established, with EKLF absent in common lymphoid progenitors and their B- and T-cell progeny, yet increased in the common myeloid progenitor (CMP). At this point there is another clear demarcation in expression within the CMP progeny, because EKLF levels become 5-fold higher in the megakaryocyte/erythroid progenitor (MEP) but decline further in the GMP. EKLF expression within the GMP does not develop any further, because neither monocytes, macrophage, nor dendritic cells express it (Figure 1B). EKLF expression is also much higher in the Flt3− than in the Flt3+ population. These data show that there is a gradual restriction in expression of EKLF as hematopoiesis proceeds, ending with high levels within the MEP population (Figure 1C).
cDNA downstream of the tetO promoter in the pLox-GFP plasmid. Cotransfection with a CRE plasmid into the Ainv ES cell line results in single-copy, unidirectional insertion of the plasmid into a single site after selection in neomycin. Treatment of this tetO-EKLF-GFP stable ES cell line with doxycycline results in dose-dependent expression of EKLF and GFP (Figure 2A)\(^2\). A control cell line, containing tetO-GFP without EKLF, was established in the same way.

We used this system and induced EKLF in differentiating EBs at day 4, which is the normal onset of endogenous EKLF expression,\(^4\) and we harvested each of these treated cells at day 8. The data in Figure 2B show that, as with ES cells, the EBs also exhibit little leakiness, because GFP levels remain extremely low in the absence of induction. In addition, doxycycline treatment leads to robust induction of GFP-expressing cells in the differentiating EBs. We monitored the effects of EKLF induction on hematopoietic cell populations by fluorescence-activated cell sorting (FACS) analysis of selected cell-surface proteins. Of note is the decrease in CD41 (7-fold) after EKLF induction, which could arise from a specific effect on megakaryocytic cells or from a general negative effect on primitive and definitive precursor formation. The increase in c-Kit (6-fold) makes it less likely to be the latter, particularly because c-Kit and CD41 are coexpressed in primitive and definitive hematopoietic progenitors.\(^4\) However, the concomitant decrease in CD42b (20-fold), a marker exclusively expressed in megakaryocytes, is most consistent with an effect on megakaryopoiesis. An issue that might have been anticipated, given EKLF’s antiproliferative properties,\(^2\) was that all hematopoietic markers would decrease on EKLF induction. However, the increase in c-Kit shows that EKLF induction does not generally down-regulate hematopoietic cell gene expression. This is further supported by additional experiments showing that Grl (a myeloid/granulocyte marker) is also increased and by the minimal changes seen in CD71 expression after EKLF induction (Table S2). Finally, the number of cells per EB and the level of apoptosis (not shown) are unaffected by doxycycline treatment. Similar results are obtained when the analysis is performed at day 9, or induction is performed at day 3 and analyzed at day 8 or 9 (Table S2). Doxycycline treatment of the control cell line does not have a significant effect on expression levels of these markers (Table S2).

To investigate the effect on megakaryopoiesis more precisely, we used a variation of the OP9 cell system that enables ES cells to differentiate toward specific lineages.\(^3\) After a preliminary EB formation step to increase hematopoietic progenitors (Deborah French and Gordon Keller, unpublished, April 2005),\(^3\) lineage generation is guided by the presence of selected cytokines after disaggregation of EBs and replating onto OP9 stromal cells, which are macrophage colony-stimulating factor (M-CSF) deficient and serve as feeder cells. In the present case, we used our tetO-EKLF-GFP stable ES cell line and differentiated them into EBs for 6 days before plating onto the OP9 cells in the presence of thrombopoietin (TPO) to direct the expansion of megakaryocytic cells.\(^3\) Typically, platelets are observed at day 10 after the start of ES cell differentiation, such that more than 70% of the cultured cells express megakaryocytic markers by day 12 (not shown). Doxycycline treatment (0.5 μg/mL) of OP9-growing cells showed that GFP\(^+\) cells were highly induced within 24 hours, remained high (90%) at 48 hours before a slight decrease by 4 days (50%).

We used this system to investigate the specific effects of EKLF induction on different stages of megakaryocyte production. In all cases, EBs were formed for 6 days before plating on OP9 cells. However, we varied the time of addition of doxycycline: at day 4 during the early stages of EB formation, at day 6 when hematopoietic progenitors are enriched in the EB, or at day 8 or 12 after megakaryopoiesis had already begun. Cells were monitored every day after plating on OP9. Addition of doxycycline at day 4 or 8 has no effect on the shape or numbers of cells compared with the control (untreated) cell population (Figure S1). However, a dramatic effect of doxycycline addition at day 6 was clear from visual inspection of the cells, because the cells do not form colonies of large megakaryocytes but remained small, floating, and rounded (Figure S1). This is even more apparent by performing a kinetic analysis of megakaryocyte formation after day 6 by following double-positive CD41/CD42d cell-surface expression as indicative of mature megakaryocyte formation. Because CD41 is expressed not only in megakaryocytes but also in other hematopoietic progenitors, we additionally used CD42d expression as a megakaryocyte-specific marker and compared the percentage of positive cells between uninduced and induced cultures (Figure 3). In a typical assay, CD42d\(^+\) cells, present at a level of 1.5% at day 6, expand to 11% of the population 24 hours later at day 7 and to greater than 40% by day 9. However, in the doxycycline/EKLF-induced cultures, CD42d\(^+\) cells accumulate to 4% after 24 hours (equivalent to day 7) and only reach 15% by 72 hours (equivalent to day 9) of culture. Additional data indicate that the doxycycline treatment had no effect on cell viability at any time point (as measured by trypan blue exclusion) and that the total cell numbers are similar in all cultures (data not shown). Consistent with the morphologic observations, doxycycline treatment at day 8 has little or no effect on the levels of CD42d\(^+\) cells compared with controls when harvested 1, 2, or 3 days later (not shown). These data show that EKLF has a negative effect on megakaryopoiesis. But more precisely, the OP9 studies suggest that EKLF action can only occur within a limited time frame during this process, later than day 4 but earlier than day 8. This immediately suggests that EKLF action plays a role in early, rather than late, megakaryocytic differentiative decisions.

Additional FACS analyses also show that, under conditions in which doxycycline treatment leads to lower levels of CD42d,
Loss of EKLF leads to expansion of megakaryocytes

One prediction from the in vivo expression pattern and the gain-of-function studies is that the converse will occur; that is, loss of EKLF expression will lead to an increase in megakaryocyte expansion. We tested this idea by culturing E13.5 fetal liver cells from wild-type and EKLF-null embryos in the presence of TPO and monitored levels of megakaryocyte expression. Morphologic examination (not shown) shows that day 4 cultures derived from the EKLF-null fetal livers contain a greater number of megakaryocytes (17%) than those derived from wild-type cells (6%). We defined this more precisely by monitoring CD41, CD42d, and CD42b expression by FACS analysis (Figure 5A). Even before incubation, CD41/42b and CD41/42d double-positive cells are greater in the EKLF-null cells; this becomes significantly more evident after liquid incubation, when the CD41/42b population is more than 2-fold higher in the day 4 cultures from the EKLF-null cells than from the wild-type cells. In addition, the EKLF-null cells appear to be expanding more rapidly than the wild-type cells after day 2.

To understand whether the greater numbers of megakaryocytes in the EKLF-null cultures have resulted (at least in part) from an increased presence of megakaryocyte precursors in the fetal liver, we assayed megakaryocyte colony formation in Megacult collagen cultures. Although such colonies are relatively dispersed, this assay enables a more accurate means than methylcellulose to determine their presence. We used the IB5 monoclonal antibody (directed against megakaryocyte CD41/CD61 complex [GPIIb-IIIa]51) to stain these colonies and to provide an unambiguous determination of their presence. Two things are readily apparent from these studies (Figure 5B). First, a 3-fold greater number of colonies result from the EKLF-null cultures relative to wild-type cells; second, the colonies from the null cells are more expanded in size and number of cells compared with the relatively compact colonies derived from the wild-type cells. These larger proliferating colonies are similar to that seen in Megacult cultures derived from GATA1s variant-expressing fetal liver cells.39

Fetal liver cellularity was carefully monitored and was within 10%, comparing E13.5 wild-type with EKLF-null fetal livers, making it unlikely that the increase in megakaryocyte cell numbers is an indirect result from (for example) loss of erythroid cells in the total population. However, to further clarify this point, we compared megakaryocyte colony formation in sorted populations. As seen by others,4,40 we did not obtain efficient megakaryocyte colony formation from E13.5 fetal liver MEPs even though formation of other myeloid colonies was fine; however, because the CMP population gave robust numbers of megakaryocyte colonies, we used these as our source of enriched progenitor cells for comparison. We find that the frequency of megakaryocyte colony formation is up to 4-fold greater within enriched lin-depleted or CMP-sorted hematopoietic progenitor subpopulations when isolated from EKLF-null fetal liver cells compared with wild-type cells (Figure 5C). In total, the studies in Figure 5 show that, in contrast to its overexpression, EKLF loss-of-function leads to a dramatic
expansion of megakaryocyte colonies and cells in either un-
sorted and sorted E13.5 fetal liver populations. Differentiated
Ter119
cells (R3-R5 49) do not accumulate in the absence of
EKLF (Andre Pilon, P.G.G., and David Bodine, manuscript in
preparation), concurring with the notion that EKLF is required
for full erythroid maturation. Together with the gain-of-function
studies, these results suggest that EKLF levels play a role in
2 lineages during hematopoiesis, with its presence stimulating
erythroid terminal differentiation while inhibiting megakaryo-
cyte expansion.

EKLF negatively regulates Fli1 expression

To gain a molecular understanding of how EKLF might be causing
a repressive effect on megakaryopoiesis, we comparatively ana-
alyzed expression arrays between EKLF wild-type and EKLF-null
fetal liver cells, particularly focusing on genes that may be
important for megakaryocyte differentiation. Inspection of the data
immediately showed a number of genes fitting this criterion that are
up-regulated at least 2-fold in the absence of EKLF (Table 1). This
was not due to a general effect, because not all megakaryocyte
genes are affected (eg, c-mpl, PBP, RANTES, and ENA78). However, of particular interest for the present studies is Fli1, an
ETS-related transcription factor that is critical for megakaryocyte
differentiation,52-54 and EKLF-Fli1 interactions have been noted.55
As a result, its down-regulation in the presence of EKLF could
provide a molecular framework for the cellular effects that we
have observed.

To better determine the timing of Fli1 repression after EKLF
induction, we returned to our doxycycline-induced EB/OP9 cell
system and performed quantitative analysis of RNA expressed
under various conditions. The addition of 0.5 g/mL doxycycline
at day 6 stimulates EKLF expression within 24 hours (by day 7) to
substantially higher levels than that seen in the absence of inducer
and, although attenuated, remains so at day 8 (Figure 6A).
Concomitant with this, at day 7 the Fli1 mRNA levels are
dramatically lower in the EKLF-induced samples than in the
uninduced samples and remain lower at day 8. As expected from
the known ability of EKLF to stimulate β-globin expression, β maj
mRNA levels are significantly increased when EKLF is induced in
these samples. The same results are observed when 1 µg/mL
doxycycline is used to induce EKLF (not shown). Other hematopoietic genes such as Runx1, GATA1, and GATA2 are unaffected by EKLF induction (Figure 6B). These data show that EKLF expression levels are critical for differentiative decisions, because EKLF causes divergent effects on specific target gene expression. The cellular and molecular data strongly suggest that EKLF expression leads to inhibition of megakaryocyte differentiation (by repressing Fli1) and expansion of erythroid differentiation (as monitored by increased β maj expression).

These results compelled us to revisit our sorted cell populations (from Figure 1). We find that, although commonly expressed with EKLF in the MEP, the subsequent normal expression pattern of Fli1 is opposite to that of EKLF; that is, the bipotential differentiation of MEPs leads to an almost 60-fold greater level of expression in megakaryocyte progenitors compared with erythroid (Figure 7A,B). Interestingly, GATA1 levels vary less than 3-fold in all populations.

To garner evidence that EKLF might play a direct role in Fli1 expression, we performed transient cotransfection assays with a Fli1 promoter construct linked to a luciferase promoter in the erythro-megakaryoblastic leukemia K562 cell line.56 Activity of this construct in the absence of a phorbol ester inducer depends on exogenous Fli1. We find there is a dose-dependent repression of the activated promoter by EKLF (Figure 7C). Although this does not distinguish direct (EKLF binding to the promoter as a repressor) or indirect (EKLF protein interacting with Fli1 and preventing its activity) modes of regulation, it is consistent with the idea that EKLF can negatively alter Fli1 promoter activity.

Discussion

Our studies suggest that EKLF plays 2 roles in hematopoiesis by repressing the onset of megakaryopoiesis and promoting gene expression in the erythroid lineage. This unexpected property...
distinguishes it from other commonly expressed transcription factors that play critical positive roles for both the megakaryocyte and erythroid lineages and is directly relevant to issues of bipotential mechanisms of differentiation. Knowledge of EKLF molecular mechanisms of action raises intriguing models of how EKLF may be affecting these consequences.

Hematopoietic bipotential lineage decision mechanisms share common features with those made during early stages of development. Select transcription factors can effectively tip the balance in these decisions. In hematopoiesis, the myeloid/megakaryocyte-erythroid pathway exemplifies one well-studied paradigm whose outcome relies on the cross-antagonism between PU.1 and GATA1, an idea originally suggested by gain-of-function studies (reviewed in Graf and Laiosa et al). The molecular mechanism relies on the ability of each protein to decrease each other’s activity by protein-protein interactions that result in down-regulation of their normal activation targets. Positive autoregulation then reinforces the initial outcome. Theoretical modeling suggests that, in addition to cross-inhibition of the opposing factor’s activity and autoregulation, repression of the opposing factor’s message levels solidifies the resultant differentiation program within the progeny of the original bipotent cell. In the present case, the first step suggesting that such a mechanism is in place is that EKLF expression has a significant repressive effect on endogenous Fli1 expression, a critical megakaryocytic activator. Second, transient assays have shown that Fli1 can inhibit EKLF-dependent transcription of the β-globin gene in cotransfected mouse erythroleukemic (MEL) cells, and EKLF can inhibit Fli1-dependent transcription of the megakaryocyte GPIX gene in cotransfected COS cells and of the Fli1 promoter (present data). Third, these proteins can physically interact in vitro and thus may negatively affect each other’s transcriptional activity. Together, these observations raise the intriguing possibility that the cross-antagonistic paradigm observed in other cases may also be operant here, although the individual players and subsequent consequences are different.

Consistent with the biology, Fli1 interactions with GATA1 yield a dramatically different effect, because they synergistically activate megakaryocytic genes.

These models directly imply that expression levels of the regulators must be properly down- or up-regulated to correctly orchestrate such decisions. Indeed, in the present case it is the mis-expression of EKLF that suggests a role for it in the MEP, a postulate supported by the opposite effects seen after EKLF ablation. Consistent with the molecular antagonism model, EKLF
misexpression represses Fli1 message levels, and its removal leads to an increase of Fli1 message. A study designed to monitor the effects of EKLF overexpression on β-globin in transgenic mice found, as a side effect, that circulating platelet levels were negatively affected. The complementary prediction is that Fli1 levels should similarly affect EKLF expression. Although EKLF levels were not directly assessed, analysis of cultured erythroid cells from Fli1-disrupted mice contained primarily polychromatophilic and orthochromatophilic cells and lower numbers of proerythroblasts and basophilic cells, similar to the effect seen after EKLF overexpression in the present studies. Conversely, Fli1 overexpression inhibits erythroid differentiation. As a result, an antagonistic genetic regulatory network between EKLF and Fli1 within the MEP can be postulated.

The present studies not only show that EKLF plays a negative role in megakaryopoiesis but also a positive role in promoting erythroid differentiation as judged by morphologic changes and cell-surface protein (Ter119) and target gene (βmaj) expression patterns. Because these are relatively late events in the erythroid differentiation program, it is difficult to tell whether EKLF also plays a directive role in earlier events that establish the lineage. However, EKLF must be playing a role early in megakaryopoietic decisions, given the critical window of its effect and its modulation of a crucial early megakaryocyte gene target. Consistent with this is the increase in megakaryocyte colony formation observed in its absence.

One recent study provides evidence that c-myb may also be playing a similar role to EKLF in MEP lineage decisions. A fortuitous genetic disruption of an upstream enhancer by transgene insertion resulted in a hypomorph level of c-myb expression that led to an increase in megakaryocytes and a decrease in erythroid cells, a result foreshadowed by an earlier knockdown study.

Known molecular properties of EKLF suggest at least 2 ways in which it could affect these bipotential decisions as an activator or as a repressor. One is by changes in EKLF protein partners. EKLF has been primarily characterized as a transcriptional activator, particularly at the adult β-globin locus and in the erythroid cell environment, where EKLF interacts with coactivators such as P300/CBP. However, EKLF also exhibits transcriptional repression properties in vivo. It achieves this by interaction with corepressors such as Sin3a and HDAC1 and possibly others (Miroslawa Siatecka and J.J.B., manuscript submitted; April 2007), suggesting one molecular means by which EKLF can repress megakaryocytic promoters such as Fli1 and others that appeared in the microarray analysis. The results of anti-EKLF chromatin immunoprecipitation using total cells from EBs differentiated at day 6 as source material (selected based on our present characterization and on their minimal manipulation) suggest that EKLF may be bound to the Fli1 promoter (Figure S2). A phylogenetic comparison points to a small number of conserved potential EKLF DNA binding elements within the tested regions that may be playing a role (P.F. and J.J.B., unpublished observations; July 2006). However, it is difficult to ascertain the relevance of CACC elements to repression, because their functional basis arose from studies directed at EKLF transactivation, and in any case not all putative elements are used in vivo. However, a more fundamental and complex problem is that repression by EKLF occurs in the absence of DNA binding. At this point our evidence does not yet favor a direct (DNA) or indirect (protein) model for EKLF repression of Fli1.

The second way for EKLF activity to be altered follows from its post-translational modifications. Phosphorylation of T41 and acetylation of K288 are required for optimal transactivation activity. Acetylation of K288 in particular leads to increased association of EKLF with chromatin remodeling complexes in vitro, which lead to open chromatin and transcription of the adult β-globin promoter in vitro and in vivo. However, EKLF acetylation may also play a role in its repressive properties, because K302, which is the other EKLF acetylation site, is critical for EKLF interaction with Sin3a. In other concurrent studies we have recently discovered that EKLF is sumoylated at a single lysine, thus adding to the possible post-translational modifications that may be involved in its regulation (Miroslawa Siatecka and J.J.B., manuscript submitted; April 2007). Of particular interest, we find that the sumoylation state of EKLF has an effect on megakaryopoiesis; that is, mutated EKLF that is sumo-deficient is less inhibitory toward megakaryocyte development than is wild-type EKLF. EKLF modification status and the identity of its protein partners are thus linked and may be directly relevant to any directive EKLF role in bipotential decisions from the MEP. This can be the result of subtle changes in the cellular environment; for example, EKLF protein partners and its repression versus activation function in an erythroid cell can be altered within 48 hours by simply changing the cytokine mix that leads to primitive or definitive erythropoiesis.

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Authorship

Contribution: P.F., D.M., and J.J.B. were actively involved in the conceptual design and direction of the experiments as the project unfolded; D.M. and M.G. constructed the clone and established the inducible EKLF ES cell line; P.F. performed the experiments in Figures 2A, 3, 4A,B, 6, 7C, and Figure S1; D.M. performed the experiments in Figures 2A,B, 4C,D, 5C and in Table S2; M.G. performed the experiments in Figures 2A,B and 5A,B, and in Table S2; H.K. performed the experiments in Figures 1 and 7A,B and in Table S1; F.L. in performed the experiment in Figure S2; P.G.G. performed the experiment in Table 1; J.J.B. established the collaborations with H.K. and P.G.G., and wrote the paper with input from P.F. and D.M. This project was initiated based on observations made by D.M.

P.F. and D.M. contributed equally to this study.

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Novel role for EKLF in megakaryocyte lineage commitment

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