Regulation of Erythroid Gene Expression by the Transcription Factor EKLF

Roy Drissen

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Regulation of Erythroid Gene Expression by the Transcription Factor EKLF

Regulatie van gen-expressie in rode bloedcellen door de transcriptie factor EKLF

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Chapter 1

Introduction

Red blood cells

Aerobic organisms require oxygen for their viability. Through the oxidation of nutrients, such organisms generate energy. As organism size and complexity increased through evolution, not all the cells of an organism were directly exposed to available oxygen. To overcome this problem, vertebrates have evolved a circulatory system that actively delivers oxygen to the cells. Red blood cells (RBCs) in the circulating blood are the carriers, taking up oxygen in the lungs or gills and delivering it to internal tissues. The actual oxygen-binding molecule in the red blood cells is hemoglobin, an iron-containing protein that gives blood its red colour. One millilitre of human blood contains approximately 5 milliard RBCs that have a lifetime of about four months. Aging cells are removed from the circulation and destroyed by macrophages in the spleen and liver. To replace them, an adult produces about 10^{11} RBCs daily.

Hematopoietic stem cells

All blood cells derive from hematopoietic stem cells (HSCs) that are at the base of a complex hematopoietic cell differentiation hierarchy. These stem cells possess a high proliferative potential, are self-renewing and remain active over the lifespan of the individual.[133,144,159]
The incidence of HSCs is very low. In adult bone marrow only 1 to 10 in 100.000 cells are estimated to be HSCs.[27,95,96] HSCs self-renew by producing daughter cells that retain stem cell characteristics. Alternatively, daughter cells divide and differentiate into one of the blood lineages. HSCs are pluripotent, which means they can give rise to every type of mature blood cell; erythrocytes, neutrophils, basophils, eosinophils, platelets, monocytes, macrophages, osteoclasts, and the T and B lymphocytes (Figure 1). Once lineage choices are made, progenitors display increasingly limited lineage potentials at each branch point. To maintain their multipotentiality, stem cells are thought to have a wide-open chromatin structure. This is progressively quenched during the differentiation process, concomitant with a progressive restriction of developmental potential.[1]
Thus, stem cells are a unique cell type in that they can produce more stem cells, as well as a population of cells that can undergo further development and/or differentiation.[176,177] Although the model proposes that differentiating cells become more committed after each branch point, there is evidence that the loss of developmental potential is reversible.[155] In addition, a number of recent reports suggest that HSCs from murine bone marrow may be more plastic than previously thought. Depending on culture conditions, HSCs can dedifferentiate or be reprogrammed, giving rise to non-hematopoietic tissues including neural cells, hepatocytes, myocytes, muscle tissue and multiple organ tissues.[84,85,130] However, the plasticity of HSCs is still under question, since tissue-specific stem cells could be mislabelled as HSCs and contribute to non-hematopoietic cell tissues. Furthermore, two reports challenge the concept of transdifferentiation by showing the spontaneous fusion of mouse bone marrow or brain cells with ES cells after which they take on the ES cell characteristics.[204,233]

The clinical importance of HSCs is reflected in the nearly forty years of bone marrow transplantations for the treatment of inherited and acquired disorders of the blood system. A large amount of clinical and fundamental research has focused on the biology of bone marrow and more recently, umbilical cord blood HSCs. Despite these intense efforts, the cellular characteristics of HSCs are still poorly described. In experimental mouse models, the activity of HSCs is determined by their potential to long-term repopulate irradiated adult recipients.[144,152] They are generally present in the Sca-1^{+} c-kit^{+} CD34^{+} Lin^{-} population of adult bone marrow.[106,127,162,198] Sca-1 and c-Kit are expressed on HSCs but also on other cell types. Generally, no HSC activity is found in cell populations negative for Sca-1 or c-

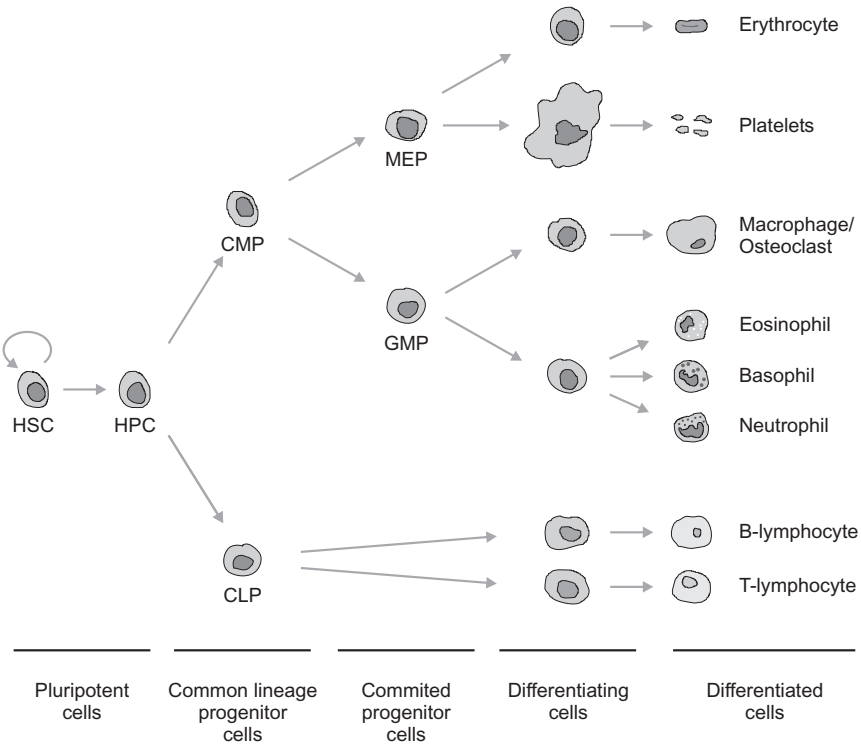


Figure 1. Schematic representation of the hematopoietic hierarchy
The hematopoietic stem cell gives rise to all blood cells. During differentiation, cells loose their pluripotent capacity and become progressively more restricted to one of the blood cell lineages. HSC= hematopoietic stem cell; HPC= hematopoietic progenitor cell; CMP= common myeloid progenitor; CLP= common lymphoid progenitor MEP= myeloid and erythroid progenitor and GMP= granulocyte monocyte progenitor. Adapted from Gilbert.[73]

Kit. CD34 has proven to be a useful HSC marker mainly in mouse embryos,[186] whereas in adult mice most HSC activity was found in a CD34$^-$ cell population.[142] The lineage marker negative cell (Lin$^-$) fraction consists of cells negative for a mixture of antibodies that recognize differentiated cells. Thus, HSCs are enriched by using a combination of antibodies recognizing surface markers and such cells are characterized as Sca-1$^+$ c-kit$^+$ Lin$^-$ cells. The dye rhodamine can be used for further enrichment. Rhodamine stains highly active mitochondria.[45] Since most HSCs are inactive, they are found in the rhodamine dull fraction.[216] A recent paper demonstrates that endoglin, an ancillary TGF- receptor, is another useful marker for HSC enrichment. HSCs were exclusively found in the Endo$^+$ Sca-1$^+$ Lin$^{-/low}$, but not in the Endo$^-$ Sca-1$^+$ Lin$^{-/low}$ population. Furthermore, the Endo$^+$ Sca-1$^+$ Rh^{dull} phenotype defines an almost pure population of HSCs from mouse bone marrow.[40]

The bone marrow is established as the principle hematopoietic tissue around birth, being seeded with HSCs that are generated earlier in the embryo. In the mammalian embryo, several tissues serve as reservoirs and/or generators of hematopoietic activity: the yolk sac,[150] the para-aortic splanchnopleura (PAS),[82,83] the aorta-gonad-mesonephros (AGM),[143,145,152] the placenta,[3] the liver,[100,113] the spleen and the thymus[151] (reviewed by Dzierzak et al[63]).

The first hematopoietic tissue in the developing mouse is the yolk sac. Around day 7 of gestation (E7) extra-embryonic blood islands appear in the yolk sac, from which primitive erythroblasts and endothelial cell differentiate. Primitive erythroblasts enter the newly formed vascular system and continue to divide for several days.[21] They differentiate within the blood stream, accumulating hemoglobin and becoming less basophilic with a progressively condensed nucleus.[12,187] At this stage primitive erythrocytes are distinct from the definitive erythrocytes found in the adult. The majority of the primitive erythrocytes retain their nucleus, whereas definitive erythrocytes enucleate at the end of their differentiation program. Primitive cells are bigger and contain about four times the amount of hemoglobin found in definitive erythrocytes.[199] Moreover, primitive erythrocytes differ in their globin gene expression patterns from adult erythrocytes (see 'β-globin locus').[12,31] It is thought that these first differentiated hematopoietic cells are critical for delivering oxygen to the rapidly growing embryo and thus, the yolk sac serves as an efficient supplier of these first circulating blood cells.

The PAS/AGM region is the first intra-embryonic hematopoietic tissue. Although it does not contain erythropoietic foci, functional hematopoietic assays have indicated the presence of hematopoietic stem cells, starting at E10.5 until E13.[83,145] These HSCs are exclusively and autonomously generated in the AGM at E10/E11, independent of the yolk sac and are the first definitive HSCs. Around late E11, they are thought to colonize the fetal liver, giving rise to all adult hematopoietic lineages including adult erythrocytes.[143] Although erythrocytes and hematopoietic progenitors are first found in the fetal liver around E9, these hematopoietic cells are thought to be derived from the yolk sac. At E12, the number of HSCs in the fetal liver increases dramatically, suggesting that this microenvironment supports the expansion of HSCs.[65,152] The liver is considered to be the principle hematopoietic tissue during the fetal stage of mouse development. Around the time of birth, stem cells from the liver are thought to populate the spleen and the bone marrow, which then becomes the major site of blood formation throughout adult life.[28]

Erythropoiesis

HSCs can differentiate into red blood cells via a differentiation program called erythropoiesis (see Figure 2). The erythroid differentiation program takes place through a series of numerous and distinct cell intermediates, some of which are morphologically recognizable and some which require *in vitro* functional assays to retrospectively be identified. Following the HSC, the first slightly differentiated cell in this series is the CFU-S (colony forming unit - spleen). *In vivo* these cells form macroscopic erythroid-myeloid colonies on the spleens of lethally irradiated recipient mice 9 to 14 days post-injection. Although CFU-S are still multipotent, these cells have lost their long-term repopulation capability. The next more differentiated progenitor in the series is the lineage restricted CFU-GEMM (colony forming unit - granulocyte, erythrocyte, macrophage, and megakaryocyte). These cells differentiate *in vitro* to all erythroid-myeloid cell types, but have no lymphoid potential.[112] The first erythroid lineage restricted progenitor is the BFU-E (burst-forming unit, erythroid). These cells are named after the large *in vitro* colonies they form in 7-10 days in semi-solid medium under the influence of erythropoietin (EPO), interleukin-3 (IL-3) and stem cell factor (SCF). These colonies consist of up to 5000 mature erythrocytes.[146] The next erythroid lineage restricted progenitor in the series is the CFU-E (colony-forming unit, erythroid). CFU-Es form small *in vitro* colonies (8-64 cells) when cultured for a short time in semi-solid medium (3-4 days). When BFU-Es develop into CFU-Es, they lose responsiveness to IL-3 and SCF, but remain dependent on EPO.[188-190]

CFU-Es produce the first morphologically recognizable differentiated member of the erythroid lineage, the pro-erythroblast. From this stage on, the erythroid progenitors can be distinguished by microscopic examination because they are smaller than cells at earlier stages of differentiation. Around the pro-erythroblast stage, cells become positive for the TER-119 surface antigen.[121] The TER-119 specific antibody is widely used to detect this exclusive marker of erythroid lineage cells.

The completion of the erythroid differentiation program in pro-erythroblast cells includes about four rapid cell divisions.[2] First, the size of the nucleus decreases somewhat and the cytoplasm becomes more basophilic due to an increase in ribosomes. Hence, at this stage the cells are called basophilic erythroblasts. Subsequently, the cells start to produce high amounts of hemoglobin. As a result, the cytoplasm attracts both basic and eosin stains, and the cells are called polychromatophilic erythroblasts. Eventually, the cytoplasm becomes more eosinophilic, and the cells are then called orthochromatic erythroblasts. In these last differentiation steps the cells continuously decrease in size. Following the expulsion of the nucleus, orthochromatic erythroblasts become reticulocytes. Reticulocytes take their name from the reticular networks of polyribosomes that are formed at this differentiation stage. Despite the absence of the nucleus, these cells still translate globin mRNA into functional protein. As a final differentiation step, reticulocytes loose their polyribosomes to become mature red blood cells. These erythrocytes are small cells with up to 25% of their cytoplasmic volume taken up by hemoglobin. In humans, erythrocytes have a diameter of 7-8 μm and their shape is the visually distinct biconcave disk.[66]

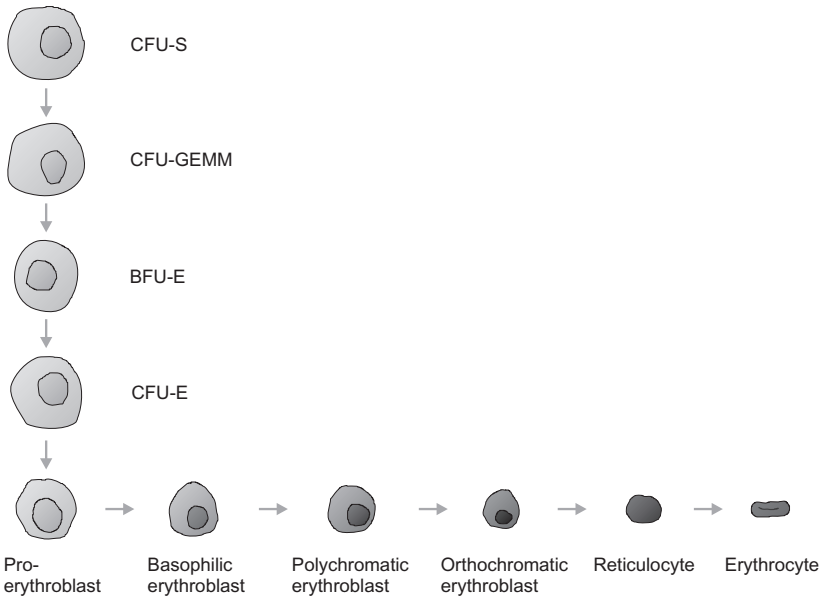


Figure 2. Erythropoiesis
Schematic drawing of the various stages in the erythroid differentiation programme. Vertical direction indicates cells in differentiation stages that require *in vitro* functional assays to retrospectively be identified. Horizontal direction indicates cells that are morphologically recognizable. CFU-S= colony forming unit – spleen; CFU-GEMM= colony forming unit – granulocyte, erythrocyte, macrophage, and megakaryocyte, BFU-E= burst-forming unit, erythroid and CFU-E= colony-forming unit, erythroid

Erythropoiesis *in vitro*

To study erythropoiesis at the molecular level, a reliable *in vitro* culture system is highly desirable. For many years, the colony forming unit assays have been used to study progenitors in the erythroid lineage differentiation hierarchy.[200] Single BFU-E cells grow in 7-10 days in semi-solid medium in the presence of EPO, IL-3 and SCF to large colonies, whereas CFU-E cells form smaller colonies in 3-4 days. CFU-Es have lost responsiveness to IL-3 and SCF.[146] These assays are useful for the detection of erythroid cells that have developed to the various stages in differentiation. However, the small number of cells and lack of synchronous differentiation does not allow using these cultures for studying molecular events. Other differentiation models include erythroid cell lines that have an erythroleukemic origin. To some extent these cells are useful models to study erythropoiesis, although they do have their shortcomings; erythroleukemic cell lines generally fail to efficiently execute the

normal erythroid differentiation program (four to five terminal cell divisions, massive size reduction, nuclear condensation and enucleation).[2,59,189] A frequently used model to study erythropoiesis is the Friend virus murine erythroleukemia (MEL) cell line.[77] However, these cells do not recapitulate an *in vivo*-like differentiation program; differentiation is induced by non-physiological agents such as dimethyl sulfoxide and hemoglobin levels are much lower than found in erythrocytes of peripheral blood. Another erythroid cell line is the J2E cell line. These cells were immortalised by transformation of the oncogenes v-myc and v-raf. Although they start differentiating in response to the physiological agent EPO, the culture continues to proliferate after induction and only half of the cells accumulate hemoglobin.[123]

A highly advantageous approach is to use primary erythroid cells that recapitulate normal erythroid differentiation more faithfully. Only recently have the conditions for culturing and differentiating large numbers of primary erythroid cells been achieved. A culture method was developed in which erythroid cells are strictly dependent on physiological agents.[220] Primary mouse cells, isolated from fetal livers, proliferate in a serum-free medium under the control of EPO, SCF and dexamethasone (Dex). These proliferating cells are characterised as pro-erythroblasts. When exposed to the physiological differentiation factors EPO and insulin, in absence of SCF and Dex, the cells undergo synchronously an *in vivo*-like differentiation program. Within approximately two days, they mature terminally into enucleated erythrocytes and express stage-specific erythroid genes in the expected temporal order.[60] In addition to the primary cell cultures, an immortalised cell line has been made using this method. Fetal liver cells from mice lacking the tumor suppressor gene p53 were maintained under proliferative conditions, leading to the immortalised cell line I/11.[220] This cell line behaves similar to primary cells when cultured under proliferating conditions; in serum free medium with EPO, SCF and Dex the cells proliferate at an erythroid progenitor stage. After changing the proliferation medium with differentiation medium, the cells undergo a synchronous differentiation program. This differentiation program lasts slightly longer than for the primary cells, as enucleated erythrocytes appear after approximately three days.

In our group, yet another culture method has been developed to study erythropoiesis. It is called the hanging drop culturing method. This method has been described for a variety of cell types, most commonly for the differentiation of T lymphoid progenitors.[30] Fetal liver cells are resuspended in a medium containing fetal calf serum, EPO, insulin and hemin. The cells are cultured in small drops, hanging from the lid of a Petri dish. Cells sink to the bottom of the droplet, resulting in a high concentration of cells and the cells are in physical contact with each other. Differentiation and enucleation is evaluated on the basis of cell size by cytospin- and FACS analysis. The majority of wild type fetal liver cells differentiate and enucleate within two to three days (unpublished). This method allows a swift study of erythroid differentiation of fetal liver derived progenitors. Possibilities for molecular analysis of these cultures are limited; the small number of cells that are typically used suffices e.g. for real time PCR, but not for extensive Western blot analysis. Furthermore, the cells are at various differentiation stages at the beginning of an experiment. Synchronisation of the cells by culturing them in proliferation medium as described above is therefore advantageous for the study of the cells at various intermediate differentiation stages.

Diseases

Hemoglobinopathies are the most prevalent of all human genetic diseases. Most of these disorders are caused by mutations or deletions in the genes on the α- or β-globin locus or in their regulatory elements. Extensive clinical and fundamental investigations motivated by the need to improve therapies for such a large group of patients have contributed to the current understanding of globin gene regulation and regulation of gene expression in general.[170] The two most common hemoglobinopathies are thalassemia and sickle cell anemia. Less common is hereditary persistence of fetal hemoglobin (HPFH), which is a condition rather than a disease.

Thalassemias

The name 'thalassemia' is derived from the Greek word 'thalassa' ('sea'), because it is well recognized that there is a high incidence of people suffering from this hemoglobinopathy near the Mediterranean Sea. The curious geographical distribution of thalassemia, as well as sickle cell anemia (see below) resembles that of malaria. It was found that heterozygous carriers of these diseases appear to have a slightly improved resistance to malaria. A small but highly significant degree of protection against malaria may be the result of an accelerated destruction of infected erythrocytes.[9] This results in a selective survival advantage, explaining why these disorders are maintained at a high frequency in areas where malaria used to be endemic.[224]

Thalassemias are caused by a disturbance in the expression of either the α- or β-globin genes. A shortage in α- or β-globin expression causes α- or β-thalassemia respectively. Normally the production of the α- and β-globins is in balance and two of both globins join to form a tetrameric hemoglobin molecule with a central iron moiety. Because of a reduced expression of one of the globins, thalassemia patients have unbalanced quantities of α- and β-globin proteins. This leads to the formation of erythrocytes lacking sufficient functional hemoglobin and results in anemia. Moreover, the precipitation of the excess of free globin chains make the red cells vulnerable to degradation, further contributing to the anemia.[206] The severity of the phenotype can range from mild to lethal anemia, depending on the extent of the disturbance between α- and β-globin protein levels. Many deletion- and non-deletion type β-thalassemia mutations have been described.[47] The deletion type thalassemias usually have large segments of the globin gene locus missing, including genes and/or regulatory elements. The identification of these segments and correlation with disease phenotype and severity has played a role in the discovery of important regulatory elements. For example, the genetic characterization of Dutch deletion type β-thalassemia led to the discovery of the Locus Control Region. These patients have an intact β-globin gene, including the promoter. However, they have a large deletion of DNA upstream of the β-globin gene that contains regulatory elements required for its transcription.[89,122,202,217]

Non-deletion type thalassemias give rise to frameshifts, nonsense mutations, RNA processing mutants or a decrease in transcription of globin genes. Some of these molecular changes have led to the identification of important promoter elements, such as the β-globin CACC box. This is a binding site for the erythroid-specific transcription factor EKLF. Mutations within the CACC box result in severe down regulation of the β-globin gene expres-

sion.[73,128,161] Apart from the mutations in the EKLF binding site, a variety of other, non-deletion mutations have been described that lead to β-thalassemia.[104]
The development of therapies for thalassemias is facilitated by the establishment of mouse models. Both the adult mouse β-globin genes have been deleted. Heterozygous mice of this deletion show characteristics typical for thalassemia, whereas homozygous mice for the deletion die of anemia.[44,231]

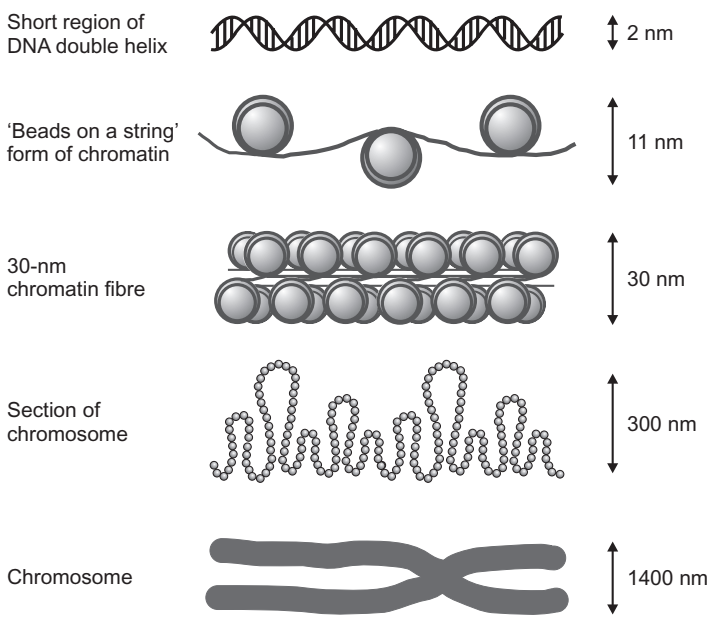
Sickle cell anemia
Sickle cell disease is caused by a single base pair mutation in the β-globin gene, resulting in a substitution of valine for glutamine at position 6 of the β-globin chain.[107] This alteration markedly reduces the solubility of the deoxygenated, but not the oxygenated form of hemoglobin. Deoxygenated mutant globins form large polymers and precipitate. This leads to deformation of the red blood cells into sickle-shaped cells, which have now lost their deformability. Patients have modest to severe anemia and crisis of peripheral tissues. Such a crisis is caused by a vicious cycle: vessel blockage (that can be caused by sickled cells) leads to a local low oxygen concentration. This results in a transition of more hemoglobin into the deoxygenated form, which again causes more sickling, thus resulting in an extension of the original lesion and necrosis. Although sickle cell anemia was the first hereditary disease to be understood at the molecular level, there is still no adequate treatment. The establishment of a mouse model for this disease has facilitated the development of therapies. Transgenic mice that express human sickle hemoglobin were mated with mice having knockout mutations of the mouse α- and β-globin genes, resulting in mice that express only the mutated human hemoglobin.[185]

Hereditary persistence of fetal hemoglobin
Hereditary persistence of fetal hemoglobin (HPFH) is characterized by the presence of a substantially elevated level of fetal hemoglobin in adults. It is considered a condition rather than a disease, since the fetal hemoglobin can substitute the adult hemoglobin without clinical consequences. However, HPFH is often found in combination with β-thalassemia or sickle cell anemia. The increased levels of fetal hemoglobin ameliorate the clinical course of these diseases.[50,118,230] Similar to the thalassemias, there are both deletion- and non-deletion types of HPFH. The non-deletion types of HPFH are characterized by the presence of point mutations in the promoter regions of the Gγ or Aγ genes, which are thought to alter interactions between various transcription factors and the promoter. For instance, in transgenic mice it was found that the point mutation found in the Greek non-deletion HPFH causes loss of binding of transcription factor GATA1 to the γ-globin promoter, resulting in persistence of γ-globin expression.[20] A number of deletion types of HPFH are described[75] and studied in mouse models.[4,6,116] In most cases, the β-globin gene is deleted, bringing distal hypersensitive sites in close proximity of the γ-genes.[117]

Chromatin and transcription

The DNA in the nucleus is highly organized[72] (Figure 3). It is bound to proteins, together called chromatin. In order to fit all the chromatin into the nucleus, it is organized at different levels. In the first place, the DNA is wrapped around histones. Each histone forms a symmetrical octamer of two of each of the H2A, H2B, H3 and H4 proteins. About 147 bp of DNA is wrapped twice around each histone, resulting in a 'beads on a string'-like structure that compacts the DNA five- to ten-fold.[124,125] The histones, especially their N-terminal tails, are subject to many covalent modifications. These modifications include lysine acetylations, lysine and arginine methylations, serine phosphorylations, lysine ubiquitinations, as well as other modifications about which less is known.[19,105,126,137,182,221] Two distinct, but non-exclusive models explain the influence on chromosome function by these modifications. Firstly, nearly all modifications alter the electrostatic charge of the histone, which could change the structural properties of the histone or the binding to the DNA. Secondly, the modifications might create a special surface that is recognized by specific proteins.

Figure 3. Chromatin packing
Various levels of chromatin packing that leads to a highly organised chromosome. Adapted from Alberts et al.[2]



Therefore, functional complexes can be recruited to their proper sites of action. As a result, chromatin can be involved in both activation and repression of gene expression, depending on the nature of the complexes attracted.
The space between nucleosomes can vary in length (~10 - 80 bp) and is a binding target for histone H1, which is required for the next level of compaction. H1 stabilises a polynucleosome string folded into a compact fibre with a diameter of ~30 nm. This results in an approximately 40-fold compaction of the linear DNA.[86,207] The chromatin is thought to be further organized by non-histone proteins. The resulting chromatin structure is roughly divided into two types: euchromatin and heterochromatin (reviewed by Dillon and Festenstein[57]). Heterochromatin stays condensed throughout the cell cycle and is in general transcriptionally inactive. Euchromatin undergoes a typical cycle of condensation and unravelling. It defines more accessible and transcriptionally active portions of the genome.
In general, the higher order chromatin structures are associated with gene inactivation. Loss of higher order chromatin structure is often detectable by an increase in sensitivity to nucleases such as DNAseI. DNA is cut more easily by DNAseI when it is present in an open, accessible chromatin structure. Therefore, susceptibility to DNAseI can be used as an assay to measure the general accessibility of DNA regions in chromatin. Sensitivity can extend over the entire region of a transcribed gene, including regions up- and downstream of the genes. This type of general DNAseI sensitivity has been attributed to more common chromatin changes caused by the absence of histone H1, the acetylation of histones or hypomethylation of DNA sequences.[102,115,191,203,219] In addition to sensitivity regions, some regions are particularly susceptible to DNAseI digestion and are called hypersensitive sites (HS). These HS are reliable indicators of *cis*-regulatory elements: DNA sequences that regulate gene expression such as promoters and enhancers.[90]
Polymerase II promoters are found immediately upstream of the transcription initiation site. They are about 100 bp in length and are the minimal requirement for accurate and efficient initiation of transcription of a gene. A number of sequence motifs can be found in most promoters: The TATA box and initiator are often referred to as the core promoter. A third element is found in a subset of TATA box-deficient promoters approximately 30 nucleotides downstream of the RNA start site and is termed the downstream promoter element (DPE).[37] These elements recruit the basal transcription machinery and determine the precise site of transcription initiation. Additional elements are found in promoters, consisting of typical recognitions sites such as CCAAT and CACC boxes. Both ubiquitous and specific *trans*-acting factors recognize these sites. Many of these *trans*-acting factors recruit chromatin-remodelling complexes to the promoter and open up the chromatin.[71]
Enhancers are *cis*-regulatory elements with a size that ranges from 50 base pairs to 1.5 kilo base pairs, containing binding sites for transcription factors. They are functionally defined by their ability to stimulate transcription, independent of their orientation and with some flexibility in distance to the promoter.[10,25] Enhancers are found up to several kilo bases upstream, downstream and within genes. Enhancers cannot initiate transcription by themselves, but they cooperate with one or a number of promoters. It appears that this cooperation results in an increase of establishing and maintaining an active transcriptional state, rather than increasing the transcription rate.[139,222,225]

Like enhancers, Locus Control Regions (LCRs)[135] have a strong, transcription-enhancing activity on linked genes. However, unlike enhancers, LCRs possess all the properties necessary for opening a chromosome domain and preventing heterochromatinisation at ectopic sites. LCRs are defined by their ability to drive high-level expression of linked genes in a tissue-specific and copy number-dependent manner. The components of LCRs are hypersensitive to DNAseI in cells that actively transcribe the linked genes. These components contain multiple ubiquitous and lineage specific transcription factor-binding sites. An LCR was first identified in the human β-globin locus.[89] Since then, several others have been identified and described in a broad spectrum of mammalian gene systems, suggesting that they play an important role in the control of tissue-specific eukaryotic gene expression.[135]
In addition to these *cis*-regulatory elements, *trans*-acting factors are necessary for transcription. The central component required for the transcription of the DNA into RNA is the enzyme RNA polymerase (RNAP). Eukaryotes contain three highly related enzymes: RNAPI, RNAPII and RNAPIII. Each of these enzymes is dedicated to the transcription of a specific set of genes. RNAPI recognizes a single promoter structure and transcribes only the ribosomal RNA genes.[91] The genes transcribed by RNAPIII have in common that they are short (< 400 bp) and encode structural or catalytic RNAs, such as transfer RNA (tRNA) and small nuclear RNA (snRNA).[192] Protein-encoding messenger RNA (mRNA) is transcribed by RNAPII. Transcriptional regulation depends on an RNAPII-protein complex that consists of numerous proteins.[134] General transcription factors (GTFs; e.g. TFIID, TFIIB, TFIIF, TFIIE, TFIIH and TFIIA) are necessary for accurate promoter recognition and transcriptional initiation. Transcription is preceded by the formation of a pre-initiation complex (PIC) that consists of the GTFs, the template DNA and RNAPII. The PIC formation starts with the binding of TFIID to the core-promoter elements, usually a TATA box.[87,183] Fine tuning of gene transcription is accomplished by cooperation of the RNAPII protein complex with sequence-specific activators (e.g. tissue specific transcription factors) and co-regulators (e.g. chromatin modifying- and chromatin remodelling complexes).[134]

Transcription factors

Since basically all cells in an organism have identical genetic information, differences between cell types are established through differential regulation of gene expression. Transcription factors play an important role in this regulation. They integrate physiological and environmental signals and make a cell respond correctly to these signals by enhancing or repressing the expression of particular genes.
A number of domains can be found in transcription factors. One of these is the domain that physically interacts with the DNA, usually at promoter and/or enhancer sites. The DNA binding domain is one of a number of common structural motifs including zinc-fingers, helix-turn-helix, helix-loop-helix and leucine zipper motifs.[97,164] The surface of these motifs is such that it strongly binds specific DNA sequences. Small differences in the amino acid chain can alter the affinity and/or the DNA sequence that they recognize.

Apart from the DNA binding domain, transcription factors usually contain a separable domain that either activates or represses gene transcription.[36,148] The activation or repression is accomplished by binding to proteins or protein complexes. For instance, they can bind the basal transcription machinery, recruiting this complex to the correct transcription initiation site.[154] They can also bind proteins that influence the structure of chromatin. As described earlier, chromatin structure has an important role in the accessibility of DNA to proteins, and therefore in gene expression. Modifications of histones (acetylations, methylations, phosphorylations or ubiquitinations) by transcription factor-binding proteins modulate the accessibility of DNA and specific recognition sites for nuclear factors can be created.[19,105,126,137,182,221] Chromatin remodellers form another class of protein complexes that transcription factors can bind to in order to influence chromatin structure. In an ATP-dependent manner, they alter DNA-histone contacts within nucleosomes, thus influencing the accessibility of the DNA sequence.[14] On the basis of the similarities of their ATPase subunits, these complexes can be divided into three classes: the Swi2/Snf2, Isw1, and Mi-2 chromatin remodelling machines.

In addition to the DNA binding and activation/repression domains, most transcription factors have sites that are involved in post-translational modifications. These modifications include phosphorylation,[99] acetylation,[11] sumoylation,[79] glycosylation[109] and ubiquitination.[226] Numerous aspects of transcription factors have been shown or postulated to be the result of modifications, such as protein-protein interaction, protein stability, subcellular localisation and DNA binding.

In a cellular differentiation process, such as erythropoiesis, quantitative and temporal control of gene expression is a prerequisite. This is established by the presence of the appropriate transcription factors, the fine tuning by posttranslational modifications and cooperation of multiple housekeeping- and tissue-specific transcription factors functioning in different complexes.[134,195] One of the many transcription factors that are of vital importance for normal erythropoiesis is the erythroid Krüppel-like factor.

EKLF

History

The erythroid Krüppel-like transcription factor EKLF was initially discovered in a fishing expedition for novel erythroid genes. It was isolated by enriching genes that are expressed in a mouse erythroleukemia cell line but not in a mouse monocyte-macrophage cell line.[147] The cDNA encodes a protein of approximately 38kD. Initially its expression was found in bone marrow and spleen, the two hematopoietic organs of the mouse. Further studies showed that the expression of EKLF is restricted to erythroid cells.[156,169,197]

Sp/XKLF family

Most transcription factors are grouped into families on the basis of common DNA- or protein-binding domains. EKLF has been classified to the Sp/XKLF (specificity protein/Krüppel-like factor) family of transcription factors.[29,174,215] These transcription factors bind

and act through GC and GT/CACC boxes that are present in promoters, enhancers and locus control regions of numerous genes. An 81 amino acid DNA binding domain, found close to the C-terminus, defines the transcription factors of the Sp/XKLF family. It consists of a combination of three conserved Cys2His2-type zinc fingers.[174] The name of this family of proteins derives from the observation that a similar arrangement of zinc fingers was found in the Drosophila segmentation gene Krüppel.[193] In the human genome, 24 genes have been classified as belonging to this family of transcription factors. Eight of them belong to the sub-family of Sp-factors (Sp1-Sp8). In addition to the zinc-fingers, Sp-factors contain a conserved motif immediately N-terminal to the zinc finger domain.[29] The Krüppel-like factors are more heterogeneous, but form a subgroup on the basis of additional conserved residues between each finger.[23] The 'X' usually indicates the major expression site of the factor, i.e. erythroid cells (EKLF), gut (GKLF),[194,232] intestine (IKLF),[48] lung (LKLF),[5] ubiquitous (UKLF)[141] or fetal (FKLF).[8] Another ubiquitously expressed factor has been named basic (BKLF) because its N-terminus is rich in basic residues.[52] Although these abbreviations are commonly used for the proteins, the human gene nomenclature committee has assigned different official names to the factors. For EKLF, LKLF, BKLF, GKLF and IKLF these are respectively KLF1 to KLF5, UKLF is named KLF7 and the official name at this moment for FKLF is TIEG2.

EKLF in erythropoiesis

Expression of EKLF is largely restricted to the erythroid cell lineage and is expressed both in primitive and in adult erythropoietic cells.[197] The promoter of the EKLF gene contains a functional binding site for the transcription factor GATA-1, suggesting that the expression of EKLF is dependent on and downstream of GATA-1.[51] GATA-1 is expressed and functional in primitive and adult erythroid cells.[67,78,211]

EKLF binds DNA at CACC-boxes, which are found in many erythroid gene promoters.[181] EKLF specifically binds the sequence 5'-CCA CAC CCT-3'.[147] β-thalassemia patients that carry mutations in the EKLF binding site in the β-globin promoter illustrate the importance of this high affinity site. The loss off affinity for binding of EKLF reduces the expression of β-globin strongly.[70,73,128,160,209] Furthermore, EKLF has a higher affinity for the human β-globin promoter than the γ-globin promoter, which has a similar but not identical CACC box.[61]

To determine the role of EKLF *in vivo*, the gene has been knocked out in mice.[156,169] Mice heterozygous for the EKLF gene appear completely healthy. In the absence of EKLF however, mice die around E14. The primitive blood cells function sufficiently for normal survival of the EKLF knockout mice during embryonic development up to approximately E12. The expression of embryonic globins εy and βH1 is not influenced by the absence EKLF. So far, no abnormalities have been described for EKLF$^{-/-}$ primitive cells. However, the gene is expressed in wild type primitive cells and the low expression of the βmaj gene in these cells is dependent on EKLF.[210] Furthermore, an EKLF-dependent reporter gene has been used to demonstrate that EKLF is a transcriptional activator in embryonic erythropoiesis.[205] In Chapter 3, we describe the first endogenous target genes for EKLF in primitive cells. These are the Alpha Hemoglobin Stabilising Protein (AHSP) and Erythroid Protein Band 4.9 (Epb4.9). AHSP is an abundant erythroid-specific protein that protects cells from precipitation of free α-globin by forming a stable complex with it.[120] Epb4.9 is an actin-

binding and bundling protein of the erythrocyte membrane skeleton and it is required for the maintenance of the erythrocyte shape and membrane mechanical properties.[119]
When hematopoiesis has switched to the fetal liver, EKLF knockout mice develop a fatal anemia that is caused by strongly reduced β-globin expression. In contrast, α-globin gene expression does not appear to be dependent on EKLF. Furthermore, fetal liver-derived erythroid cells of EKLF$^{-/-}$ mice have an abnormal morphology. In particular, most of the cells retain a nucleus. Interestingly, definitive erythroid cells of mice with deleted βmin and βmaj genes have morphological abnormalities that are more like those found in human β-thalassemia than those found in EKLF$^{-/-}$ fetuses.[231] This was the first indication that the β-globin genes are not the only target genes of EKLF. Stronger evidence that non-globin EKLF target genes contribute to the definitive red blood cell abnormalities and prenatal death in EKLF$^{-/-}$ fetuses came from a transgenic rescue study. EKLF$^{-/-}$ mice could not be rescued by expression of exogenous human γ-globin. Despite efficient production of hybrid hemoglobin, consisting of mouse α- and human γ-globin in the fetal livers, hemolysis was not corrected and survival was not prolonged.[168] This strongly suggests that EKLF regulates more essential genes in erythropoiesis. In Chapter 3, we describe a number of such non-globin EKLF target genes.

Domains in EKLF
The gene coding for EKLF in the mouse is located at chromosome 8.[110] Three exons code for a 357 amino acid protein of approximately 38 kD.[147] The cDNA contains two potential translation start sites. The second ATG site, corresponding to Met_{19} in the open reading frame appears to be exclusively used as the start codon. In the literature, the amino acids are commonly numbered from the first ATG site as in Figure 4.
The genomic structure is highly conserved between mice and human.[22,218] The DNA binding domain, consisting of three Cys2His2 zinc fingers, is the most conserved part. The homology of this domain between both genes is about 90%. Consequently, the proteins are believed to recognize the same DNA sequences with very similar affinity. In mice, the zinc fingers span the region from aa293 to aa376.
Since EKLF is a nuclear protein, it has to be imported from the cytosol to the nucleus. The C-terminal domain, more precisely aa275-aa376, contains two separable Nuclear Localisation Signals (NLS).[167,180] The first NLS is a basic domain adjacent to the zinc fingers, aa275-aa296, and is called NLS1. The zinc finger domain itself (aa293-aa376) is called NLS2 and is more efficient than NLS1. However, the most efficient nuclear localisation is found with the entire domain. Importins, that are involved in the import of proteins from the cytosol to the nucleus,[136] can bind this domain. Basic amino acids are essential for this binding; after mutation of the basic amino acids in the NLS of EKLF to alanine, the protein accumulated in the cytosol. Loss of the tertiary zinc finger structure could not account for the altered localisation; EKLF with histidine to alanine point mutations in the first zinc-chelating histidine of all three fingers was exclusively found in the nucleus.[167] These mutations are known to destabilise the zinc finger tertiary structure.[46] Interestingly, the basic residues of all mammalian Krüppel zinc fingers are highly conserved, indicating that they form a common NLS shared by all Krüppel family members.[167] The N-terminal basic region is only shared by EKLF, LKLF and GKLF.[23]

Figure 4. Structure of mouse EKLF
The amino acids of the EKLF protein are numbered 19 to 376. The DNA binding domain (aa293-aa376) consists of three zinc fingers (ZF1 to ZF3). The C-terminal domain also contains Nuclear Localisation Signals (aa275-aa376). aa19-aa292 is proline rich and contains two transactivation domain (TD). CKII recognizes the consensus TQED and phosphorylates T41. Both K288 and K302 are acetylated by CBP. The posttranscriptional modifications of T41 and K288 play an important role in the transactivation potential of EKLF.

The proline-rich amino-terminal domain, aa19-aa292, is called the transactivation domain and can be separated from the DNA binding domain.[24] Two sub-domains are reported to enhance transactivation.[41,166] The first transactivation domain is located in the first 40 amino acids of the protein. The second consists of 85 amino acids at position aa158-aa243. Both transactivation domains, bound to the DNA binding domain, activate a reporter gene to wild type levels. This indicates that these domains are functionally redundant.[166] In this respect, EKLF is similar to another Sp/XKLF family member, Sp1, which also contains two potent transactivation domains that are functionally redundant.[49]
The transactivation domain contains serine and threonine residues that are phosphorylated *in vivo*.[163] The phosphorylation status of EKLF is thought to play an important role in the control of activity of the transcription factor. Threonine41 lies within a consensus that is recognized by casein kinase II (CKII) (see Figure 4). CKII is ubiquitously expressed and modifies the activity of a range of substrates by phosphorylation, including transcription factors. In the human EKLF protein, a CKII target sequence is found at the same position,[218] suggesting an important function for this site. Mutations in the consensus site indicate that threonine41 is indeed phosphorylated by CKII, and that phosphorylation of this site is essential for EKLF's transactivation activity. Although serine residues in EKLF are also phosphorylated, the significance of these modifications has not yet been investigated.

E-RC1
DNA is packaged in chromatin (see 'Chromatin and transcription'), which has a repressive effect on transcription. A number of complexes have been described that counteract this repressive effect. One of these complexes is the SWI/SNF complex.[138] It is an ATP-dependent chromatin-remodelling complex that changes the chromatin structure by altering DNA-histone contacts within nucleosomes. It has been shown that EKLF requires a SWI/SNF-related chromatin-remodelling complex for transactivation of the β-globin gene when the DNA template is packaged in chromatin. The EKLF-SWI/SNF complex generates a

DNAseI hypersensitive, transcriptionally active β-globin promoter *in vitro*. The SWI/SNF-related complex is called EKLF co-activator-remodelling complex 1 (E-RC1). Factors in this complex are homologues of yeasts BRG1, BAF170, BAF155, BAF47 and a subunit that is unique to higher eukaryotes, BAF57.[7]
EKLF is necessary for recruitment of two E-RC1 subunits, BRG1 and BAF170 near the transcription initiation site of the β-globin promoter, suggesting that the complex uses EKLF for specific targeting.[132] Further studies showed that the two subunits BRG1 and BAF155 are necessary and sufficient for targeted remodelling and transcriptional activation by EKLF *in vitro*. The DNA binding domain of EKLF is sufficient for targeting the two subunits to the β-globin promoter. However, to activate transcription the EKLF activation domain is required.[114]

Acetylation
The acetylation of histones results in general in a more open chromatin structure. Hypo-acetylated histones on the other hand are associated with transcriptionally inactive or silent chromatin.[129] The acetylation pattern of histones is controlled by two families of proteins with opposing activities; histone acetyl-transferases (HATs) and histone de-acetylases (HDACs). Surprisingly, EKLF appears to interact with proteins of both families. Transcriptional repression is often accomplished by binding with corepressors, such as mSin3a, and recruiting HDACs to the site of interest.[131] EKLF interacts with both mSin3a and HDAC1, suggesting that EKLF can function as a transcriptional repressor.[42] The two proteins bind EKLF at its DNA binding domain. It is important to note that the zinc finger domain, when bound to mSin3a and HDAC, has lost its capacity to bind DNA. Gene repression was only observed when the mSin3a-HDAC complex was targeted to a promoter by another DNA binding domain fused to EKLF or its zinc fingers. So far, there is no evidence that EKLF functions as a transcriptional repressor *in vivo*.
Since EKLF is better known as a transcriptional activator, it is less surprising that a number of studies reported interactions with acetyl-transferases. CREB-binding protein and p300 are two widely expressed factors that are believed to regulate gene expression in most cell types with their histone acetylation activity.[26] However, they can acetylate non-histone nuclear proteins as well. CBP and p300 have been shown to interact with EKLF. They bind its zinc finger domain, as well as its transactivation domain.[234] As a result of the binding of CBP and p300, EKLF is acetylated. EKLF contains 11 lysines that are potential acetylation targets. However, only K288 in the transactivation domain and K302 in the first zinc finger of the DNA binding domain are acetylated by CBP (see Figure 4).[235] Interestingly, these lysines are conserved; K302 is conserved across all KLF family members, whereas K288 is shared by EKLF, LKLF and GKLF. Thus, in addition to the phosphorylation events described earlier, acetylation is a second post-translational modification of the EKLF protein. Maximum activity of EKLF is dependent on its acetylation status. However, the DNA binding capacity of EKLF is not influenced by acetylation. Mutagenesis of K288 to alanine indicated that acetylation of this lysine in particular plays an important role in the transactivation potential of EKLF. This is explained by a higher affinity for the SWI/SNF chromatin remodelling complex.[235]

β-globin locus

The β-globin locus has been widely used as a model to study gene regulation. An important contribution to the understanding and interest in the β-globin locus is the high incidence of genetic mutations and deletions, leading to hemoglobinopathies described earlier. The strictly tissue-specific and developmentally regulated expression of the genes makes the β-globin locus an interesting model for the studies of gene regulation.

Both the human and mouse β-globin locus contain a number of genes, well-characterised promoters, enhancers and a locus control region. The genes encode for the β-globin-like proteins. Together with two α-globin-like proteins and four heme groups they form the tetrameric molecule hemoglobin, the oxygen binding protein in red blood cells.[171] All the globin genes originate from one ancestral globin gene. About 450 million years ago, this ancestral globin gene duplicated, leading to the separate α- and β-globin genes.[92] After duplication, these genes evolved independently of each other, ending up on separate chromosomes in birds and mammals. Both the α- as well as the β-globin genes duplicated several times.[80,94] For the mouse β-globin locus this led to four functional genes; εy, βH1, β-major (βmaj) and β-minor (βmin). The expression of the genes is strictly regulated during development. The genes are arranged from 5' to 3' in the same order as their developmental expression (see Figure 5); Embryonic, yolk sac-derived erythroid cells express primarily εy- and βh1-globin. Expression of βmaj and βmin is at a very low level in these primitive erythroid cells. Their expression becomes high around E11 when erythropoiesis commences in the fetal liver.[31,223,227] From this stage onwards, the embryonic genes are shut down. In the early adult erythroid cells, the βmaj to βmin ratio is about 60% to 40%. This changes to a ratio of 80% to 20% in later stages of development. These two genes appear to compete with each other for transcription, since only one of the genes is actively transcribed at any time point[210] and deletion of the βmaj-promoter and gene results in a 50% increase in βmin-gene transcription.[53,196]

A necessity for the switch in globin expression may be the competition for oxygen between blood cells from the embryo/fetus and the maternal erythrocytes. Adult-type hemoglobin would not be able to take up oxygen from the maternal blood efficiently. The early expressed globin-like genes form a type of hemoglobin that has a higher affinity for oxygen than the adult type. Therefore, oxygen can efficiently be extracted from the maternal blood.[179]

A number of *cis*-regulatory elements are important for the erythroid-specific and developmentally controlled expression of the genes in the β-globin locus. All the genes contain promoters within 200 bp 5' of the transcriptional start site. The functional promoters can be detected as DNAseI hypersensitive sites when they are active. In mammals, the globin promoter sequences are highly conserved. All promoters contain three conserved, although not completely identical regions: CACC, CCAAT and TATA boxes. Complete deletion of the embryonic gene promoters results in elimination of embryonic gene expression.[101] Single base mutations in these sequences leads to a significant decrease in level of transcription.[153] The significance of the promoters is further demonstrated by mutations that lead to thalassemia or HPFH.[157,160,230] In fact, some thalassemias are caused by mutations in the CACC box, that influence the binding capacity of EKLF.[73,128] This indicates that binding of EKLF to the CACC box in necessary for normal adult globin expression.

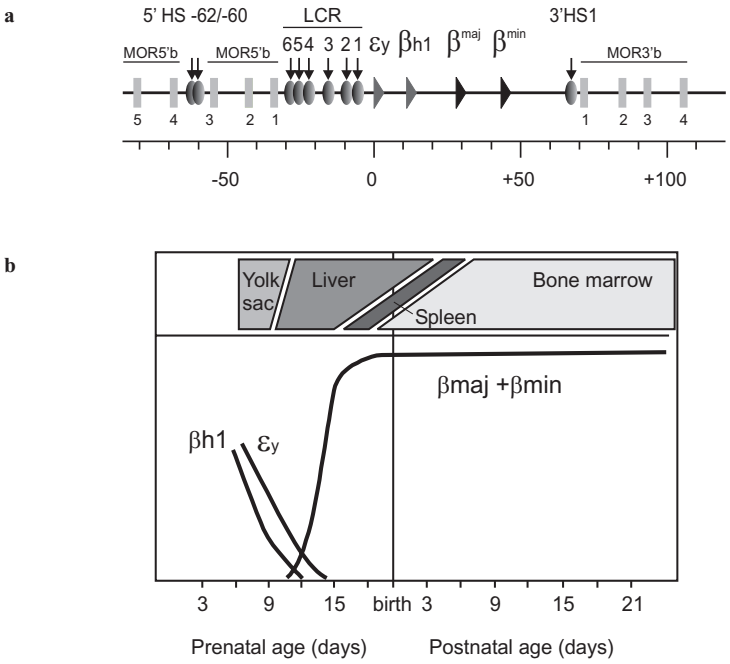


Figure 5. Mouse β-globin locus and β-globin expression
a The mouse β-globin locus contains four globin like genes (triangles) whose expression is controlled by the Locus Control Region (LCR) that consists of 6 Hypersensitive sites (HS) (ovals). Furthermore it contains 3 more distally located HS and a number of genes that are not expressed in erythroid cells (rectangles). **b** Embryonic, yolk sac derived cells express mainly εy and βh1. High expression of the definitive βmaj and βmin genes starts around E11 when erythropoiesis commences in the fetal liver. After birth, blood is mainly formed in the bone marrow.

The promoters alone are not sufficient for a controlled expression of the globin genes. The first indications that other sequences apart from the genes are necessary for normal expression came from the study of some β-thalassemia patients. The Dutch deletion-type thalassemia patients have a deletion of about a 100 kb upstream of the β-globin gene. The β-globin gene including its promoter is left intact but is not transcribed.[122] Further study of the deleted sequence revealed 5 DNAseI hypersensitive sites.[213] These HS together are called the Locus Control Region (LCR), which is necessary for normal globin gene expression. Transgenic mice with a DNA fragment in which the LCR is linked to the human β-globin gene show high level, tissue-specific and copy-number dependent expression.[89]
The LCR is an evolutionary conserved region. On the basis of sequence homology, the LCR in the mouse β-globin locus was found.[93,111,149] The mouse LCR consists of 6 HS, spread over ~30 kb upstream of the εy-globin gene. The cores of these HS contain binding sites

for transcription factors and comprise the functional components of the LCR. Complete deletion of the LCR leads to a severe reduction in expression of all mouse β-like globin genes, showing that the LCR is necessary for high-level transcription.[16] The functions of the individual HS of the mouse LCR are less extensively studied than those in the human LCR. However, the deletions of every mouse HS have been described. Individual deletion of the first four HS, 5'HS1 to 5'HS4, all have a comparable effect on the expression of the endogenous globin locus; the expression levels of the embryonic genes, εy- and βh1, are hardly reduced. However, all four deletions decrease the expression levels of the adult genes βmaj and βmin with approximately 25-30%.[18,74,103] Deletion of 5'HS5 and 5'HS6 does not result in a decreased level of gene expression.[17] Although the expression levels of the adult genes are reduced, none of the HS deletions perturbs the developmental gene expression pattern or affects the DNAseI hypersensitivity of other HS.[18] Because of the homology between the human and mouse LCR, it is assumed that the function of comparable HS is maintained during evolution. Although the individual roles of the HS remain unclear, both in the human and mouse β-globin locus, 5'HS2 and 5'HS3 appear to be the most important sites for efficient transcription.[35,64,74,76,103,172,173,175,201] The 5'HS2 of the human locus has been shown to function as a classical enhancer in both transfection and transgenic mouse studies.[54,184,214]
The core regions of the HS, several hundred base pairs, and a number of binding sites for erythroid-specific and ubiquitous transcription factors within this region are highly conserved.[13,93,158] Among these, potential EKLF binding sites are present and at least one potential binding site in human 5'HS3 is a functional EKLF binding site *in vivo*.[81,205] In transgenic mice that carry a human β-globin locus, EKLF is necessary for the formation of DNAseI hypersensitivity at 5'HS3 and the β-globin promoter.[228]
Apart from the HS in the LCR, some HS are found more upstream of the LCR and downstream of the genes. The HS found downstream of the genes in mouse and human is called 3'HS1.[213] In the mouse, two HS are found at 60.7 and 62.5 kb upstream of the εy-promotor (5'HS-60/-62), homologous to a HS found 111 kb upstream of the human ε-globin gene promoter.[68] So far, their function remains enigmatic. Deletion of 5'HS-60/-62 did not effect globin expression.[68] Interestingly, the distal HS contain sequence motifs that appear to bind CTCF.[34,69] CTCF (CCCTC-binding factor) is believed to play a central role in many chromatin insulators.[15] Insulators can block the communication between gene promoters and enhancers, and can mark a boundary between open and condensed chromatin. Therefore, these distal HS may define a physical border of the β-globin locus.

Active Chromatin Hub

Since the discovery of the LCR, a number of models have been proposed for the regulation of developmental control and high level of gene expression over such a long distance. The three basic models are the accessibility model, the scanning model and the looping model. The accessibility model proposes that the function of the LCR is merely to establish an open chromatin structure.[32,140] This would enable stage-specific factors to bind the more proximal regulatory elements of the genes. After opening of the chromatin, the genes would be acti-

vated in a stochastic manner, depending on the trans-acting factor environment. In the scanning (or tracking) model, the LCR binds the transcriptional machinery, which then scans the DNA in a unidirectional way in order to find the gene promoters.[98,212] The looping model proposes a direct interaction between the LCR and promoter, with the intervening DNA sequence looping out of the LCR-promoter complex.[178] The looping model best explains a number of properties of the (human) β-globin locus. Dillon and colleagues described the transcriptional effects of an additional marked β-globin gene in the human β-globin locus in transgenic mice.[58] The marked β-globin gene was inserted at varying positions. Comparing the expression levels of the β-globin gene and the marked β-globin gene showed that the most proximal gene to the LCR has the highest expression. Furthermore, the relative expression of the most proximal gene decreased when positioned more closely to the more distal gene. The accessibility model would predict an equal transcription of the two genes, irrespective of their position. The scanning model cannot explain differences in expression at varying distances between the genes. After insertion of the marked β-globin gene, the combined expression of the two genes equals that of the expression of the β-globin gene in the wild type locus. This suggests that the two genes compete for expression, which is best explained by the looping model. Two other papers further demonstrate that multiple genes in the globin locus are transcribed alternately rather than simultaneously.[88,229] These findings have resulted in the proposal of a flip-flop mechanism, in which the genes alternately form a complex with the LCR to be actively transcribed.

In 2002 two techniques were developed with which it was finally demonstrated that the LCR is in close proximity to the actively transcribed gene.[39,208] One approach is called RNA TRAP (tagging and recovery of associated proteins).[39] Primary transcripts are hybridised with a horseradish peroxidase (HRP) labelled probe. Biotin-tyramide is added, which, under the control of the localized peroxidase becomes a highly reactive radical intermediate that covalently attaches to chromatin proteins in the immediate vicinity. This deposition on chromatin can be quantified and is an indication for its vicinity to the transcribed gene.

The second technique is called chromosome conformation capture (3C).[56] Cells are crosslinked with formaldehyde, followed by restriction enzyme digestion of the DNA. After de-crosslinking, the samples are ligated under conditions that favour the ligation of DNA fragments that are physically connected through the crosslinks. Quantitative PCR across the junctions is used to determine the relative crosslinking frequencies between restriction fragments in the locus. This is an indication of the nuclear proximity of DNA fragments *in vivo*. The advantage of the first technique is that it uses primary transcripts. Therefore, the results are obtained only from cells that are actively transcribing the gene of interest. An advantage of the 3C technique is that interactions between DNA fragments over an entire locus can be analysed, regardless whether it is transcriptionally active or not. With this technique it was shown that the actively transcribed gene is not only in close proximity to 5'HS2, but to all of the HS in the LCR and to the three distal HS; 5'HS-62/-60 and 3'HS1.[208] This complex is called the Active Chromatin Hub (ACH) (Figure 6). In erythroid precursors that do not express globin yet, a substructure of the ACH is found. This consist of (at least) the distal HS and the HS at the 5' site of the LCR. For active transcription, the gene that will be transcribed and the HS at the 3' site of the LCR participate to form the

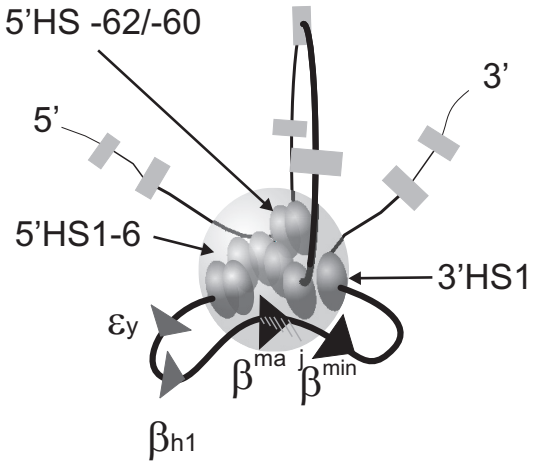


Figure 6. Active Chromatin Hub
In erythroid cells, the β-globin locus is spatially organised into an ACH that consists of the hypersensitive sites and the actively transcribed gene.

complete ACH. In embryonic cells the actively transcribed gene is either εy or βH1, in adult cells βmaj or βmin.[165]

An important function for the ACH may be the spatial clustering of binding sites for *trans*-acting factors. As a result, the local concentration of these factors will increase, which is necessary for efficient transcription.[38,43,55,62,108] Thus, the ACH is proposed to represent a nuclear compartment dedicated to RNA polymerase II transcription of β-globin genes.

It is not known how the separated *cis*-regulatory elements establish an ACH. Three models have been proposed, random collision, tracking[25] and linking.[33] The random collision model proposes that the flexibility of the chromatin permits transcription factors, bound to regulatory sequences, to randomly contact each other. Complex formation depends on the affinity between the proteins.[55] The tracking and linking models are basically combinations of the earlier proposed models for enhancer function, with the addition of the end result: i.e. that the LCR is in close proximity to the actively transcribed gene. Both models assume a linear propagation of a signal along intervening DNA from the LCR to the promoter. The tracking model proposes that this signal is an enhancer-bound protein complex, the linking model proposes a growing chain of proteins. Distinguishing between these models is quite difficult. So far, very little is known about the dynamics of ACH formation, or the proteins involved. In Chapter 4 we show that EKLF is indispensable for the completion of the ACH. In the absence of EKLF, an ACH substructure similar to that observed in erythroid precursor cells was found. Apparently this substructure is formed independent of EKLF. However, the completion of the ACH, which entails the participation of the actively transcribed gene and the HS at the 3' site of the LCR, requires the presence of EKLF.

Scope of the thesis

This thesis describes experiments that shed light on the role that the transcription factor EKLF plays in erythroid gene regulation.
Chapter 2 describes an *in vitro* culture method with which molecular aspects of erythropoiesis can be studied. Primary mouse erythroid cells are kept proliferating as progenitors. The cells undergo synchronous terminal differentiation after induction with physiological agents.
Chapter 3 describes genes whose expression is controlled by EKLF. Using the *in vitro* culture method, described in Chapter 2, and micro array experiments, we found a number of genes that are not upregulated during terminal differentiation of adult erythroid cells in the absence of EKLF. Apart from the target genes in adult erythrocytes, we found target genes, AHSP and Epb4.9, whose expression is EKLF dependent in primitive cells as well. The absence of AHSP and Epb4.9 in primitive cells in EKLF null fetuses causes precipitation of globin chains and an instable cell membrane.
The βmaj gene is a known EKLF target. Its transcription requires a spatial conformation of the globin locus called the Active Chromatin Hub. This consists of the *cis*-regulatory HS sites and the actively transcribed gene. In **Chapter 4** we describe that in the absence of EKLF a substructure of the ACH is formed, similar to the substructure found in erythroid progenitor cells that do not yet express globin. Thus, we propose a model in which EKLF is necessary for the completion of the substructure into a complete and fully functional ACH.

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Chapter 2

Establishment of an EKLF knock out cell line

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Introduction

To study molecular processes in erythropoiesis, it is desirable to have a culture system in which cells undergo a synchronous, *in vivo*-like differentiation program. Terminal differentiation includes a limited number of cell divisions, cell size reduction, accumulation of hemoglobin and eventually enucleation of the cells.[1] Most established erythropoietic cell lines have a leukemic origin. Therefore, it is unlikely that their differentiation program is equivalent to that of differentiating cells *in vivo*. This is in fact indicated by a relative low accumulation of hemoglobin. In Friend-virus induced murine erythroleukemia (MEL) cells, hemoglobin constitutes only 10% of total protein after 96 hours of differentiation,[8] whereas protein extracts from erythrocytes of peripheral blood contain about 95% hemoglobin.[7] Furthermore, MEL cell differentiation is induced by non-physiological reagents such as dimethyl sulfoxide (DMSO). Another cell line, the v-myc/v-raf transformed J2E cell line, does initiate differentiation upon induction by the physiological agent erythropoietin (EPO). However, these cells do not perform an *in vivo*-like differentiation program with respect to a limited number of terminal cell divisions; the cultures continue to proliferate at the time of highest globin expression.[9]

Another culture system was developed in which erythropoietic progenitors proliferate and can undergo a synchronous terminal differentiation program. Originally this culture method was applied to avian erythropoietic progenitors[22] and later to mouse erythropoietic progenitors.[5,20] In this culture system, pro-erythroblasts are kept proliferating under the control of EPO, Stem Cell Factor (SCF) and the glucocorticoid hormone dexamethasone (Dex). EPO is necessary during proliferation of pro-erythroblasts, as well as during the terminal differentiation of the cells. It prevents the cells from undergoing apoptosis. Erythropoietic cells become EPO-dependent after the CFU-E-stage of differentiation; mice lacking the EPO-receptor die around day 12.5 of gestation from failure of definitive erythropoiesis.[25] The fetuses produce normal BFU-E and CFU-E progenitors, but fail to produce hemoglobinised erythrocytes.[10] The combination of SCF and Dex supports cell proliferation. The receptor for SCF, c-Kit, is expressed on the first erythropoietic progenitors, up to the stage of the basophilic erythroblast.[19] In the absence of SCF or c-Kit, mice suffer from severe anemia.[24] SCF increases the number of cell divisions before progenitors mature in erythrocytes.[14] Dex regulates gene expression via the glucocorticoid receptor (GR). The GR is not erythroid-specific and mice lacking the GR die at birth with impaired development of several organs.[4] Glucocorticoids (GCs) are expressed mainly as a result of stress to maintain homeostasis.[2] Situations in which stress erythropoiesis is induced are blood loss, hemolysis and conditions of oxygen deprivation. As a result of the released GCs the number of erythroid progenitors is expanded through additional cell divisions.[3] Therefore, the proliferation of pro-erythroblasts *in vitro* under the control of EPO, SCF and Dex, is thought to be related to stress erythropoiesis. Terminal differentiation is induced by removal of Dex and SCF and a tenfold increase of the EPO concentration. Under these conditions, cells undergo four to five rapid cell divisions, accumulate hemoglobin, the nucleus condenses and eventually the cells enucleate.[5,20] The pro-erythroblasts are typically isolated from day 12.5 mouse fetal livers. Hence, this is a culture system in which primary pro-erythroblasts undergo a reliable *in vivo*-like differentiation following expansion and synchronisation.

Using this culture system, primary erythroblasts can be kept proliferating for 15-20 days. This results in an expansion of more than 10,000-fold. When cells are kept longer in the proliferation medium, they undergo abortive differentiation and/or cell death. To overcome this problem, an immortalised pro-erythroblast cell line was established from fetal liver cells of $p53^{-/-}$ mice. p53 is a tumor suppressor gene; in absence of p53 hematopoietic cells are immortalised rapidly and spontaneously.[12] After long term proliferation and cloning an immortalised pro-erythroblast cell line was established, termed the I/11 cell line. Like the short term proliferating cells, these immortalised cells are characterized as pro-erythroblasts by their high expression of c-Kit and transferrin receptor, low levels of Ter-119 and lack of expression of markers for myeloid, lymphoid, or multipotent progenitors.[20] Proliferation of I/11 cells, like primary pro-erythroblasts, dependents on EPO, SCF and Dex. Furthermore, upon induction the cells undergo the characteristics of terminal differentiation such as size reduction, massive hemoglobinisation and enucleation.[20] The gene expression profiles of the primary erythroblasts and the I/11 cells have been studied revealing very similar expression patterns during differentiation. Genes with known functions follow the expected expression pattern.[5] Examples are; the up regulation of Bcl-X_L, an anti-apoptotic member of the Bcl-2 gene family;[6] down regulation of the receptor for SCF, c-Kit[19] and the glucocorticoid receptor.[23] The transcription factors GATA-1[26] and EKLF are both up regulated during the initial stages of differentiation and down regulated at late stages of differentiation.
We have used this culture system to study the erythroid-specific transcription factor EKLF in erythropoiesis. $EKLF^{-/-}$ mice die around day 14 of fatal anemia, because of a deficit of β-globin expression.[15,17] However, the phenotype cannot be rescued by exogenous expression of human γ-globin, despite production of sufficient amounts of hemoglobin,[16] indicating that EKLF regulates other essential genes during definitive erythropoiesis. Although EKLF is necessary for normal definitive erythropoiesis, fetuses lacking the transcription factor develop a fetal liver with normal numbers of CFU-E progenitors. This recently established culture system provides an opportunity to expand $EKLF^{-/-}$ erythroid progenitors and study terminal differentiation in the absence of EKLF. In the current chapter we describe our efforts at generating an $EKLF^{-/-}$ immortalised cell line while in Chapter 3 we describe the phenotype of $EKLF^{-/-}$ definitive erythroblasts and the discovery of a number of novel EKLF target genes.

Results and Discussion

Concordant to the establishment of the I/11 culture, we attempted to make an immortalised $EKLF^{-/-}$ cell line by using p53-deficient cells. We therefore crossed heterozygous $EKLF^{+/-}$ mice with heterozygous $p53^{+/-}$ mice. The $EKLF^{+/-}$ mice carried a copy of the human -globin locus.[18] Subsequently, $EKLF^{+/-}$ $p53^{+/-}$ mice were crossed and fetal livers were isolated at day 12.5 of gestation from a number of litters. The litter that we focus on in this chapter consisted of 7 embryos, including 1 $EKLF^{+/+}$ and 1 $EKLF^{-/-}$. These two embryos were also deficient for p53. The fetal livers were resuspended in proliferation

medium. During the first 12 days of proliferation, the p53 genotype did not appear to have an effect on the cell cultures. In all cultures the cells were kept at a density of about 2 million cells per ml, and the number of cells doubled every 24 hours. The EKLF$^{-/-}$ cells grew slightly better than wild type and heterozygous cells; the culture expanded slightly faster and fewer smaller, differentiating, cells were formed compared to the controls. During proliferation of the pro-erythroblasts, a small number of cells escape the self-renewing program and undergo 'spontaneous' terminal differentiation. This is indicated by the appearance of small cells in the culture. In the absence of EKLF and its target genes the cells are apparently less prone to start their terminal differentiation programme, resulting in slight proliferative advantage. There are no signs of increased apoptosis as has been observed in pro-erythroblasts of some other key regulators of erythropoiesis such as GATA-1[21] and the erythropoietin receptor.[10]

After 10 days of proliferation, parts of the cultures were induced to terminally differentiate. Wild type and EKLF$^{+/-}$ cells displayed *in vivo*-like differentiation, based on the observation that they underwent 4 to 5 rapid cell divisions accompanied by cell size reduction, accumulated hemoglobin and eventually enucleated. EKLF$^{-/-}$ cells underwent rapid cell divisions as well, but their cell size did not decrease as much as that of wild type and heterozygous cells. Furthermore, in the absence of EKLF there was hardly any accumulation of hemoglobin (data not shown; similar to data in Chapter 3).

In several experiments, cultures of cells containing functional p53 became stationary after about two weeks of proliferation, showing increased terminal differentiation and cell death, in agreement with results from von Lindern et al.[20] (data not shown). We therefore only continued with cultures of p53 null cells (cultures 1, 2 and 5, EKLF$^{+/+}$, EKLF$^{-/-}$ and EKLF$^{+/-}$ respectively). Mature cells were removed by centrifugation through Ficoll at regular intervals. After 5 weeks of proliferation, the cultures were again tested for their differentiation capabilities. Cell size distribution and hemoglobin content were measured at regular time points during differentiation. The differentiation results of these cells after 5 weeks of proliferation were comparable to the results after 10 days of proliferation. Both EKLF$^{+/+}$ and EKLF$^{+/-}$ cells accumulated hemoglobin, whereas the EKLF$^{-/-}$ cells failed to do so (see Figure 1). The cell size distribution during the first 24 hours is similar for all three genotypes. After

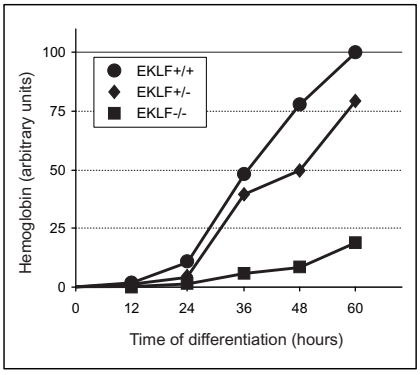

Figure 1. Hemoglobin accumulation
Hemoglobin accumulation of pro-erythroblasts after 5 weeks of proliferation upon induction is comparable to the one found in primary erythroblasts (see Chapter 3). Hardly any hemoglobin is formed in EKLF$^{-/-}$ cultures compared to EKLF$^{+/-}$ and EKLF$^{+/+}$ cultures. The values in the graph represent the amount of hemoglobin per cell volume in arbitrary units (see materials and methods).

24 hours, the cell size in EKLF$^{+/+}$ and EKLF$^{+/-}$ decreases and subsequently small, enucleated cells are observed by the appearance of a second peak. In the EKLF$^{-/-}$ cultures, the mean cell size decreases less and no enucleation is observed (see Figure 2).
Next, we wished to establish stable, clonal cultures for all three genotypes. After six weeks of continuous growth, EKLF$^{+/+}$, EKLF$^{+/-}$ and EKLF$^{-/-}$ cells from the mass cultures were seeded in serum-free semi-solid medium (StemPro-Methocult). After 10 days, 60 colonies of each genotype were isolated and expanded in proliferation medium. Half of these colonies, the ones that proliferated best, were partially induced for differentiation. After 2.5 days, hemoglobin content was measured. On the basis of proliferation rates and hemoglobin accumulation after induction, we continued with cultures of 7 EKLF$^{+/+}$, 6 EKLF$^{+/-}$ and 8 EKLF$^{-/-}$ colonies. In an additional differentiation experiment, the EKLF$^{+/-}$ cultures and 3 of the EKLF$^{+/+}$ cultures did not accumulate significant amounts of hemoglobin and these cultures were discarded.

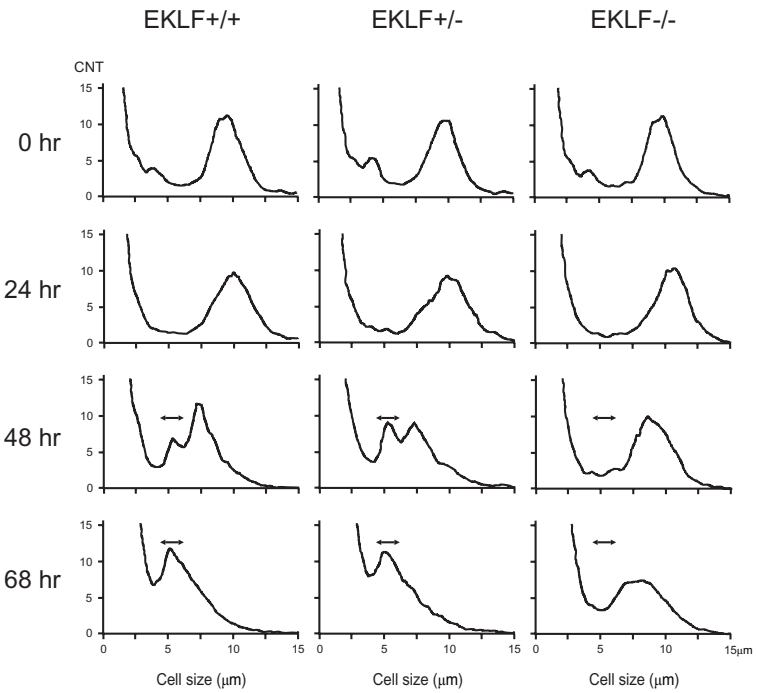

Figure 2. Cell size distribution
Pro-erythroblasts that have been cultured for 5 weeks show a similar cell size distribution upon induction as primary pro-erythroblasts (compare with Figure 1a in Chapter 3). The arrow indicates the cell size of enucleated cells. All cultures have a similar cell size distribution regardless of their genotype. Upon induction, the size of wild type and heterozygous cells decreases and after 48 hours enucleated cells appear. This is not found in absence of EKLF.

To test whether the EKLF$^{-/-}$ phenotype in the cloned cultures could be rescued, we wanted to rescue the cells with exogenous expression of EKLF. Therefore a construct was cloned in the retroviral expression vector pBABE-puro.[13] The construct codes for EKLF fused in frame with the hemagglutinin (HA) epitope tag. Two EKLF$^{+/+}$ and 4 EKLF$^{-/-}$ cultures were transfected with HA-EKLF-pBABE-puro or empty pBABE-puro virus. Unfortunately, all the cultures failed to undergo a complete differentiation program. The EKLF$^{-/-}$ cultures transfected with the HA-EKLF transgene did not accumulate more hemoglobin during differentiation than cultures transfected with empty vector. Moreover, in the same differentiation assay, a non-transfected EKLF$^{+/+}$ culture accumulated the same amount of hemoglobin as a non-transfected EKLF$^{-/-}$ culture. Based on these negative results, we decided to focus on the short-term cultures, as we obtained very promising results with such cultures (see Chapter 3). However, because we are now more experienced in culturing pro-erythroblasts, it might be worthwhile to perform a new series of experiments aimed at the derivation of immortalised EKLF$^{-/-}$ erythroid cell lines. The I/11 cell line is an immortalised cell line derived from a p53$^{-/-}$ fetal liver, that can still undergo a faithful differentiation program.[5,20] During the selection of this cell line, a considerable number of cells were found to be aneuploid. In the attempts to derive immortalised EKLF$^{-/-}$ cell lines described here, we did not check the karyotypes of the cells. Therefore, aneuploidy may have contributed to the disappointing differentiation results of the cloned cells.

Apart from the I/11 cells, another indication that p53$^{-/-}$ pro-erythroblasts are relatively easy to immortalise is the establishment of the R1 and R10 cultures. These cultures were started in the same period as the cultures described in this chapter. None of the embryos were both EKLF- and p53-deficient. The R1 and R10 cultures are derived from EKLF$^{+/+}$ p53$^{-/-}$ embryos. Furthermore they carry the human β-globin locus. These cultures have been cloned successfully and have been proliferating for more than a year. All the cloned cultures still perform a reliable terminal differentiation upon induction. In our laboratory, these cultures are now frequently used as an alternative to the I/11 cells, having the benefit of the presence of the human β-globin locus. This indicates that p53$^{-/-}$ pro-erythroblasts are relatively easy to obtain, providing the means to generate erythroid cell cultures with different genetic make-ups with an unlimited life span, yet retaining primary erythroid progenitor behaviour with respect to differentiation characteristics. The availability of mice carrying a transgene coding for a tamoxifen-inducible EKLF and the human β-globin locus provides a particularly attractive system for the generation of immortalised erythroid cell lines, since such mice could be crossed with the EKLF knockout. Tamoxifen inducibility of the EKLF could then be used to full advantage to study the kinetics and sequence of events leading to β-globin gene activation by EKLF (see Chapter 4), and activation of other EKLF target genes (see Chapter 3). Such a cell line would be amenable to use the complete array of tools available in modern biomedical sciences, such as chromatin immunoprecipitation assays, DNA micro arrays, and RNA interference experiments. However, the important issue of γ- to β-globin switching would still have to be addressed in mice, since cultured cells almost invariably switch to the expression of adult-type globins after a few days in culture.

Materials and methods

Cultivation of murine erythroid progenitors
Fetal livers of day 12.5 mouse fetuses were disrupted and seeded into Stem-Pro-34™ medium supplemented with human recombinant erythropoietin (Erypo, Cilag AG, Switzerland, 1 U/ml), murine recombinant stem cell factor (SCF, R&D Systems, Minneapolis, MN, USA, 100 ng/ml) and dexamethasone (Dex, Sigma, 10^{-6} M). Cell density was kept at approximately at $2x10^6$ cells/ml by adding fresh medium. Typically the cultures were diluted twofold daily. Cell numbers and size distributions were determined daily, using an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany). Pro-erythroblasts were enriched through Ficoll (lymphocyte separation medium, 1078 g/cm^3, Eurobio, France) by centrifugation, when the cultures contained >40% dead and/or differentiated cells as estimated by size distribution.

Cloning of immortalised cell lines
After six weeks in culture, $p53^{-/-}$ erythroblasts were seeded into semisolid medium (Methocel-containing StemPro-34 SFM, Life Technologies, Gibco BRL) containing SCF, EPO and Dex as above. After 7-10 days of incubation, large colonies were obtained. Of each genotype ($EKLF^{+/+}$, $EKLF^{+/-}$ and $EKLF^{-/-}$) 60 clones were picked from dishes with well-separated colonies, and cultivated in 96-well plates. After ~4-6 days, colonies were inspected visually. Clones containing either a high proportion of mature cells, cells of irregular size/shape or exhibiting a slow proliferation rate were discarded. The remaining clones (15, 20 and 34 respectively) were expanded and partially set up to differentiate. On the basis of hemoglobin accumulation in the differentiation experiment and proliferation kinetics, 8 clones of each genotype were kept in culture.

Terminal differentiation
Pro-erythroblasts were washed twice in PBS and seeded at $1.5x10^6$ cells/ml in differentiation medium (Stem-Pro-34™ medium supplemented with EPO (10 U/ml) and iron-saturated human transferrin (Sigma, 1 mg/ml). Cells were maintained at densities of $2\text{-}4x10^6$ cells/ml, requiring daily dilution with fresh medium, between 24 and 50 h requiring dilution twice a day. Cell number and size distribution were determined using an electronic cell counter (model CASY1; Schärfe Systems). Hemoglobin content was quantified in a photometric assay as described.[11] In short, 2–4 x 10^4 cells were washed in PBS, lysed in 20 µl H_2O, and frozen until further analysis. 100 µl of reagent mix (0.5 mg/ml o-phenylenediamine (Sigma-Aldrich) and 0.03% H_2O_2 in 0.1 M citrate/phosphate buffer, pH 5.0) was added to thawed samples. The reaction was stopped after ~2 min. with 20 µl 8 N H_2SO_4. The 492 nm extinction of the reaction product was measured with an ELISA spectrophotometer. Extinction/(cell number x average cell volume) was taken as a measure for hemoglobin content.

Transfection of EKLF construct
A construct coding for a fusion protein of EKLF with an HA-tag was cloned in the retroviral expression vector pBABE-puro. The construct was transiently expressed in Phoenix cells. 40 h after transfection, cells were treated with 10 µg/ml mitomycinC (Kyowa Hakko

Kogyo) for 1 h and washed with PBS, followed by two additional washes after an interval of >4h. Two $EKLF^{+/+}$ and four $EKLF^{-/-}$ cloned cell cultures (0.5 x 10^6/ml) were added and co-cultured for 24 h in proliferation medium. The cells were removed from the Phoenix cells and grown in 2 µg/ml puromycin-containing proliferation medium. The capacity to terminally differentiate was determined as described above.

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Chapter 3

The erythroid phenotype of EKLF null mice: defects in hemoglobin metabolism and membrane stablility

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Abstract

Development of red blood cells requires multiple cellular processes including changes in cell morphology, globin expression and heme synthesis. Gene ablation studies have revealed transcription factors critical in this process, such as erythroid Krüppel like factor (EKLF or Klf1). Mice lacking EKLF die around embryonic day 14 because of defective definitive erythropoiesis, partly caused by a deficit in β-globin expression. To identify additional genes contributing to the severity of the EKLF knockout, we analysed the phenotype and gene expression profiles of wild-type and EKLF null primary erythroid progenitors that were differentiated synchronously *in vitro*. We show that EKLF is dispensable for the expansion of erythroid progenitors, but required for the last steps of erythroid differentiation. We identify novel EKLF-dependent genes involved in hemoglobin metabolism and membrane stability, providing an explanation for the severity of the EKLF null phenotype. Strikingly, expression of these genes is also EKLF-dependent in primitive, yolk sac derived blood cells. Consistent with lack of upregulation of these genes we find previously undetected morphological abnormalities in EKLF null primitive cells.

Introduction

The anemias, caused by failure of erythropoiesis, constitute the most common human genetic disorders. Most of these disorders are caused by mutations or deletions in the coding sequences or regulatory elements of the α- or β-globin genes.[41] A number of these mutations have marked the importance of specific promoter elements, such as the β-globin CACC box. Mutations within this box result in severe down regulation of β-globin gene expression.[8,9,20] The CACC box motif is found in many erythroid gene promoters;[33] it is a binding site for the transcription factor erythroid Krüppel-like factor (EKLF or KLF1), a member of the SP/XKLF transcription factor family.[9,25] An 81 amino acid DNA binding domain, found close to the C-termini of all members, defines this family. The binding domain consists of a combination of three conserved Cys2His2-type zinc fingers.[31] The expression of EKLF is largely restricted to the erythroid lineage,[25,36] although expression in macrophages has been reported recently.[23] Mice lacking EKLF die *in utero* around embryonic day 14 (E14) from severe anemia associated with a marked deficit in β-globin expression.[28,30] The time of death coincides with the stage of development in which the fetuses become dependent on definitive, fetal liver-derived, erythroid cells, that take over the oxygen transport from the primitive, yolk sac-derived cells. So far, no abnormalities in EKLF$^{-/-}$ primitive cells have been described. However, EKLF is expressed in these cells.[36] Although the expression of the embryonic εy and βh1 globin genes is unaffected by the absence of EKLF, the low expression rate of the adult βmaj-globin gene in these cells is EKLF-dependent.[39] Furthermore, LacZ reporter transgenes have been used to demonstrate that EKLF can act as a transcriptional activator in embryonic erythropoiesis.[38]

The fatal anemia caused by the absence of β-type globin protein in definitive erythrocytes, can not be rescued by expression of exogenous γ-globin (the human fetal β-type globin) despite an efficient production of hybrid $\alpha_2\gamma_2$ hemoglobin.[29] Moreover, Lim et al. demonstrated that EKLF$^{-/-}$ ES cells injected into blastocysts do not contribute to the mature erythrocyte compartment, although EKLF$^{-/-}$ cells were found as erythroid progenitors. This phenotype was ameliorated but not completely rescued by γ-globin expression in these EKLF$^{-/-}$ cells.[22] The failure of γ-globin to restore hemoglobin synthesis and maturation of erythroid progenitors could be due to a failure in heme synthesis. Heme synthesis is tightly coupled to globin gene expression.[26] Heme is synthesized from succinyl CoA and glycine in seven enzymatic steps. It has been suggested that EKLF plays a role in the control of expression of some of these genes.[37] Two critical enzymes of the heme synthesis pathway, Alas2 and Pbgd, contain potential EKLF binding sites.[32,35] Alas2, the erythroid-specific isoform of Alas, is upregulated during erythroid differentiation.[10] Expression of Alas2 is critical for erythropoiesis and cannot be compensated for by expression of Alas1.[42]

In addition, EKLF may regulate other genes that are of vital importance in definitive erythropoiesis. To study the role of EKLF in erythropoiesis and identify novel EKLF-dependent genes, we employed *in vitro* cultures of primary erythroid progenitors that undergo a synchronous differentiation program.[6,40] Progenitors from mouse fetal livers proliferate in serum-free medium under the control of Erythropoietin (Epo), Stem Cell Factor (SCF) and the glucocorticoid hormone dexamethasone (Dex). When exposed to Epo in absence of SCF and Dex, the cells undergo synchronous terminal differentiation. Differentiation of wild type progenitors mimics *in vivo* erythropoiesis as the cells undergo three to four rapid cell divisions accompanied by a decrease in cell size and the accumulation of hemoglobin. Finally the cells expel their nucleus.[40] In addition, erythroid genes are expressed in the appropriate temporal order during this process.[6] This differentiation model of primary cells provides a unique opportunity to study gene expression in the absence of EKLF.

In this paper we describe the phenotype of differentiating primary definitive erythrocytes in the absence of EKLF. Our data suggest that EKLF is dispensable for erythropoiesis up to the pro-erythroblast stage, but is essential for completing the terminal differentiation program. We demonstrate that EKLF is required for the activation of erythroid-specific genes that are important for stabilization of the cells. Furthermore, we show that expression of these novel EKLF-dependent genes is severely reduced in primitive erythroid cells, strongly indicating that EKLF is a positive regulator of endogenous genes in these cells. We find morphological abnormalities in EKLF$^{-/-}$ primitive cells that are consistent with the reduced expression of these genes. Collectively, our data provide an explanation for the hitherto unexplained severity of the EKLF null phenotype in definitive cells. The genes identified in this study are good candidates for modifier genes affecting the severity of the clinical symptoms of thalassemia and sickle cell anemia patients.[3,41]

Results

EKLF is required for the in vitro differentiation of primary progenitors

To study the role of EKLF in definitive erythropoiesis, we expanded erythroid progenitors from fetal livers and studied their expansion and differentiation kinetics.[6,40] E12.5 fetal livers of wild type and EKLF$^{-/-}$ embryos were resuspended in serum-free medium supplemented with Epo (1U/ml), SCF (100ng/ml) and Dex (10^{-6}M) and erythroid progenitors were expanded for 10 days. The absence of EKLF did not influence the growth proliferation capacity of the cultured progenitors. EKLF$^{-/-}$ cells proliferated with similar vigour as wild type cells (data not shown). Differentiation of the cultures was initiated by replacing the renewal factors SCF and Dex with transferrin and high concentrations of Epo (10U/ml). For the wild type erythroid progenitors this resulted in the execution of the terminal differentiation program, which is characterized by three to four accelerated 'differentiation' divisions, cell size reduction, accumulation of hemoglobin and finally enucleation. Although EKLF$^{-/-}$ cells proliferated normally, they failed to undergo normal terminal differentiation (Fig. 1). First, the size of wild type cells decreased significantly. After 48 hours of differentiation, enucleated cells with a diameter of about 5 μm started to appear. In absence of EKLF, the cell size distribution barely changed upon induction of differentiation and the cells failed to undergo enucleation (Fig. 1a). Second, wild type cells accumulated large amounts of hemoglobin after 24 hours of differentiation. In the absence of EKLF however, the amount of hemoglobin formed was 10-fold lower than in wild type cells (Fig. 1b). Third, cytospins of the differentiating cultures show that there are no morphological differences between wild type and EKLF$^{-/-}$ cells under renewal conditions (T=0; Fig. 1c). Upon induction of differentiation, however, wild type cells showed all hallmarks of differentiation such as decreased cell size, hemoglobin accumulation and enucleated cells, while the EKLF$^{-/-}$ progenitors initially retained a large cell size. Eventually, their nuclei became pycnotic (Fig. 1c). In conclusion, the data show that the phenotype of EKLF-deficient mice[28,30] is not due to a failure of EKLF-deficient erythroid progenitors to expand, but that EKLF is specifically required to execute the terminal differentiation program. Furthermore, our results validate the use of this culture system for the detection of EKLF-dependent genes.

TER-119 positive erythroid cells are lacking in EKLF$^{-/-}$ fetal liver

The TER-119 specific antibody is widely used to detect and enrich erythroid cells. This antibody specifically stains erythroid cells that are well advanced in terminal differentiation. TER-119-positive cells show no erythroid blast-forming unit (BFU-E) and erythroid colony-forming unit (CFU-E) activities, and therefore represent cells at later differentiation stages.[17] We have used the TER-119 antibody during our studies of erythropoiesis in EKLF null mice. Cells of E12.5 fetal livers were analysed by FACS using the TER-119 antibody, and 7-amino-actinomycin D (7-AAD) as a marker for dead cells. In wild type fetal livers, the majority of the cells were positive for TER-119, and negative for 7-AAD (~66%) (Fig. 2). However, in EKLF$^{-/-}$ fetal livers, <1% of the cells were TER-119 positive. In liver cells of heterozygous fetuses, the percentage of TER-119 positive, 7-AAD negative cells was reduced (~49%) compared to the wild type fetal livers. We also tested embryonic blood of E11.5 and E12.5 fetuses for TER-119 staining. In wild type fetuses ~75% of the blood cells

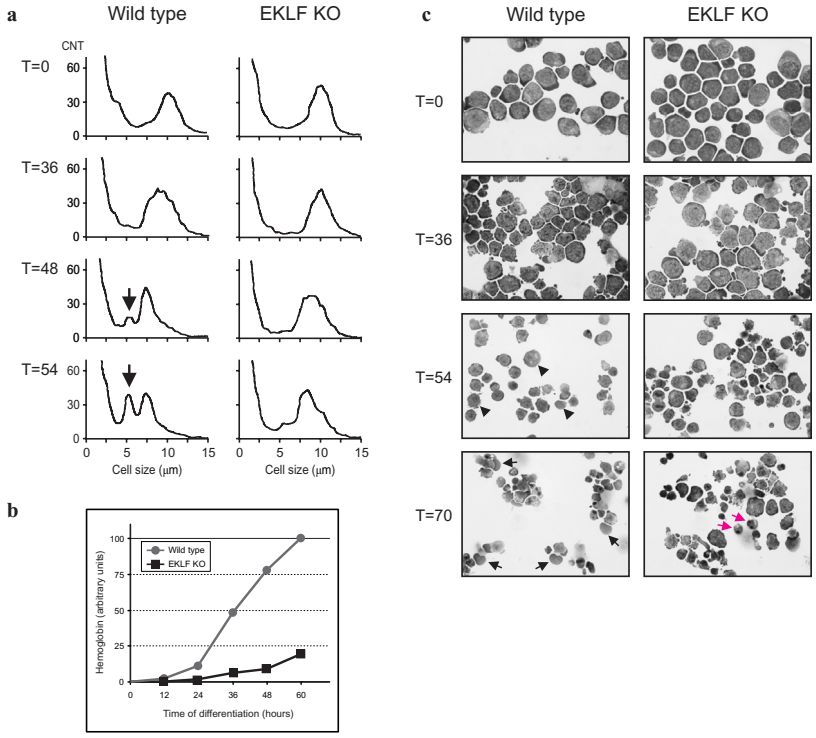


Figure 1. Differentiation of primary erythroid progenitors
a Cell size distribution of wild type and EKLF$^{-/-}$ erythroblast cultures during differentiation. The size of wild type cells decreases during differentiation and after 48 hours enucleated cells appear, indicated by an arrow. The cell size of EKLF$^{-/-}$ cells hardly decreases during differentiation. **b** Hemoglobin per cell volume was measured with intervals of 12 h. in wild type and EKLF$^{-/-}$ cultures after induction for differentiation. **c** Morphological analysis of wild type and EKLF$^{-/-}$ erythroblasts during differentiation. Aliquots of the cultures were cytocentrifuged onto glass slides and stained with both cytological dyes and with neutral benzidine for hemoglobin (brownish stain). The absence of EKLF does not affect progenitors (T=0). At T=36 a decrease of cell size compared to cells at T=0 is observed in the wild type culture, but not in the EKLF$^{-/-}$ culture. Only in the wild type cultures, hemoglobin is detected, first at T=54 (arrowheads), and these cells can complete the terminal differentiation to enucleated erythrocytes (black arrows). EKLF$^{-/-}$ cells do not accumulate hemoglobin and their nuclei become pycnotic (red arrows). Original magnification is 100X. T = time in hours; KO = knockout.

were TER-119 positive, 7-AAD negative. In contrast to the EKLF null fetal liver cells, a subpopulation of the EKLF null embryonic cells was TER-119 positive, although this fraction was strongly reduced (~20%). Embryonic blood of heterozygous fetuses also contained a reduced number of TER-119 positive cells (~41%) compared to wild type blood (Fig. 2). We conclude that definitive cells in absence of EKLF do not express the antigen surface marker recognized by TER-119 and that fewer embryonic blood cells express this antigen in the absence of EKLF.

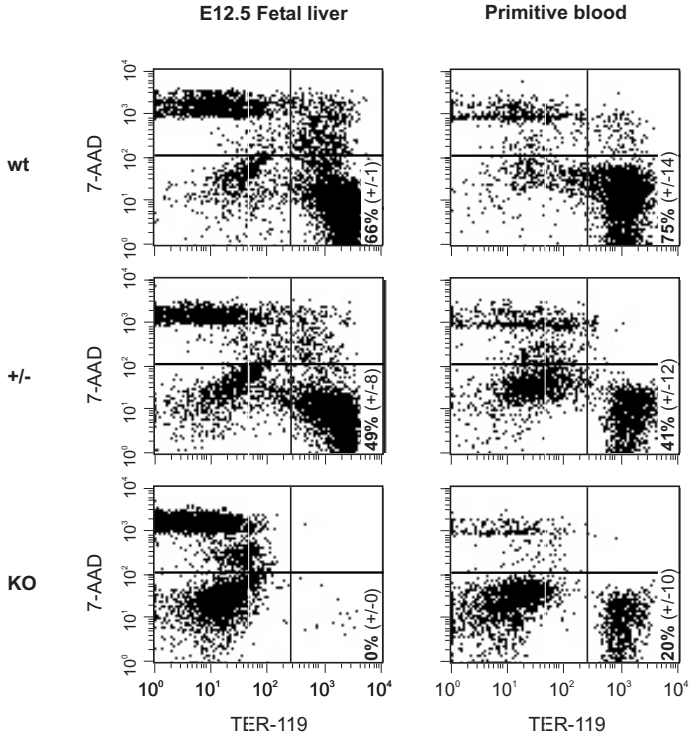


Figure 2. FACS analysis
Fetal liver- and embryonic blood cells of wild type, heterozygous and EKLF$^{-/-}$ embryos were stained with TER-119 and 7-AAD and analysed by FACS. Numbers give the average of TER-119 positive, 7-AAD negative cells in percentage of the counted cells. The standard deviations are included.

Reduced expression of genes involved in heme synthesis in EKLF$^{-/-}$ erythroid cells

The *in vitro* expansion and synchronous induction of terminal differentiation of erythroid progenitors provides a unique opportunity to study EKLF-dependent gene expression. In addition to insufficient β-globin synthesis, the failure of EKLF-deficient cells to accumulate hemoglobin could be due to reduced heme synthesis, as both processes are tightly linked. Heme is synthesized from succinyl CoA and glycine in seven enzymatic steps, catalysed by aminolevulinic acid synthase (Alas), aminolevulinate dehydratase (Alad), porphobilinogen deaminase (Pbgd), uroporphyrinogen decarboxylase (Urod), coproporphyrinogen oxidase (Cpox), protoporphyrinogen oxidase (Ppox) and ferrochelatase (Fech) respectively. We performed real time PCR experiments to detect the expression of these genes in *in vitro* expanded erythroid progenitors 36 hours after induction of differentiation and in fresh E12.5 fetal livers (Fig. 3a). Expression of Alas2 and Alad is significantly decreased in the absence of EKLF both in *in vitro* expanded progenitors and in fetal livers. Because the first step catalysed by Alas2 is the rate-limiting step in the heme synthesis pathway, we were particularly interested in its expression. Fig. 3b shows the expression pattern of Alas2 during the first 36 hours of differentiation in wild type and EKLF null cells. As expected, the gene is upregulated during erythroid differentiation of wild type cells. Although the gene is also upregulated in the absence of EKLF, the expression is reduced compared to wild type cells. Furthermore, expression of Alas2 in EKLF$^{-/-}$ fetal livers is strongly reduced compared

to wild type fetal livers. No obvious difference in Alas2 expression is found between wild type and heterozygous fetal livers. We conclude that the expression of enzymes involved in heme synthesis is not completely dependent on EKLF. However, dysregulation of the rate-limiting enzyme Alas2 might contribute to the failure of EKLF-deficient cells to accumulate hemoglobin.

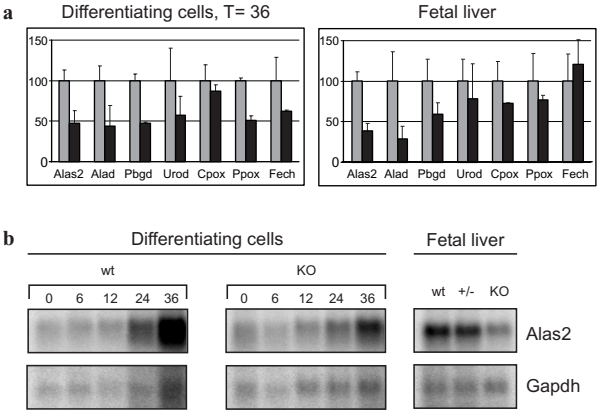


Figure 3. Expression of genes involved in heme synthesis in EKLF-deficient erythroid cells
a Expression of genes involved in heme synthesis was measured by real time PCR on cDNA of wild type and EKLF KO cultures 36 hours after induction of erythroid differentiation, and of fresh E12.5 fetal livers. Values were normalised to Hprt. The wild type values were set to 100% in grey, EKLF KO values in black. Error bars indicate the standard deviation between experiments. **b** Northern blot showing the expression of Alas2 in differentiating wild type and EKLF KO cultures and in E12.5 fresh fetal livers of wild type, heterozygous and EKLF KO embryos. Gapdh was used as a loading control. Numbers indicate time of differentiation in hours. wt = wild type; +/- = heterozygous for the EKLF knockout; KO = EKLF knockout. Abbreviations for the heme synthesis enzymes are given in the text.

Micro-array screening reveals EKLF-dependent genes

The failure to upregulate Alas2 and βmaj globin gene expression is consistent with the observation that EKLF$^{-/-}$ mice lack functional definitive erythrocytes. However, the *in vitro* differentiation of expanded erythroid progenitors indicates that differentiation of EKLF-deficient progenitors is impaired prior to hemoglobin accumulation (Fig. 1c). To find other genes that may be regulated by EKLF we performed gene expression profiling of primary fresh E12.5 fetal liver cells and *in vitro* expanded and differentiated cells. We used cDNA micro-arrays made by the BioOptics department at the IMP in Vienna, containing approximately 17,000 different EST sequences (representing individual genes once or maximally twice). These sequences were obtained from 'the German Genome Initiative' in Berlin. Furthermore, we used custom-made micro-arrays containing approximately 9000 cDNAs enriched for erythroid- and T cell-specific transcripts.[18] This custom-made micro-array has the advantage that it contains multiple copies of erythroid-specific genes, thus enabling multiple measurements of the expression of these genes in a single experiment. Primary progenitors of wild type and EKLF$^{-/-}$ fetal livers were expanded for 10 days and subsequently induced to differentiate. RNA was collected from non-induced cells and from cells 12 hours after induction. RNA was also isolated from fresh E12.5 wild type and EKLF$^{-/-}$ fetal livers. cDNAs were generated from these RNAs, Cy5- or Cy3-labelled and hybridised

to both 9K and 17K micro-arrays. The Cy3/Cy5 ratios were calculated for each spot as ^{2}log value. Table 1 lists these values for genes that appeared to be regulated by EKLF. The largest differences in gene expression between wild type and EKLF$^{-/-}$ RNA samples were found in fresh fetal livers. Spots representing genes involved in heme synthesis did not give high ratios between wild type and EKLF$^{-/-}$ samples, which could be due to the fact that these genes are only upregulated later in differentiation. The erythroid-specific Alas2 gene was not present on the micro-arrays used. Many of the genes that appeared to be upregulated by EKLF can be classified as erythroid-specific and/or cell membrane-specific. Amongst the erythroid-specific genes is the known EKLF target gene βmaj globin. As expected, high ratios are found since the expression of the βmaj gene is dependent on EKLF.[28,30]

Regulation of target genes by activating EKLF

To study the direct involvement of EKLF in the expression of a number of these listed genes, we made use of a tamoxifen (4-OHT) inducible fusion protein consisting of EKLF and a modified estrogen-receptor ligand-binding domain (EKLF-lbd).[7] In transgenic mice this construct is expressed under the control of an α-globin promoter. We wished to demonstrate expression of the target genes by activating the EKLF-lbd protein. We isolated EKLF$^{-/-}$ fetal liver cells carrying the EKLF-lbd transgene and cultured the cells for 16 hours in differentiation medium. To demonstrate direct EKLF-dependent expression of the genes, we added the protein inhibitor cycloheximide (CHX) to the cultures. CHX prevents synthesis of new proteins. Therefore, gene expression of these cultured cells cannot be due two secondary pathways. Gene expression of these cultures was studied by real time quantitative PCR. The differences in PCR cycles at which the PCR products reach the threshold level are shown in Figure 4. The expression of βmaj, AHSP, Kcnn4 and Epb4.9 are upregulated after 16 hours of differentiation in the presence of 4-OHT and CHX, indicated with the dark bars. These genes are also upregulated in the absence of CHX (light bars). Ca1 may be downregulated by EKLF (Table 1). However, in these experiments Ca1 is only downregulated in the absence of CHX, indicating that EKLF is indirectly involved in its downregulation. Next, we focussed our attention on two newly identified EKLF-dependent genes in the erythroid-specific- and membrane-specific categories, AHSP and Epb4.9 respectively.

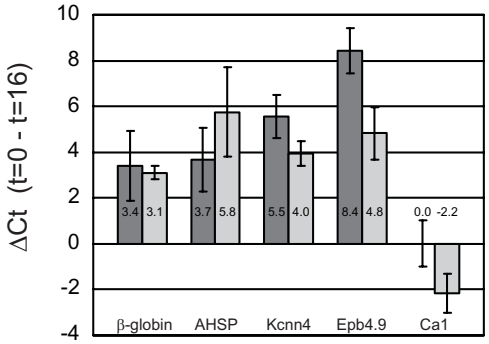


Figure 4. Gene regulation by activating EKLF
EKLF *null* fetal liver cells carrying an EKLF-lbd transgene were cultured for 16 hours. Gene expression was measured by real time quantitative PCR. The graph shows the difference in PCR cycles at which the PCR products reach the threshold level between T=0 (fresh liver cells) and T=16 (cultured cells). The medium of the cultured cells contained 4-OHT with (dark bars) or without (light bars) CHX. Error bars indicate SEM.

	Genes down-regulated in EKLF KO	Symbol	Cultured cells T=0	Cultured cells T=12	Fetal liver
	Heme synthesis				
●	aminolevulinic acid synthase 1	Alas1	0.1	0.2	0.2
●	hydroxymethylbilane synthase/porphobilinogen deaminase	Hmbs/PBGD	0.2	0.5	1.1
●	uroporphyrinogen decarboxylase	Urod	-0.1	0.3	0.6
●	coproporphyrinogen oxidase	Cpox	0.0	-0.3	0.0
●	protoporphyrinogen oxidase	Ppox	0.7	0.3	0.8
●	ferrochelatase	Fech	-0.2	0.0	0.6
	Erythroid specific				
●	hemoglobin, beta adult major chain	Hbb-b1	0.9	0.8	3.5
▲	Hemoglobin, beta adult major chain	Hbb-b1	0.7	0.5	3.0
●	hemoglobin Z, beta-like embryonic chain	Hbb-bh1	0.7	0.3	1.6
●	hemogen	Hemgn	-0.2	0.6	1.5
▲	Alpha Hemoglobin Stabilizing Protein	Eraf	1.0	2.5	3.6
	Cell membrane				
●	erythrocyte protein band 4.9	Epb4.9	0.4	0.5	3.7
●	K^+ intermediate/small conductance Ca^{2+}-activated channel, subfamily N, member 4	Kcnn4	0.8	1.0	2.5
▲	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2	Slc3a2	0.2	1.2	2.9
●	solute carrier family 2 (facilitated glucose transporter), member 1	Slc2a1	-0.2	-0.6	1.5
●	solute carrier family 22 (organic cation transporter), member 4	Slc22a4	0.2	0.3	1.7
●	solute carrier family 43, member 1	Slc43a1	0.5	0.7	1.8
●	Kell blood group	Kel	-0.1	0.4	1.9
●	CD24a antigen	Cd24a	0.3	0.3	1.5
●	transferrin receptor	Tfrc	0.2	0.8	2.0
●	intercellular adhesion molecule 4, Landsteiner-Wiener blood group	Icam4	-0.1	0.0	1.7
	Other				
●	protease, serine, 25	Prss25	0.9	1.0	4.7
●	sin3 associated polypeptide	Sap30	0.2	0.8	2.1
●	interferon-induced protein with tetratricopeptide repeats 3	Ifit3	0.4	0.4	2.1
●	ubiquitin associated domain containing 1	Ubadc1	0.4	0.4	1.6
●	unc-84 homolog A (C. elegans)	Unc84a	0.8	0.6	2.2
●	RIKEN cDNA 1110063G11 gene	1110063G11Rik	0.1	0.8	1.9
●	RIKEN cDNA 1200006I17 gene	1200006I17Rik	-0.4	0.1	1.8
●	monoglyceride lipase	Mgll	0.0	0.3	1.4
●	cat eye syndrome chromosome region, candidate 2 homolog (human)	Cecr2	1.0	0.7	1.8
●	lectin, galactose binding, soluble 1	Lgals1	0.7	1.0	1.5
●	myeloid differentiation primary response gene 116	Myd116	0.3	0.2	1.4
●	guanine nucleotide binding protein, alpha inhibiting 2	Gnai2	1.2	0.9	4.0
▲	guanine nucleotide binding protein, alpha inhibiting 2	Gnai2	1.0	0.9	3.2
▲	mitogen activated protein kinase kinase 5	Map2k5	0.7	0.7	3.1
▲	hypothetical Guanylate-kinase-associated protein	Dlg7	1.2	1.0	3.6
▲	CCR4-NOT transcription complex, subunit 7	Cnot7	0.4	0.4	4.0
●	Mus musculus RIKEN cDNA 2700084L06 gene	2700084L06Rik	0.7	1.0	1.3
●	peroxiredoxin 2	Prdx2	0.4	0.6	1.2
●	microsomal glutathione S-transferase 3	Mgst3	0.0	0.3	1.9
●	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Nfkbia	0.6	1.0	1.6
●	glutathione peroxidase 4	Gpx4			1.4
	Genes up-regulated in EKLF KO				
●	coagulation factor II (thrombin) receptor	F2r	-1.0	-0.4	-1.3
●	enolase 1, alpha non-neuron	Eno1	0.0	-0.4	-1.2
●	heme oxygenase (decycling) 2	Hmox2	0.0	-0.2	-2.9
●	phosphoglycerate mutase 1	Pgam1	-0.1	-0.1	-1.2
●	RIKEN cDNA 1110014J01 gene	1110014J01Rik	-0.3	0.0	-2.3
●	kelch domain containing 3	Klhdc3	0.1	-0.1	-1.8
●	24-dehydrocholesterol reductase	Dhcr24	-0.3	0.3	-1.9
●	RIKEN cDNA 6720456B07 gene	6720456B07Rik	0.0	0.1	-2.1
●	thrombospondin 1	Thbs1	0.1	-0.4	-2.5
●	guanosine monophosphate reductase	Gmpr	-0.7	-0.2	-1.6
●	ethanolamine kinase 1	Etnk1	-0.7	-0.5	-1.6
●	metastasis suppressor 1	Mtss1	0.2	-0.1	-1.6
▲	carbonic anhydrase 1	Car1	-1.2	-1.3	-2.5
▲	pyruvate kinase, muscle	Pkm2	-1.6	-1.1	-1.7

Table I. Genes that are differentially expressed in the absence of EKLF
Gene expression in wild type and EKLF knockout (KO) cultured cells at T=0 and T=12 hours after differentiation, and in wild type and EKLF KO fetal livers was determined with cDNA micro arrays. Values are the ^{2}log of the ratio wild type/EKLF KO. The average of values is taken when genes are represented more than once on the chips. The list includes values from the 17k (circles) and the erythroid-enriched 9k (triangles) micro-arrays. Genes with ratios higher than 1.2 or lower than -1.2 in fetal livers, and genes involved in heme synthesis, are displayed.

Alpha Hemoglobin Stabilising Protein

AHSP (Eraf, Edrf) is an abundant erythroid-specific protein that forms a stable complex with free α-globin.[16] An imbalanced production of α- and β-globin, as observed in thalassemia patients, can lead to precipitation of the free globins forming cytotoxic Heinz bodies.[27] By binding free α-globin, AHSP prevents α-globin denaturation and precipitation. AHSP was represented 10 times on the erythroid-specific micro-array chip and the average ratio between wild type and EKLF$^{-/-}$ expression at T=0 was 2 fold, at T=12 6 fold and in uncultured fetal livers 12 fold. Thus, AHSP expression appears to be consistently down-regulated in EKLF$^{-/-}$ erythroid cells.

The micro-array data for AHSP expression were confirmed by Northern blot analysis (Fig. 5a). AHSP mRNA is not detected in undifferentiated wild type progenitors (T=0), but increases to high levels during differentiation. However, in the absence of EKLF AHSP is not detected at any time during differentiation. Similarly, AHSP is not expressed in uncultured EKLF$^{-/-}$ E12.5 fetal livers. No obvious difference in expression in EKLF$^{+/-}$ compared to wild type uncultured fetal livers is found.

Since the expression of AHSP appears to be strongly dependent on EKLF, we were interested in the expression of AHSP in primitive erythroid cells in embryonic blood. Primitive cells express α-globin and therefore expression of AHSP can be expected. Notably, EKLF is also expressed in these cells, although to date, no endogenous EKLF-dependent genes have been described in primitive cells. We performed Northern blot analysis for AHSP on RNA isolated from EKLF$^{+/+}$, $^{+/-}$ and $^{-/-}$ primitive blood cells. Figure 5a (right panel) shows that AHSP expression is readily detected in wild type and heterozygous primitive blood cells. However, no expression of AHSP is observed in the absence of EKLF (Fig. 5a).

In adult blood of AHSP$^{-/-}$ mice, precipitates of α-globin are detected as Heinz bodies.[16] Because of the absence of β-globin in EKLF$^{-/-}$ fetuses, the presence of Heinz bodies in erythrocytes would not provide useful information on the consequences of the absence of AHSP. The excess of α-globin chains would result in the formation of globin chain precipitates regardless of the functional status of AHSP. However, since EKLF has no effect on the expression of the embryonic globin genes, we examined whether precipitates could be found in EKLF$^{-/-}$ primitive blood cells. E12.5 yolk sacs (7 wild type or EKLF$^{+/-}$ and 8 EKLF$^{-/-}$) were fixed, embedded in epon and 1 μm sections were stained with toluidine/methylene blue. We observed blue spots, generally located in apparent close proximity to the cell membrane, only in the EKLF$^{-/-}$ yolk sacs (Fig. 5b). These blue spots most likely represent inclusion bodies resulting from the precipitation of α-globin chains in the absence of

AHSP. Morphologically they appear to be analogous to the Heinz bodies found in definitive erythrocytes of AHSP-deficient mice.[16] Collectively, our results demonstrate that EKLF positively regulates the expression of AHSP in both definitive- and primitive erythroid cells. Absence of AHSP in EKLF$^{-/-}$ primitive blood cells results in the formation of α-globin precipitates and, together with the formation of such precipitates in definitive blood cells, may contribute to the severity of the phenotype of the EKLF knockout.

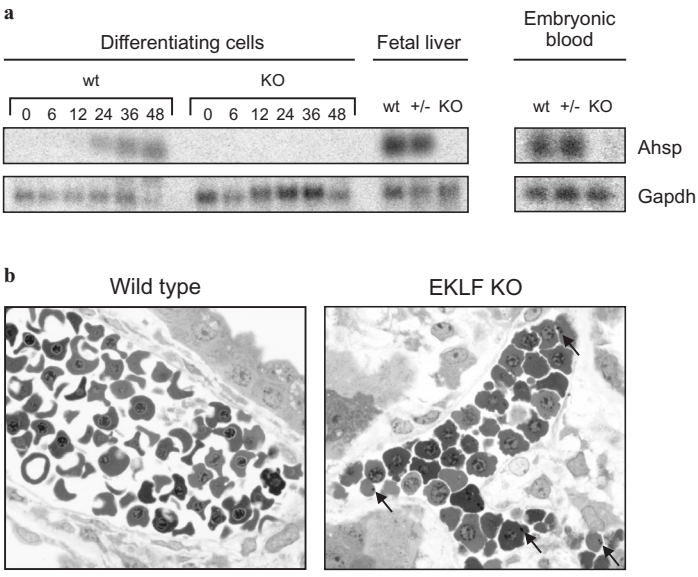
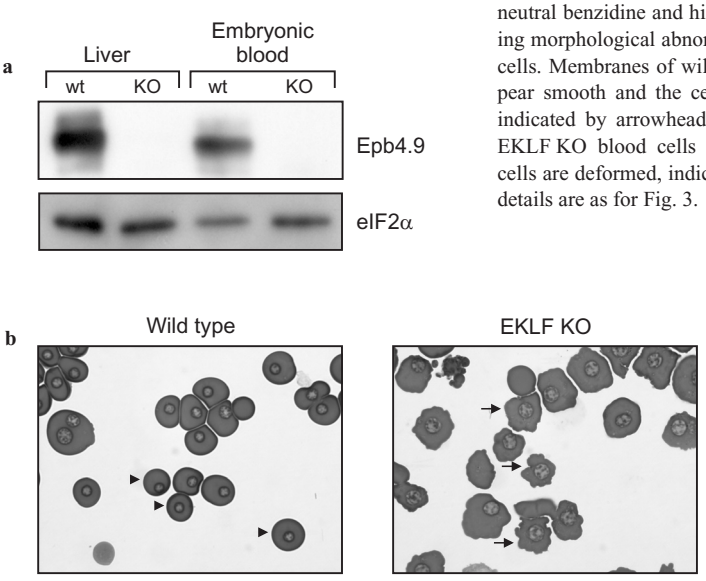


Figure 5. Ahsp expression in EKLF null erythroid cells
a Northern blot showing accumulation of Ahsp in wild type cells during differentiation, but not in EKLF$^{-/-}$ cultures. Furthermore, Ahsp expression is EKLF-dependent in E12.5 fetal livers and in embryonic blood. Gapdh was used as a loading control. **b** Apparent inclusion bodies detected as dark spots (indicated by arrows) in EKLF$^{-/-}$ embryonic blood cells in toluidine/methylene blue stained semi-thin sections of fixed E12.5 yolk sacs. These dark spots were not detected in wild type embryonic blood cells. Original magnification is 100X. Other details are as for Fig. 3.

Erythrocyte protein band 4.9

Epb4.9 is the most differentially expressed membrane-specific gene found in the micro-array experiments comparing wild type- and EKLF$^{-/-}$ fetal livers (Table I). Epb4.9, or dematin, is an actin-binding and bundling protein of the erythrocyte membrane skeleton. It is required for the maintenance of the erythrocyte shape and mechanical properties of the erythrocyte membrane.[15] Epb4.9 expression is not limited to erythroid cells,[34] but mice expressing a truncated form of the protein (dematin headpiece null mice) show signs of compensated anemia and spherocytosis,[15] indicating that erythroid cells require this protein. To confirm the micro-array data, we performed a Western blot with whole cell protein extract from E12.5 fetal livers. Epb4.9 expression is completely EKLF dependent (Fig. 6a). Interestingly, Epb4.9 expression in primitive blood cells is also dependent on the presence of EKLF (Fig. 6a). Since Epb4.9 is required for the stability of the erythrocyte membrane,[15] we analyzed the morphology of EKLF$^{-/-}$ primitive cells on cytospins of blood isolated from E12.5 embryos (Fig. 6b). The membrane of wild type cells appears smooth and, in general, the cells are round-shaped (arrowheads). In contrast, the membranes of EKLF$^{-/-}$ cells appear

Figure 6. Epb4.9 expression in EKLF knockout erythroid cells
a Western blot of whole cell protein extract. Expression of Epb4.9 is detected in E12.5 fetal livers and in embryonic blood of wild type embryos, but not in EKLF$^{-/-}$ embryos. Antibody against translation elongation factor eIF2α is used as a loading control. **b** Cytospins of primitive blood cells, stained with neutral benzidine and histological dyes, showing morphological abnormalities of EKLF KO cells. Membranes of wild type blood cells appear smooth and the cells are round shaped, indicated by arrowheads. The membranes of EKLF KO blood cells are wrinkled and the cells are deformed, indicated by arrows. Other details are as for Fig. 3.



to be wrinkled and the cells are deformed (arrows). This suggests that the cell membrane is less stable and malleable, very similar to the morphological abnormalities observed in erythrocytes of Epb4.9 mutant mice.[15] We conclude that the expression of the Epb4.9 gene in erythroid cells is strictly dependent on the presence of EKLF. The lack of Epb4.9 expression adversely affects the functioning of the erythroid cell membrane, thus contributing to the severity of the EKLF knockout phenotype.

Discussion

EKLF is active late in the erythroid differentiation program
In primary erythroid cultures we found that that EKLF is dispensable for the expansion of erythroid progenitors. However, activation of EKLF-dependent genes is required to execute the terminal differentiation program completely. Upon induction of differentiation wild type cells rapidly differentiate to hemoglobinised, enucleated erythrocytes, whereas EKLF$^{-/-}$ progenitors initially retain their blast-phenotype, fail to enucleate and their nuclei finally become pycnotic. This is in agreement with the observation that EKLF$^{-/-}$ ES cells contribute to erythroid progenitors but not to the mature erythrocyte compartment in chimeric mice.[22] It was postulated that this phenotype was due to the lack of β-globin expres-

sion. However, expression of γ-globin in these cells only partially rescued the phenotype, suggesting that EKLF regulates more genes that are important for erythroid differentiation. Here, we describe a number of novel EKLF-dependent genes that have important functions in the physiology of erythroid cells. In definitive erythroid cells these genes display an expression pattern similar to that of β-globin, since they become highly expressed late in erythroid differentiation. Thus, EKLF is an activator of essential erythroid genes that are upregulated during terminal erythroid differentiation.

Is TER-119 a target gene of EKLF?

TER-119[14] is an antibody that specifically recognizes erythroid cells that have progressed in the differentiation hierarchy beyond the progenitor stage.[17] Its antigen is associated with glycophorin A, although it does not bind it directly.[17] The gene and function of the protein are unknown. The finding that no EKLF$^{-/-}$ fetal liver cells express this antigen raises the question whether its gene is a direct target of EKLF or whether the absence of EKLF causes a defective terminal differentiation of erythroid cells. Since TER-119 positive primitive blood cells are found in EKLF$^{-/-}$ fetuses, a defect in differentiation is the most likely explanation. This is further supported by the fact that the intensity of TER119 staining (Fig. 2) is not decreased in EKLF$^{-/-}$ primitive blood cells. Hence, the expression per cell is not affected by EKLF but only the number of cells expressing the TER-119 antigen is decreased.

Interestingly, the percentage of TER-119 positive cells in heterozygous fetal livers cells and in heterozygous embryonic blood cells is lower than in wild type fetuses. This can be explained by a shortened life span of the cells, caused by reduced expression of EKLF, concordant to EKLF null cells.[22] A reduced expression of some EKLF target genes (e.g. AHSP and Epb4.9) may cause this shortened life span. This would imply that heterozygous cells are more affected by the presence of only a single EKLF gene than previously thought. However, it remains to be considered whether the TER119 gene is a direct target of EKLF.

Heme synthesis enzymes

The effect of the absence of EKLF on the expression of heme synthesis genes is rather subtle. Apparently these genes are not completely dependent on the presence of EKLF. This is not surprising since the enzymes of the heme synthesis pathway are ubiquitously expressed. Heme is an important molecule in many metabolic processes. Cell growth and in particular mRNA translation are controlled by the availability of nutrients and mitogenic factors, and also by the availability of heme. Heme-regulated eIF2α kinase (HRI) phosphorylates and inactivates eukaryotic initiation factor 2α (eIF2α).[4] HRI kinase activity is inhibited by heme, in a mechanism that co-ordinates heme- and globin synthesis.[12] In E12.5 EKLF$^{-/-}$ fetal livers we did not observe an elevated phosphorylation of eIF2α compared to wild type fetal liver (data not shown), indicating that heme synthesis is still sufficient for the basic metabolic requirements of erythroblasts.

However, hemoglobin synthesis in differentiating erythroblasts requires synthesis of heme at a level that largely exceeds that in normal cells. Therefore, erythroblasts express an erythroid-specific isoform of Alas, the rate-limiting enzyme in heme synthesis. Alas2-deficiency completely abrogates hemoglobin synthesis.[13] In contrast to the ubiquitously expressed Alas1, the erythroid specific Alas2 is controlled by the erythroid specific transcription

factors Gata1, EKLF and NF-E2 that also regulate β-globin expression.[19] In addition, the appropriate expression of Alas2, but not Alas1, is controlled by the availability of iron at the level of mRNA translation.[24] Notably, reticulocytes contain high levels of HRI, requiring high concentrations of heme to allow mRNA translation to proceed. Thus, a network of control mechanisms ensures the coordinate expression of the components required for the synthesis of hemoglobin. This may explain why EKLF-deficiency does not completely inhibit Alas2 and β-globin gene expression, yet almost fully abrogates hemoglobin production in differentiating erythroblasts.

EKLF is functional in primitive blood cells

In addition to the heme synthesis genes, we have focussed on two genes, AHSP and Epb4.9, that appear to be strictly dependent, for their expression in definitive erythroid cells, on EKLF. Interestingly, these genes are also dependent on EKLF for expression in primitive cells. This strongly suggests that EKLF functions as a positive regulator in both definitive and primitive cells. Previously, no abnormalities have been described for EKLF null embryonic cells. However, the absence of AHSP and Epb4.9 is likely to cause the observed phenotype of primitive EKLF-deficient cells, i.e. the presence of protein precipitates reminiscent of Heinz bodies and apparent membrane instability. Mice lacking AHSP are viable, but Heinz bodies are present in definitive erythrocytes caused by precipitated -globin chains.[16] We found similar inclusion bodies in EKLF null embryonic blood cells. Since α-globin synthesis precedes β-globin synthesis,[5] the nascent α-globin proteins have to be stabilised until they are incorporated in hemoglobin. In the absence of AHSP, α-globin precipitates inflict membrane damage on the erythrocytes leading to an increased turnover of the cells.[16]

Epb4.9 plays an important role in the stability of the erythroid membrane skeleton. Erythrocytes of Epb4.9 mutant mice have a more fragile membrane skeleton and reduced deformability, caused by reduced association of spectrin and actin to the plasma membrane.[15] Embryonic blood cells lacking EKLF and therefore lacking Epb4.9 expression, appear to have a similarly weakened cell membrane. Furthermore, this phenotype can be aggravated by the lack of AHSP. Although AHSP- and Epb4.9- mutant mice are viable, the of lack of expression of both genes, as is the case in the absence of EKLF, is likely to further reduce the half life of erythrocytes, consistent with observations on EKLF$^{-/-}$ adult erythrocytes.[22] Notably, EKLF$^{-/-}$ primitive blood cells appear to function sufficiently since EKLF$^{-/-}$ embryos die only at the time when they become dependent on definitive blood cells. This suggests that the large, nucleated primitive cells are relatively flexible in their ability to cope with disturbed functionality, compared with the highly specialized enucleated definitive cells. This, in addition to the fact that they last for only 4 or 5 days, may explain why the phenotype of EKLF$^{-/-}$ primitive cells is subtle, allowing them to carry out their essential tasks during embryogenesis.

Additional EKLF-dependent genes

Our studies focussed on EKLF-dependent genes that are particularly informative on the role of EKLF in primitive and definitive erythropoiesis. However, the micro-array analysis revealed a number of additional differentially expressed genes (Table I). Among those is

the transferrin receptor that transports iron into the cells. Reduced expression of the transferrin receptor is in line with impaired heme and globin synthesis. Peroxiredoxin 2 (Prdx2) is an important enzyme in the protection against oxidative stress. In particular, erythroid cells deficient in Prdx2 age prematurely. This is accompanied by membrane instability and the accumulation of Heinz bodies. As a result, Prdx2-deficient mice suffer from hemolytic anemia.[21]

Some of the differentially expressed genes are expressed at increased levels in EKLF$^{-/-}$ erythroblasts such as carbonic anhydrase 1 (Car1; Table 1). The ubiquitously expressed Car1 gene is downregulated during erythroid differentiation, when it is replaced by the erythroid-specific isoform Car2. This suggests that EKLF may function in transcriptional repression. The mechanism by which this is achieved remains to be investigated, because by default genes are shut down during terminal erythroid differentiation owing to progressing general heterochromatization of the genome. It would appear that EKLF counteracts this general shut down of transcription at loci that are of particular importance for erythroid maturation.

Synopsis

Collectively, we have shown that EKLF is dispensable for the generation and expansion of erythroid progenitors, but that it is essential for the differentiation of erythroid progenitors to functional erythrocytes. EKLF regulates hemoglobin synthesis through control of β-globin gene expression, but it also controls expression of enzymes of the heme synthesis pathway, for example the erythroid-specific, rate-limiting enzyme Alas2. The failure of EKLF$^{-/-}$ progenitors to undergo terminal differentiation suggests that EKLF regulates genes with an important function in the differentiation-specific gene expression program (Fig. 7). Impaired upregulation of these genes during terminal differentiation contributes to the phenotype of EKLF$^{-/-}$ cells. From several putative EKLF-dependent genes identified by mRNA profiling, we have shown that the expression of AHSP and Epb4.9 is strictly dependent on the presence of EKLF, not only in definitive- but also in primitive erythroid cells. Lack of expression of these genes adversely affects the functional properties of erythroid cells.[15,16]

In conclusion, our data show that EKLF coordinates the expression of a panel of proteins involved in differentiation of erythroid progenitors, and the maturation and survival of erythrocytes. These results explain why expression of exogenous γ-globin only partially restores the observed shortened lifespan of EKLF$^{-/-}$ adult erythrocytes, and fails to rescue definitive erythropoiesis in EKLF$^{-/-}$ mice.[22,29] In these scenarios EKLF$^{-/-}$ erythroid cells still have major defects even if the ratio of α- and β-like globin chains has been corrected. The genes identified in this study are good candidates for modifier genes affecting the severity of disease in thalassemia and sickle cell anemia patients.[3,11,41] Furthermore, our data suggest that mutations affecting the function of the EKLF transcription factor would have a major impact on the severity of these diseases. It is interesting to note that β-thalassemia resulting from dysfunction of the EKLF gene has not been found in the human population yet.[41] We suggest that a complete abrogation of EKLF function would not be compatible with post-embryonic life, because fetal liver erythropoiesis would be dysfunctional, even though γ-globin expression levels should be normal and the severe β-thalassemia observed in mice should not occur during human fetal development.

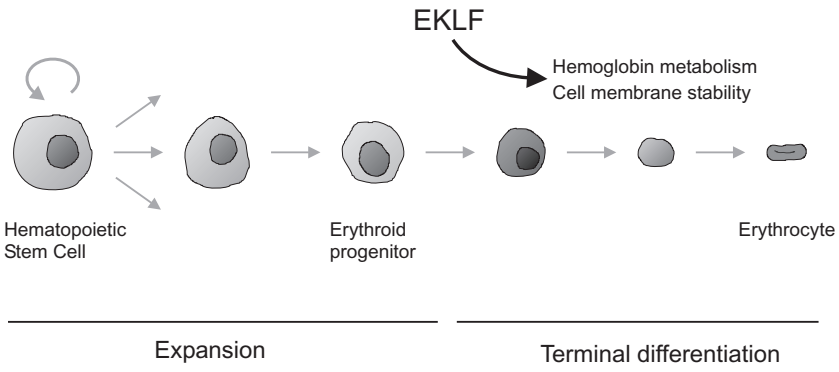


Figure 7. EKLF regulates genes necessary late in the erythroid differentiation program
The hematopoietic stem cell gives rise to all blood cell types, including the erythroid lineage. In the erythroid differentiation program, EKLF is dispensable for the expansion of erythroid progenitors. To complete the differentiation program, EKLF is required for the proper expression of genes involved in cell membrane stability (e.g. Epb4.9) and hemoglobin metabolism (e.g. β-globin, heme synthesis enzymes, AHSP), in order to enable appropriate terminal differentiation to erythrocytes.

Materials and methods

Cultivation of mouse erythroid progenitors
Fetal livers of E12.5 mouse embryos were disrupted and seeded into Stem-Pro-34™ medium supplemented with human recombinant erythropoietin (Erypo, Cilag AG, Switzerland, 1 U/ml), murine recombinant stem cell factor (SCF, R&D Systems, Minneapolis, MN, USA, 100 ng/ml) and dexamethasone (Dex, Sigma, 10^{-6} M).[40] The cultures of erythroid progenitors were subjected to daily partial medium changes and addition of fresh factors. Cell numbers and size distributions were determined daily, using an electronic cell counter (CASY-1, Schärfe-Systems, Reutlingen, Germany). Progenitors were enriched through Ficoll (lymphocyte separation medium, 1078 g/cm3, Eurobio, France) by centrifugation when the cultures contained >40% dead and/or differentiated cells as estimated by size distribution. Cell density was kept at ~$2x10^6$ cells/ml.

Terminal differentiation of mouse erythroid progenitors
Progenitors were washed twice in PBS and seeded at $1.5x10^6$ cells/ml in differentiation medium (Stem-Pro-34™ medium supplemented with Epo (10U/ml) and iron-saturated human transferrin (Sigma, 1 mg/ml). Cells were maintained at densities of 2-4x10^6 cells/ml, requiring dilution with fresh medium twice daily between 24 and 50 h. Cell number and size distribution was determined using an electronic cell counter. Hemoglobin content was quantitated in a photometric assay as described.[1] In short, 2–4 x 10^4 cells were washed in PBS, lysed in 20 µl H_2O, and frozen until further analysis. 100 µl of reagent mix (0.5 mg/ml o-phenylenediamine (Sigma-Aldrich) and 0.03% H_2O_2 in 0.1 M citrate/phosphate

buffer, pH 5.0) was added to thawed samples. The reaction time was kept identical for all reactions within each experiment and was stopped after ~2 min. with 20 µl 8 N H_2SO_4. The 492 nm extinction of the reaction product was measured with an ELISA spectrophotometer. Extinction/cell volume was taken as a measure for hemoglobin content.

Real-time PCR

The real-time PCR assay was performed on a BIO-RAD Icycler. For the PCR reactions, the Eurogentec qPCR™ Corekit for Sybr™ Green was used. Reactions were performed in 25 µl mix as described by the manufacturer. Amplification program: 10 min. 95ºC, 40 cycles of denaturation at 95ºC for 20 sec., annealing and elongation at 60ºC for 45 sec. After each elongation step, the fluorescence signal was measured at 75ºC. To confirm amplification specificity the PCR products from were subjected to melting curve analysis. Primer sequences are as follows:
Alas2: 5'-CACCTATGCTTAAGGAGCCA-3' and 5'-CAGAAGCACACAGGAAAGCA-3',
Alad: 5'-CTTTGATCTCAGGACTGCTG-3' and 5'-AACAGCTGCGGTGCAAAGTA-3',
Pbgd: 5'-TACTTCTGGCTTCCAAGTGC-3' and 5'-CAAGGTGAGGCATATCTTCC-3',
Urod: 5'-ATCCCTGTGCCTTGTATGCA-3' and 5'-AGGTTGGCAATTGAGCGTTG-3'
Cpox: 5'-CAATTTGAAGCCAGTCCGTG-3' and 5'-CTGGACTAGAACTCCCTTTG-3',
Ppox: 5'-ATTCCAGCTTCAGAGCTCAG-3' and 5'-TACTGCAGATTCACCACAGC-3',
Fech: 5'-ACCAGTGACCACATTGAGAC-3' and 5'-GGCCTTGGAGAACAATGGAT-3',
HPRT: 5'-AGCCTAAGATGAGCGCAAGT-3' and 5'-ATGGCCACAGGACTAGAACA-3',
β-globin: 5'-ATGCCAAAGTGAAGGCCCAT-3' and 5'-CCCAGCACAATCACGATCAT-3'
AHSP: 5'-GGATCAGCAGGTCTTTGATG -3' and 5'-AGAGTACTCAGCTCTTGCTG-3'
Kcnn4: 5'-AAGCACACTCGAAGGAAGGA-3' and 5'-TTCCGGTGTTTCAGCCGTA-3'
Epb4.9: 5'-TGCTCAAGACCCAAGGCTTA-3' and 5'-TCCTATCTGGTTTTGCCTGG-3'
Gapdh: 5'-CCTGCCAAGTATGATGACAT-3' and 5'-GTCCTCAGTGTAGCCCAAG-3'
CA1: 5'-AGAGTCTGCAGTTCCAGTTC-3' and 5'-GCCAGGTCATAATTGAGGAC-3'

FACS

Cells from E12.5 fetal livers and E11.5 and E12.5 embryonic blood cells were collected and washed in DMEM 10% FCS. They were stained with rat PE conjugated TER-119 antibody (Pharmingen) in a 1 in 100 dilution.[17] Dead cells were stained with 7-aminoactinomycin-D (7-AAD, Molecular Probes BV) at 1 µg/ml. FACS analysis was performed on a Becton Dickinson FACScan. Percentages of TER-119 positive, 7-AAD negative cells are averages of 3 wt, 4 heterozygous and 3 KO E12.5 livers and 4 wt, 12 heterozygous and 5 KO E11.5 or E12.5 embryonic blood cells. No significant difference was found in the percentages between E11.5 and E12.5 blood.

cDNA array hybridizations and analysis

Total RNA from cultured cells and fetal livers was extracted using TRI REAGENT (Sigma) following the manufacturer's instructions. This RNA was used to hybridise micro-arrays containing ~17.000 EST sequences obtained from the 'German Genome Initiative' in Berlin and a custom-made hematopoietic micro-array containing ~9.000 cDNAs that were enriched for erythroid- and T cell–specific cDNAs by subtracting cDNA of expanding I/11

cells and quiescent CD4+ T cells from cDNAs prepared from 3T3 fibroblasts and EpH4 epithelial cells.[18] For a single hybridization, 30 µg total RNA was reverse transcribed into cDNA using Cy5-UTP or Cy3-UTP (CyDye; Amersham Biosciences). The micro-arrays were hybridized and analyzed as described previously.[18] The scanning was performed using a Genepix 400A scanner (Axon Instruments, Inc.), and the analysis was performed using the GenePix program, Microsoft Excel and Access.

Inducible EKLF cultures

Livers were isolated from E12.5 EKLF$^{-/-}$::EKLF-lbd fetuses. Single cell suspensions were cultured for 16 hours in StemPro-34 containing 1% BSA, 1% glutamine, and 10 U/ml EPO, but without serum supplement. The EKLF-lbd was activated by supplementing the medium with 250 nM 4-hydroxy-tamoxifen with or without 20µg/ml cycloheximide. After 16 hours of culture, cells were harvested and RNA was isolated for preparing cDNA.

Western blotting

From wild type and EKLF$^{-/-}$ E12.5 fetuses, livers were disrupted and embryonic blood cells washed in PBS/1mM EDTA. The cells were spun down and lysed in RIPA buffer (150mM NaCl/1.0% NP-40/0.5% DOC/0.1% SDS/50mM Tris-HCl pH 8.0) supplemented with a protease inhibitor cocktail (Sigma). Proteins were separated on 10% SDS-PAGE gels under reducing conditions and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Blots were probed with anti-dematin (BD Biosciences) or anti-eIF2 (Santa Cruz Biotechnology) antibodies. Second-step reagents were HRP-conjugated goat anti-mouse IgG or goat anti-rabbit IgG from Dako (Glostrup, Denmark). Peroxidase activity was visualized by ECL using Western blotting detection reagents from Amersham Biosciences according to their instructions.

Cytospins, sections and staining

E12.5 embryonic blood cells were collected in PBS/1mM EDTA and cytocentrifuged. The preparations were stained with neutral benzidine and histological dyes as described.[2] For the sections of embryonic blood cells, wild type and EKLF$^{-/-}$ E12.5 yolk sacs were immersion fixed in 3% paraformaldehyde/1% glutaraldehyde in Millonig's buffer at 4°C overnight or longer. Then they were rinsed with Millonig's buffer and post-fixed in 1% OsO_4 at 4°C. The yolk sacs were dehydrated through an ascending acetone series and embedded in epon. Sections were cut at 1 µm and stained with toluidine/methylene blue. Images of the cytospins and sections were acquired with an Olympus BX40 microscope. The lens used was Olympus Plan 100X/1.25. The acquisition software used was Viewfinder Lite Version 1.0.125 and Studio Lite Version 1.0.124, (Pixera Corporation), and image processing was done in Adobe Photoshop Elements.

Acknowledgements

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Chapter 4

The active spatial organisation of the β-globin locus requires the transcription factor EKLF

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Abstract

Three-dimensional organisation of a gene locus is important for its regulation, as recently demonstrated for the β-globin locus. When actively expressed, the *cis*-regulatory elements of the β-globin locus are in proximity in the nuclear space, forming a compartment termed the Active Chromatin Hub (ACH). However, it is unknown which proteins are involved in ACH formation. Here we show that EKLF, an erythroid transcription factor required for adult β-globin gene transcription, is also required for ACH formation. We conclude that transcription factors can play an essential role in the three-dimensional organisation of gene loci.

Introduction

The mouse β-globin locus contains multiple β-like globin genes, arranged from 5' to 3' in order of their developmental expression (Fig. 1a). The adult-type β^{maj}-gene is transcribed at a very low level during primitive erythropoiesis in the embryonic yolk sac, but becomes expressed at high levels around day 11 of gestation (E11) when definitive erythropoiesis commences in the fetal liver.[29] The β-globin locus control region (LCR) is essential for efficient globin transcription.[2,14] It consists of a series of DNaseI hypersensitive sites (HS) located ~50 kb upstream of the β^{maj} promoter (Fig. 1a). We have shown that the β-globin locus forms an Active Chromatin Hub (ACH) in erythroid cells.[28] The ACH is a nuclear compartment dedicated to RNA polymerase II transcription, formed by the *cis*-regulatory elements of the β-globin locus with the intervening DNA looping out. The ACH consists of the HS of the LCR, two HS located ~60 kb upstream of the embryonic εy-globin gene (5'HS-62/-60) and 3'HS1 downstream of the genes. In addition, the actively expressed globin genes are part of the ACH.[4,28] In erythroid precursors which do not express the globin genes yet, a substructure of the ACH, called a chromatin hub (CH)[23] is found, which excludes the genes and the HS at the 3' site of the LCR.[22]

Expression of the β^{maj}-gene requires the presence of the erythroid Krüppel-like transcription factor EKLF, the erythroid-specific member of the Sp/XKLF-family.[19] $EKLF^{-/-}$ mice die of anaemia around E14, because of a deficit in β-globin expression.[21,25] The β-globin locus contains a number of EKLF binding sites, in particular in the LCR and the β^{maj}-globin promoter.[3,24] Because β^{maj}-globin expression depends on the presence of EKLF, we were interested in determining whether EKLF is involved in the formation of the ACH.

Results and discussion

We used chromatin conformation capture (3C) technology[6] to investigate the three-dimensional conformation of the mouse β-globin locus in the absence of EKLF. Cells from E12.5 $EKLF^{-/-}$ and wild type fetal livers were crosslinked with formaldehyde, followed by restriction enzyme digestion of the DNA. The samples were ligated under conditions that favour the ligation of DNA fragments that are physically connected through the crosslinks. Quan-

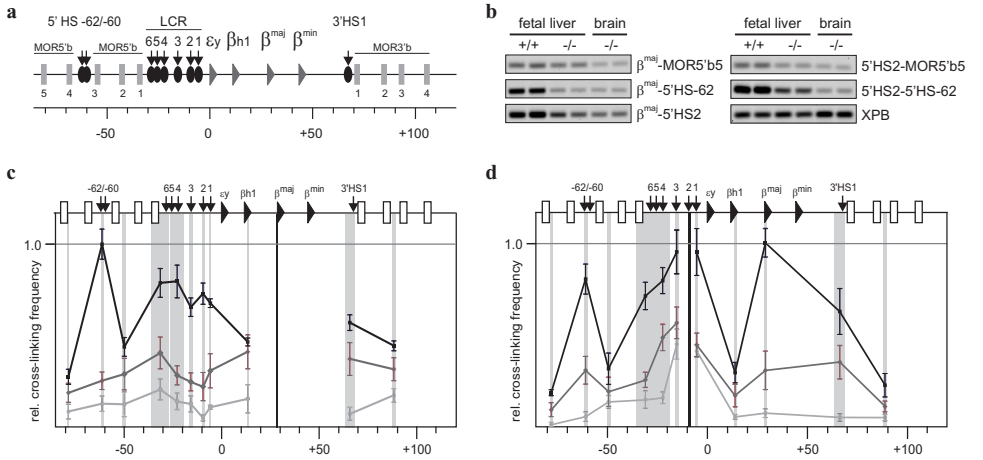

Figure 1. EKLF influences the spatial organisation of the β-globin locus.
a Schematic presentation of the mouse β-globin locus. Globin genes are indicated by triangles. Olfactory receptor genes (MOR5'b and MOR3'b) are indicated by rectangles and numbered. DNaseI HS are shown as black ovals with arrows. The scale is in kb. **b** Examples of PCR-amplified ligation products run on a 2% agarose gel. Primer combinations are shown on the right. XPB is used to standardise the amount of template.[2] +/+ = wildtype; -/- = EKLF knockout. **c**-**d** Locus-wide relative crosslinking frequencies in E12.5 fetal livers. Results obtained with wild type livers are shown in black; EKLF$^{-/-}$ livers in grey, non-expressing brains in light. The x-axis shows position in the locus. Grey shading indicates the positions and sizes of the *HindIII* fragments containing primers used in the PCR analysis. Black shading represents the position of the fragment containing the "fixed" primer in the *HindIII* fragment of the β^{maj}-gene (**c**) or 5'HS2 (**d**). Within each graph, the highest crosslinking frequency value is set to 1. Error bars indicate the standard-error-of-mean.

titative PCR across the junctions is used to determine the relative crosslinking frequencies between restriction fragments in the locus. This provides an indication of the nuclear proximity of DNA fragments *in vivo*.[6,22,28] Crosslinking frequencies were determined for a total of 66 junctions that can be formed between 12 selected *HindIII* fragments spread over ~170 kb of DNA encompassing the β-globin gene cluster (Fig. 1). Examples of quantitative PCR reactions with some of the primer combinations are shown in Fig. 1b. An overview of the locus-wide crosslinking frequencies of a restriction fragment that contains the β^{maj} promoter is shown in Fig. 1c. The brain serves as a non-expressing control tissue (light curve), in which the β-globin locus appears to adopt a linear conformation.[28] In wild type E12.5 fetal liver cells, high crosslinking frequencies are found with the LCR and 5'HS-62, indicating their proximity to the β^{maj} promoter *in vivo* (black curve). In the absence of EKLF however, these crosslinking frequencies are much lower and no interaction with a distal site stands out clearly (grey curve), showing that the β^{maj} promoter does not participate stably in a spatial clustering of chromatin. A comparable pattern is observed with locus-wide crosslinking frequencies of a fragment containing 5'HS2 (Fig. 1d). Together with 5'HS3, 5'HS2 is the most prominent transcriptional activating element of the LCR.[8-10,12,15] Interactions with 5'HS-62, β^{maj}, 3'HS1, and the other HS of the LCR are strongly reduced in the absence of EKLF, indicating that 5'HS2 requires the presence of EKLF to participate in the ACH.

The results shown in Fig. 1 demonstrate that the complete ACH is not formed in the absence of EKLF. However, the observed crosslinking frequencies in EKLF$^{-/-}$ fetal liver cells are still higher than those found in non-expressing brain cells indicating a different, non-linear, structure. To investigate this, we compared the locus-wide crosslinking frequencies of restriction fragments containing 5'HS-62 and 5'HS4/5 (Fig. 2). These sites participate in the CH present in erythroid progenitor cells before the globin genes are transcribed.[22] Examples of quantitative PCR reactions with some of the primer combinations are shown in Fig. 2a. The graphs in Fig. 2b show that in wild type fetal liver cells, 5'HS-62 is in proximity to the LCR, β^{maj} and 3'HS1. In EKLF$^{-/-}$ fetal liver cells, 5'HS-62 interactions with the HS at the 5' side of the LCR and with the distal 3'HS1 still stand out, whereas all other crosslinking frequencies are strongly reduced. This indicates the presence of a globin CH, containing 5'HS-62/-60, the HS at the 5' side of the LCR, and 3'HS1. The same structure is apparent when analysing locus-wide crosslinking frequencies of a restriction fragment containing 5'HS4/5 at the 5' side of the LCR (Fig. 2c).

There are remarkable similarities between the structure of the β-globin locus in EKLF$^{-/-}$ fetal liver cells and that observed in I/11 erythroid progenitor cells which do not yet express globin[22] (Supplementary Fig. 1). This suggests that EKLF is required for progression from the chromatin hub present in erythroid precursors to a fully active ACH. To investigate if

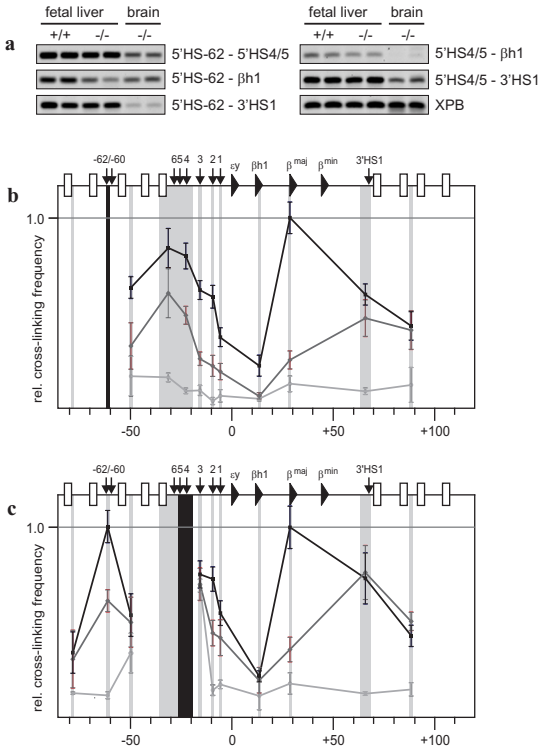

Figure 2. An ACH substructure is formed independent of EKLF.
a Examples of PCR-amplified ligation products run on a 2% agarose gel. Primer combinations are shown on the right. **b, c** Locus-wide relative cross-linking frequencies of *HindIII* restriction fragments containing 5'HS-62 (**b**), and 5'HS4/5 (**c**). See legend to Fig. 1 for other details.

this β-globin structure in EKLF *null* cells is a direct consequence of EKLF-deficiency or caused by a general differentiation failure, we analysed expression of the erythroid-specific, but EKLF-independent, α-globin gene locus. Consistent with previous observations, primary transcript *in situ* hybridisation experiments show that α-globin expressing cells are abundantly present in the EKLF$^{-/-}$ fetal liver, demonstrating that in the absence of EKLF cells are progressing to the stage of active globin expression (Fig. 3a).[17,21,25,32] We do observe that EKLF$^{-/-}$ fetal livers contain approximately 20% less α-globin expressing cells than wild type fetal livers (~55% *versus* 70% of the total number of cells in the fetal liver). Whereas this may explain the small reduction in crosslinking frequencies observed between some of the β-globin elements, it cannot account for the strongly reduced locus-wide crosslinking frequencies seen with, for example, β^{maj} and 5'HS2 (Fig. 1c,d). We conclude that the dramatically altered chromatin organisation of the β-globin locus in EKLF *null* erythroid cells is not due to a general differentiation problem.

To substantiate the specificity of the changes in the three-dimensional structure of the β-globin locus in the absence of EKLF we investigated interactions between the promoter and remote regulatory element of the erythroid-specific, EKLF-independent, α-globin locus. The mouse α-globin locus has two active genes in the fetal liver, α1 and α2, and contains a HS 26 kb upstream of the α-globin promoter that is similar to the human α-globin enhancer HS-40 (Fig. 3b).[11] It is likely that, analogous to the LCR, this element will interact with the α-like globin promoters to enhance expression. The crosslinking frequencies of the restriction fragments containing HS-26 and α2-globin are shown in Fig. 3c, d. In wild type and EKLF$^{-/-}$ fetal liver cells, the crosslinking frequencies are clearly higher than those observed

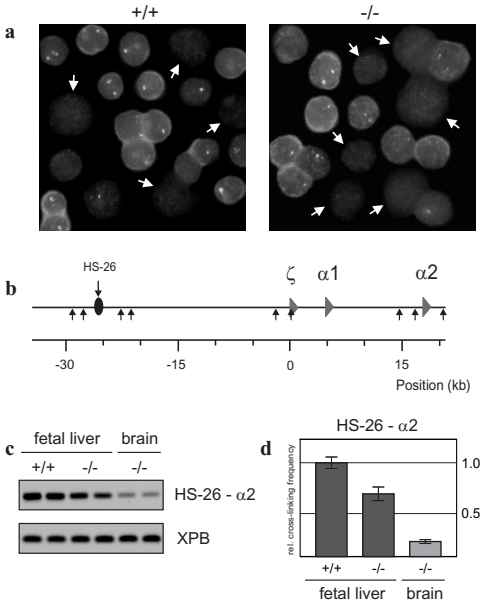

Figure 3. HS-26-promoter interactions in the α-globin locus are not affected by EKLF.
a *In situ* hybridisation of E12.5 fetal liver cells of wildtype and EKLF$^{-/-}$ fetuses, detecting α-globin mRNA (red) and primary transcripts (green). DAPI staining (blue) is used to show nuclear DNA. White arrows indicate cells that were scored negative for α-globin expression. **b** Schematic drawing of the mouse α-globin locus. The black oval with arrow depicts the position of the HS-26 distal regulatory element. The α-like globin genes are indicated by grey triangles. Small arrows = *HindIII* restriction sites. **c** Example of PCR-amplified ligation products of *HindIII* restriction fragments containing HS-26 and α2 in E12.5 day fetal liver and brain cells of wildtype and EKLF$^{-/-}$ fetuses. The XPB PCR product is used as template control. **d** Quantified data of PCR-amplified ligation products. Dark bars = fetal liver; light bar = brain. Error bars indicate standard-error-of-mean. The crosslinking frequency in wildtype fetal liver cells is set to 1.

in non-expressing brain tissue, indicating that HS-26 and the α2-globin gene are in close proximity in both types of erythroid cells. The slightly reduced interaction frequencies observed in EKLF knockout compared to wild-type fetal liver can be explained by the 20% reduction of α-globin expressing cells (see above). We conclude that major alterations in spatial organisation are restricted to the EKLF-dependent β-globin locus.

To further investigate if changes in the spatial organisation of the β-globin locus are a direct effect of the activity of EKLF, we wished to induce EKLF activation and simultanously prevent it from activating secondary pathways. For this, we used a fusion between EKLF and a modified estrogen receptor ligand binding domain (EKLF-lbd protein), that can be activated by 4-hydroxy-tamoxifen (4-OHT).[18] We wanted to test whether, in an EKLF *null* background, activated EKLF-lbd protein restores ACH formation in the presence of the protein synthesis inhibitor cycloheximide (CHX). In such a set up, genes activated by EKLF cannot be translated into protein, and therefore any structural changes would have to be attributed to EKLF acting directly on the β-globin locus. Transgenic mice carrying an expression construct of an EKLF-lbd fusion protein were generated. To ensure expression of the fusion protein in EKLF *null* erythroid cells, we used the erythroid-specific pEV3 expression vector[20] and replaced the β-globin promoter by the α-globin promoter. Western blot analysis demonstrates the presence of the HA-tagged EKLF-lbd fusion protein (Fig. 4a). We have previously shown that an EKLF-pEV3 transgene rescues the EKLF *null* mutation.[27] To test if uninduced EKLF-lbd fusion protein is inactive, we crossed the EKLF-lbd transgenics with the EKLF knockout mice. No EKLF *null*::EKLF-lbd transgene pups were born. When we dissected the fetuses resulting from this cross at E12.5, we found that the EKLF *null*::EKLF-lbd transgenic fetuses were indistinguishable from EKLF *null* fetuses, e.g. displaying signs of severe anemia and having very pale fetal livers (data not shown). We conclude that the EKLF-lbd fusion protein is inactive and does not rescue the EKLF *null* mutation.

To test the ability of activated EKLF-lbd fusion protein to rescue β-globin gene transcription, we cultured EKLF *null*::EKLF-lbd fetal liver cells in the presence of 4-OHT (Fig. 4b). After 16 hours of culturing, a subset of the cells was used to check for the activation of β-globin gene expression. Real-time RT-PCR analysis of steady-state mRNA levels shows that the β-globin gene is activated in EKLF *null*::EKLF-lbd cells in the presence of 4-OHT (Fig. 4c). The amount of β-globin transcripts in the tamoxifen-rescued cells is much lower than in wild type cells, which is is not surprising since the former cells just start to accumulate β-globin mRNA levels. We conclude that the EKLF-lbd fusion protein can be induced with 4-OHT to activate β-globin gene expression. Moreover, β-globin gene activation by 4-OHT-induced EKLF-lbd also occurs in the presence of CHX (Fig. 4c).

The remaining cells were subjected to 3C analysis using a procedure modified for use with small numbers of cells. Since the amount of material was limiting, we focussed on the analysis of interactions between 5'HS2, one of the most prominent activating elements of the LCR, and the promoter of the β^{maj} gene.[4,28] In untreated EKLF *null* fetal liver cells, we found similarly low crosslinking frequencies between 5'HS2 and β^{maj} regardless of the presence of the (uninduced) EKLF-lbd protein (Fig. 4d). In contrast, this interaction is restored in EKLF *null*::EKLF-lbd cells after culturing for 16 hours in the presence of 4-OHT. Importantly, the same effect is also observed when CHX and 4-OHT are present simultaneously

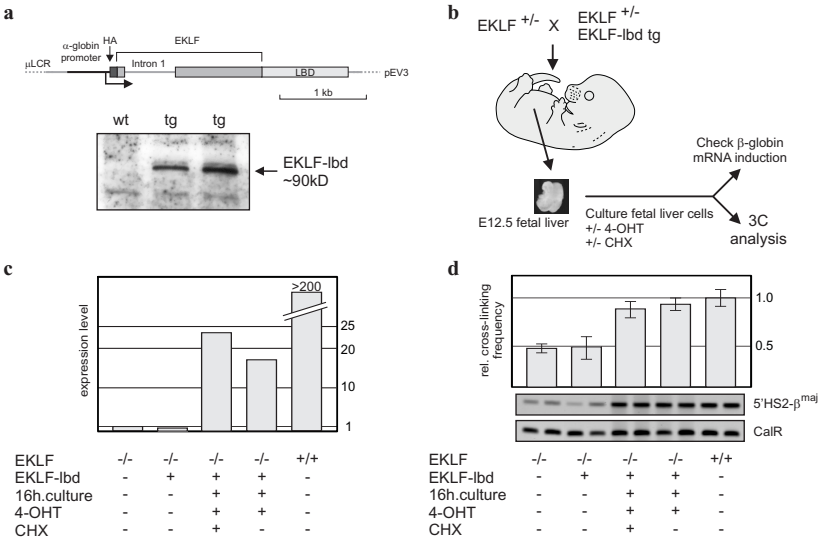

Figure 4. EKLF is directly involved in the spatial organisation of the β-globin locus.
a Schematic drawing of the EKLF-lbd expression construct used to generate transgenic mice. The Western blot shows expression of the EKLF-lbd fusion protein in the fetal livers of transgenic mice detected by an antibody recognizing the HA tag. **b** Flow chart of the experimental design. Fetal livers are isolated from E12.5 control- and EKLF null::EKLF-lbd tg fetuses, disrupted and the erythroid cells are cultured in the presence of 4-OHT with or without CHX for 16 hours. Cells are then harvested, crosslinked with formaldehyde and subjected to 3C analysis. From a portion of the cells, RNA is isolated to check β-globin gene expression. **c** Expression of β-globin analysed by real-time RT-PCR. Expression of Hprt was used to standardize the β-globin expression levels. Representative experiment is shown. **d** 3C analysis of the interactions between 5'HS2 and the β-globin promoter. Representative examples of the PCR reactions are shown. Error bars indicate the standard-error-of-mean. Calreticulin was used as template control.

(Fig. 4d). These data indicate that ACH interactions are restored in EKLF *null*::EKLF-lbd cells when the EKLF-lbd fusion protein is activated by 4-OHT. Since this also occurs when protein synthesis is inhibited through the addition of CHX, we conclude that EKLF is directly involved in the completion of ACH formation.
In conclusion, our data show that a chromatin hub is formed independent of EKLF during erythropoiesis, consisting of the 5'HS-62/-60, the HS at the 5' side of the LCR, and 3'HS1. EKLF is required for the progression to, or stabilisation of, a fully functional ACH, which includes the remaining HS of the LCR and the actively transcribed β^{maj} globin gene (Fig. 5). The β^{min} gene which is also expressed in definitive erythroid cells is known to alternate with the β^{maj} gene in the ACH in a dynamic "flip-flop" mechanism.[29,33] The EKLF-independent chromatin hub is structurally similar to that present in erythroid precursor cells, which were previously found to already contain EKLF mRNA[7] and protein (data not shown). This suggests that modifications of the EKLF protein or other protein factors are required to collaborate with EKLF in organizing a fully active β-globin ACH.

Recent work has shown that deletion of the promoter of the adult β-globin gene in the human β-globin locus mildly affects ACH formation, suggesting that in addition to the β-globin promoter other *cis*-regulatory elements in the human β-globin locus are involved in these interactions.[23] EKLF binding sites are also present in the LCR, in particular in 5'HS3, and in the 3' enhancer of the β-globin gene.[13,31] Together these data suggest that the EKLF-dependent interactions of the adult β-globin genes with the ACH involve multiple *cis*-regulatory elements.

It is also interesting to note that in the EKLF knockout absence of spatial interactions coincides with loss of chromatin accessibility at 5'HS3 and the β^{maj}-promoter.[5,32] We conclude that EKLF is necessary for hypersensitive site formation and the participation of the LCR and the β-globin promoter in the ACH, probably through interactions with a SWI/SNF-related chromatin remodelling complex.[1] Thus, EKLF is the first example of a transcription factor that is required for the proper spatial organisation of a mammalian gene locus.

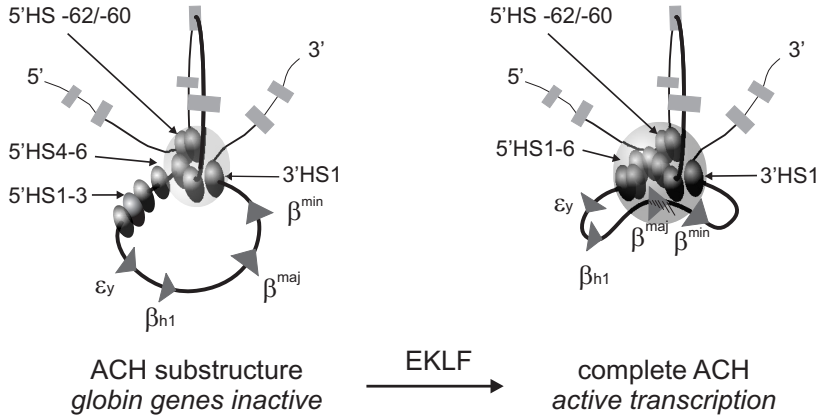

Figure 5. The formation of the complete ACH requires the presence of EKLF.
A two-dimensional representation of the proposed three-dimensional structure of the ACH is shown. The ACH is a nuclear compartment dedicated to RNA polymerase II transcription, formed by *cis*-regulatory elements of the β-globin locus.[22] In erythroid cells, a substructure of the ACH, consisting of 5'HS-62/-60, 3'HS1 and HS at the 5' side of the LCR, is formed independently of EKLF. Progression of this substructure to a fully functional ACH, including the HS at the 3' side of the LCR and the active β-globin gene, is dependent on the presence of EKLF. Grey sphere on the left: ACH substructure. Grey sphere on the right: ACH. RNA transcripts are indicated as black lines. See legend to Fig. 1a for other details.

Materials and methods

Chromosome Conformation Capture
EKLF$^{+/-}$ mice[21] were crossed and E12.5 fetal livers and brains were isolated. 3C analysis was performed as described,[26] with minor adjustments. Individual liver and brain samples were subjected to formaldehyde crosslinking. *HindIII* restriction enzyme digestion of crosslinked DNA, intra-molecular ligation, reversal of crosslinks, PCR analysis of ligation products and calculation of relative crosslinking frequencies was done with 15 pooled wildtype fetal livers, 15 EKLF$^{-/-}$ fetal livers and cells of 3 pooled EKLF$^{-/-}$ brains. Two independent samples were prepared for the analysis. Each PCR reaction was performed in duplicate and repeated at least 3 times.

α-globin
HS-26 - α2 promoter crosslinking frequencies were determined with the DNA samples described above and primers recognising the *HindIII* restriction fragment containing HS-26 (5'-GAATCTCCATCTCCAAGGG-3') and the α2 promoter (5'-AAGAGGTGCAGGTGTATTACTG-3'). *In situ* hybridisation of E12.5 fetal liver cells was performed as described before.[30] Cells were scored positive if α-globin mRNA, primary transcript, or both, was detected. >300 cells were counted to determine the percentage of α-globin-positive cells in each sample.

Generation of EKLF-lbd transgenic mice
A DNA fragment containing EKLF cDNA and the first intron was linked in frame with the HA tag sequence at the 5' side and the lbd coding sequence at the 3' side. This construct was cloned into the pEV3 vector[20] and the β-promoter was replaced by a fragment containing the α-globin promoter. The vector was linearised by AatII and transgenic mice were generated as described.[16]

Culture of primary fetal liver cells
Livers were isolated from E12.5 control- and EKLF null::EKLF-lbd tg fetuses. The genotype of the fetuses was confirmed by PCR. Single cell suspensions of individual fetal livers were cultured for 16h in StemPro-34™ containing 1% BSA, 1% glutamine and 10 units/ml EPO, but without serum supplement. The EKLF-lbd was activated by supplementing the medium with either 250nM 4-hydroxy-tamoxifen (4-OHT) alone or with 250nM 4-OHT and 20 μg/ml cycloheximide (CHX). After 16h of culture, cells were harvested and a small aliquot was taken for RNA isolation. The 16h period was chosen because it allowed detection of 4-OHT-induced β-globin gene transcription without CHX causing toxic effects. Fixation of the remainder of the cells with formaldehyde and subsequent isolation of nuclei was performed as described before.[28]

Preparation of cDNA and Real-time PCR
RNA was isolated using Trizol, according to the manufacturers guidelines (Invitrogen). The Super-script™ reverse transcriptase Kit (Invitrogen) was used for preparation of oligo-dT primed cDNA. Expression levels were determined on the Bio-Rad I-Cycler using the qPCR™ Core kit for Sybr Green 1 (Eurogentec). Expression levels of Hprt were used for normalization of β-globin expression levels. Primers used were as follows: Hprt-s,

AGCCTAAGATGAGCGCAAGT; Hprt-as, ATGGCCACAGGACTAGAACA; β-major-s, ATGCCAAAGTGAAGGCCCAT; β-major-as, CCCAGCACAATCACGATCAT.

Preparation of 3C templates

For the limiting number of cells (approximately 1.10^6) obtained from the individual EKLF null::EKLF-lbd tg fetal livers, we adapted the previously described protocol.[28] Cross-linked nuclei of E12.5 fetal livers were re-suspended in 50 µl digestion buffer containing 0.1% SDS and incubated for 1 hour at 37^0C with agitation, Triton X-100 was added to 2.6% and the nuclei were further incubated for 1 hour at 37^0C. The cross-linked chromatin was digested overnight at 37^0C with 10 units of *HindIII*. The restriction enzyme was heat-inactivated (25 minutes at 65^0C). After addition of 200 µl of 1.25x ligase buffer and 40 U of T4 ligase the chromatin was ligated for 4.5 hours at 16^0C followed by 30 minutes at room temperature. Proteinase K was added and samples were incubated overnight at 65^0C to reverse the cross-links. The following day samples were incubated for 30 minutes with RNAse and the DNA was purified by phenol extraction and ethanol precipitation using glycogen as a carrier. Locus wide cross-linking frequencies of wild type fetal livers treated with this adapted protocol were similar as those found previously (data not shown). PCR analysis of the ligation products was performed as described before.[22,28]

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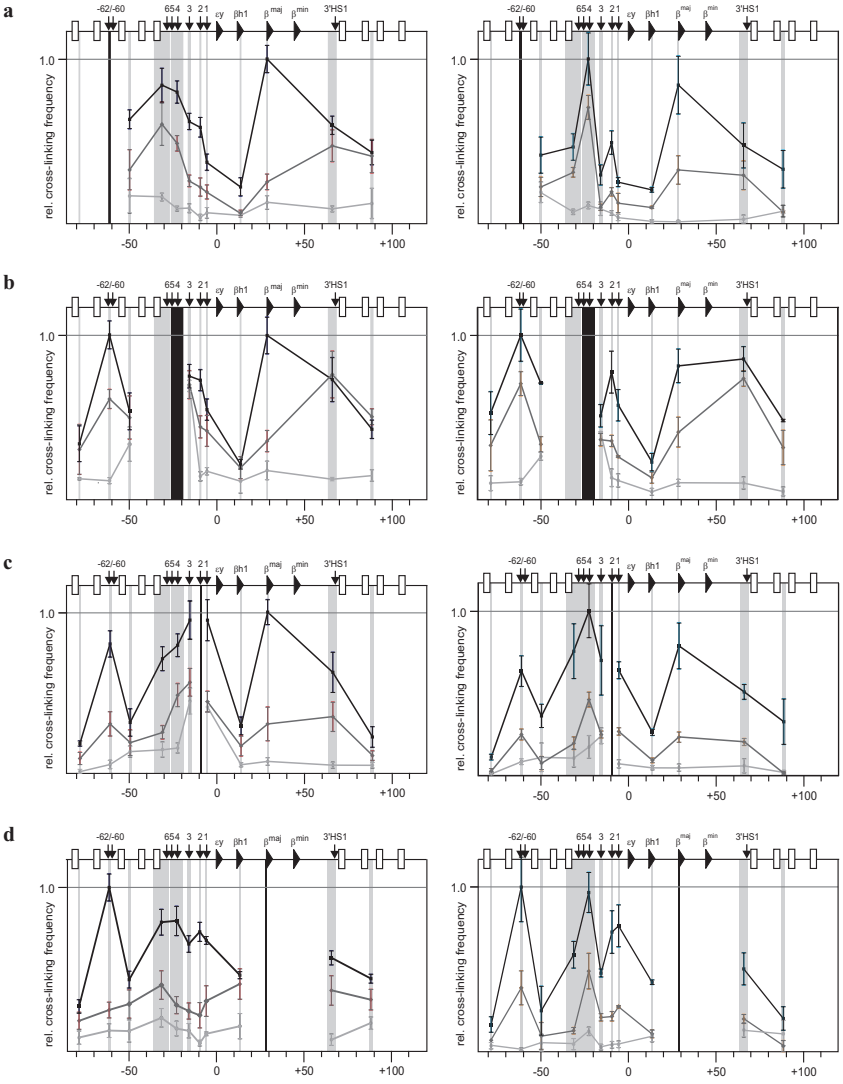

Supplementary Figure 1. The spatial organisation of the β-globin locus in EKLF$^{-/-}$ fetal liver cells is similar to that observed in erythroid progenitor cells.

(a, b, c, d) Comparison of locus-wide crosslinking frequencies of the *HindIII* restriction fragment containing 5'HS-62 (a), 5'HS4/5 (b), 5'HS2 (c), and β^{maj} (d), in fetal livers (graphs on the left) and I/11 erythroid progenitor cells (graphs on the right). Grey curves in graphs on the right: proliferating I/11 erythroid progenitor cells, not expressing globins. Black curves in graphs on the right: I/11 cells after induction of globin expression. Other details are as for Fig.1.

Chapter 5

Discussion

EKLF and globin

The initial discovery of the LCR of the β-globin locus[15] has led to an intense research effort to elaborate how this region is important for proper regulation of the genes within the β-globin locus. The LCR is required for position-independent and copy number-dependent high-level β-globin gene transcription. The mouse LCR consists of 6 HS. Deletion of any one of HS 1 to 4 leads to decreased globin expression, suggesting that the HS co-operate in a holo-complex.[4,12,18] LCRs are *cis*-acting elements that are located far away from the promoters they control. The mouse β-globin LCR is located approximately 50 kb upstream from the βmaj promoter. A number of models have been proposed to explain long-distance transcriptional control by the LCR; the accessibility-, the scanning- and the looping model. The looping model best explains a number of properties of the β-globin LCR. In studies in which a marked β-gene was placed at various positions in the human β-globin locus, it was demonstrated that the genes compete for activation by the LCR. The gene closest to the LCR has the highest level of expression.[10] Further to this it was demonstrated that the globin genes are not simultaneously actively transcribed, suggesting competition for direct contact with the LCR.[14,37] However, it was not until the application of two techniques, Chromosome Conformation Capture[30] and RNA-TRAP,[6] that it was demonstrated that the LCR is physically in close proximity to the actively transcribed gene. In fact, other more distally located HS, 5'HS-60/-62 and 3'HS1, also cluster to the LCR and the actively transcribed gene, to form a spatial organisation of the globin locus that is called the Active Chromatin Hub (ACH). Further studies showed that a substructure of the ACH is present in erythroid progenitors, consisting of the 5'HS-60/-62, the 3'HS1 and the 5' site of the LCR. Later in erythroid differentiation, when the globin genes are transcribed, the 3' site of the LCR and the globin gene co-localise with this substructure to form the ACH.

To further study the ACH, it is of interest to identify the proteins involved in its formation. A logical first choice of protein to study was EKLF. This transcription factor is required for the expression of adult β-globin genes. In the absence of EKLF, mice die of anemia around E14 due to a deficit in β-globin expression.[21,25] Furthermore, the β-globin locus contains several functional EKLF binding sites. The best studied is the one in the βmaj promoter. Some mutations in this binding site lead to β-thalassemia. Other EKLF binding sites are found in the LCR. With the 3C technique we demonstrated that EKLF is necessary for proper ACH formation (Chapter 4). An ACH substructure, reminiscent of that found in erythroid progenitors, was observed in E12.5 EKLF$^{-/-}$ fetal livers. The formation of this substructure is apparently independent of EKLF. However, the completion of the ACH is EKLF-dependent.

With the 3C technique we clearly demonstrated that EKLF is involved in the organisation of the proper spatial organisation of the β-globin locus for active transcription of the adult globin genes. However, the dynamics of the completion of the ACH remains enigmatic. A tracking model was proposed to describe the interactions of the HNF-4α promoter and its enhancer.[16] HNF-4α codes for a hepatic specific transcription factor[27] and its expression is controlled by an enhancer, 6.5kb upstream of the transcription start site. Before the gene is transcribed, proteins (such as histone acetyltransferases and chromatin remodelling proteins) are bound to the enhancer. During differentiation the enhancer/protein complex

moves along the intervening DNA towards the promoter, where upon arrival transcription starts.[16] Using R10 or I/11 erythroid progenitor cells it might be possible to show that the β-globin promoter is recruited to the LCR in a similar way. Particular proteins might be detected at the LCR before active transcription, but at the promoter only when genes are actively expressed. However, important for the demonstration of the tracking model is the binding of the enhancer/protein complex to the intervening DNA. In the Caco-cells used to demonstrate the tracking mechanism, the protein/enhancer complex is brought to the promoter over a period of days. It is not known how long this phenomenon takes for the β-globin locus. It is known that alternate transcription of the β- and γ-globin genes takes place within minutes.[14] The state of the ACH in between transcription of either of the globin genes may not be completely similar to the ACH substructure found in progenitor cells and in EKLF null cells. Nevertheless, it does indicate that recruitment of the promoter to the LCR takes place within minutes, which would complicate the demonstration of the binding of the enhancer/protein complex to the intervening DNA.

However, the finding that the distance between two genes influences the competitive advantage of the gene closest to the LCR[10] still conflicts with a tracking mechanism for the β-globin locus. Therefore, it is more likely that the gene promoter contacts the LCR in a stochastic manner. This is facilitated by the formation of the substructure of the ACH. Then, the full ACH is stabilised by the combination of DNA binding sites and protein complexes. Formation of the ACH results in a local increase of binding sites for transcription factors that is postulated to result in an increase of its cognate factors and associated chromatin modifiers, which enhances transcription.[9] The active organisation of the locus would use similar principles (but on a larger scale) as the folding of an enzyme to create an active site or "pocket".[23] The EKLF-dependent completion of the ACH leads to a stable complex, necessary for high-level transcription. However, the formation of this complex is reversible. This is demonstrated by a low expression of βmin, whose promoter competes for activation by the LCR with the βmaj promoter, leading to alternate expression of both genes in a flip-flop mechanism.[31]

For further study of the dynamics of the ACH formation, a cell line such as the one described in Chapter 2 will be very useful. In particular, an EKLF null cell line that carries a tamoxifen-inducible EKLF and the human globin locus would be advantageous. Although it has been shown that EKLF is necessary for establishment of hypersensitivity of 5'HS2 and 5'HS3 and the β-globin promoter, and for the completion of the ACH, the dynamics still have to be uncovered. For instance, HS could be formed immediately upon activation of an inducible EKLF by tamoxifen or cells may have to go through a cell division before HS are formed.

In analogy with the mouse β-globin ACH, the completion of the human β-globin locus ACH is presumably EKLF-dependent. After deletion of the β-globin promoter from the human β-globin locus, the gene is no longer transcribed. However, crosslinking frequencies between the β-globin gene and other HS are only ~30% decreased.[23] This indicates that the formation of the ACH is not a direct effect of transcription. However, since EKLF is important for the completion of the ACH, one could predict that deletion of the β-globin promoter, that contains a functional EKLF binding site, would lead to a similar incomplete ACH that is found in EKLF null mice. The difference between the ACH

structure found in absence of EKLF and in absensence of the β-globin promoter may be explained by a stabilisation of the ACH of the human locus by other sites, in particular two 3' enhancers located in the third exon and downstream of the human β-globin gene.[1] This stabilisation may be EKLF dependent since the 3' enhancer contains potential EKLF binding sites.[35]

The finding that EKLF plays an important role in the establishment of the ACH provides the basis for future work in the involvement of other factors in ACH formation. Transcription factors of interest are GATA1 and NF-E2, as it has been shown that these factors play a role in β-globin expression.[19,32,36] Another protein that is likely to play a role in ACH formation is CTCF, as this factor binds some of the HS that are clustered in the substructure of the ACH.[5]

In the work described in Chapter 4 we studied the participation of DNA fragments located on a stretch of ~130kb from 5'HS-62 to 3'HS1 in the ACH. However, the ACH may consist of more sequences. In fact, we have already demonstrated that 5'HS-80, further upstream of the locus, participates in the ACH (W. de Laat, pers. com.). Since we view the ACH as a nuclear compartment, dedicated to RNA polymerase II activity, it is not unlikely that other genes cluster to the ACH as well. Interestingly, some EKLF target genes described in Chapter 3 (AHSP and KCNN4) are located on the same chromosome as the β-globin locus. Further studies using 3C, *in situ* hybridisation or DNA-TRAP methods, could demonstrate whether these genes are in close proximity to the β-globin ACH when actively transcribed.

EKLF and other targets

The finding that the EKLF null-phenotype cannot be rescued by exogenous expression of human γ-globin[24] indicated that EKLF regulates expression of other genes important for definitive erythropoiesis, in addition to the β-globin gene. We have searched for additional target genes by micro-array screening. The way we performed the micro-array screenings can be considered as a fishing expedition; a relatively quick way of searching for differentially expressed genes, of which the significance has to be established. The micro-array data might have yielded more results if the experiments had been performed repeatedly and if dye-swaps could have been included. We were limited by the amount of RNA needed for probe production and the number of micro-arrray chips available. Nowadays protocols have been optimised for reliable RNA amplification, which allows for efficient probe production and screening multiple micro-arrays. This may not be necessary for RNA isolated from cultured cells, but for E12.5 fetal livers this is very useful. Furthermore, the micro-array chips we have used represent fewer genes than initially thought. The 9k erythroid-enriched chip was used because it was thought to represent many erythroid specific genes. However, half of the spots consist of T-cell enriched cDNAs. The other half consists of cDNAs that are enriched for genes expressed in the I/11 pro-erythroblast cell line. As a result, highly expressed genes are represented multiple times on the chip. To some extent this is an advantage, since data from multiple spots for a particular gene provide a valuable internal control. However, some genes are over-represented, as representation of a gene on the chip depends

on its expression level. Therefore, genes that are expressed at a low level are most likely not represented on the chip. The many spots (more than 1000) representing βmaj hamper the normalisation of the data, since this gene is differentially expressed in EKLF$^{-/-}$ compared to wild type cells. Nowadays chips, with more global gene representation, are available and thus, should provide an opportunity to identify additional target genes to the ones described in this thesis.

Despite all these drawbacks, we successfully discovered new target genes. Chapter 3 contains a list of genes that appear to be regulated by EKLF. We have focussed on genes that are activated by EKLF. However, it has been proposed that EKLF can also act as a repressor.[7] Our list of genes also includes genes that may be repressed by EKLF, since they are higher expressed in absence of EKLF compared to wild type. The differential expression of some of the listed genes was verified by real time PCR (AHSP, heme synthesis genes, KCNN4 (data not shown)), Northern blot (AHSP and ALAS2) or Western blot (Epb4.9) analysis. More direct evidence for the involvement of EKLF in regulating these genes would be the demonstration that EKLF binds to the promoter of these genes *in vivo*. The establishment of a transgenic mouse line, carrying a gene coding for a fusion protein of EKLF and a 23aa tag that can be biotinylated,[8] makes this experiment feasible. It has been demonstrated that a similarly tagged GATA-1 gene can be successfully immuno-precipitated from chromatin of erythroid cells (E. Katsantoni, pers. com.). In parallel, newly developed single chain llama antibodies directed against EKLF (H. Braun, unpublished data) can be used for similar chromatin immuno-precipitation (ChIP) experiments. These single chain antibodies are expected to have high affinity for their antigen.[34]

Since EKLF is an erythroid-specific transcription factor, it is not surprising that most of the (potential) target genes are either erythroid-specific or highly expressed in erythroid cells. Most of these genes have a similar expression pattern as the βmaj gene, i.e. they become highly expressed late in the differentiation program. This is in agreement with the observation that EKLF appears to have no function until the pro-erythroblast stage of definitive cells. In embryonic blood cells, lack of expression of two of these target genes leads to the appearance of Heinz bodies and an unstable membrane. Another gene that may be regulated by EKLF, KCNN4, may explain another aspect of the phenotype. This gene codes for the Gardos channel;[13] a Ca^{2+}-activated K^+ channel. It is expressed in multiple tissues, including erythroid cells, in which it is strongly upregulated late in differentiation.[17] It has been shown that this channel plays an important role in adaptation to changes in osmotic pressure.[33] In erythroid cells, KCNN4 plays an important role in cell size decrease, by excretion of KCl and water.[17] Interestingly, we found that during differentiation of primary pro-erythroblasts the size of EKLF null cells does not decrease as much as wild type cells (Chapter 2 and 3). Lack of upregulation of KCNN4 may explain this phenotype. Using the culture method described in Chapter 2, the effect of absence of EKLF and hence lack of high expression of KCNN4 on osmotic pressure adaptation could be studied.

The availability of EKLF null cells from a transgenic line containing a tamoxifen-inducible EKLF gene, allows gene expression studies in the absence or presence of active EKLF. In fact, we have performed such an experiment with primary transgenic cells. EKLF null cells carrying a tamoxifen inducible EKLF were isolated from E12.5 fetal livers and expanded in culture for 10 days before induction of terminal differentiation. 24 Hours before this induc-

tion of terminal differentiation, tamoxifen was added to half of the culture and the effect of the activated EKLF was studied by gene expression with micro-arrays 12 hours after induction. The 12 hours after induction is quite short to detect an effect of the activated EKLF as judged by the ^{2}log values in Table I, Chapter 3; The table shows that potential EKLF target genes are not extensively differentially expressed in wild type and EKLF KO cells after 12 hours of induction. However, the data suggested that the activated EKLF rescued the gene expression of EKLF target genes to some extent (data not shown). Thus, the tamoxifen-inducible EKLF is a useful tool to demonstrate EKLF dependent expression of genes.

EKLF in action

Although there had been indications that EKLF is not only present[28] but also active in embryonic cells (low expression of βmaj is EKLF-dependent[31] and a reporter gene revealed EKLF activity in embryonic cells[29]), in Chapter 3 we show for the first time that endogenous EKLF target genes (AHSP and Epb4.9) are dependent for their high expression on the presence of EKLF in embryonic cells. This raises the question whether other EKLF target genes are expressed in embryonic blood cells. In addition, our results suggest that the mere presence of EKLF is not sufficient for transcription of its target genes. This can be demonstrated in primitive cells (AHSP is expressed but βmaj is not), as well as in definitive cells. EKLF appears to be present at the pro-erythroblast stage in definitive cells, judged by the presence of RNA in proliferating I/11 cells[11] and by protein in primary pro-erythroblasts (Western blot, data not shown). However, at this stage the target genes are not expressed. Interestingly, the completion of the ACH and the expression of EKLF target genes in definitive cells appear to take place more or less simultaneously.

Another indication that the mere presence of EKLF does not necessarily lead to expression of its target gene comes from a study by Luo et al.[20] While it was previously thought that EKLF is only expressed in the erythroid cell lineage, Luo and colleagues demonstrated that EKLF is expressed in macrophages and regulates the expression of interleukin (IL)-12 p40. Depending on the activation status of the macrophages, EKLF activates or represses this gene. This is the first time that EKLF activity is demonstrated in non-erythroid cells and the first endogenous gene that can be repressed by EKLF.

The activity of EKLF can be regulated at several different levels. These include 1) the amount of protein present in the nucleus, 2) posttranslational modifications and 3) the cooperation with other proteins. It is unclear that EKLF is regulated at the first level, as preliminary data from the Western blot (data not shown) suggest that EKLF is not upregulated after the pro-erythroblast stage of differentiation.

However, a number of articles report posttranslational modifications of EKLF (phosphorylation[22] and acetylation[38,39]) and its significance for its activation capacity. For further study of post-translational modifications, the construct coding for a bio-tagged EKLF protein can be helpful. The fusion protein could be isolated from mice carrying this construct from various cells, such as embryonic cells, pro-erythroblasts and differentiating cells (using the culture technique described in Chapter 2). Analysis of the purified protein by mass

spectrometry may reveal differences in modifications in various cells at various differentiation stages. Alternatively, the llama antibodies could be used, although the affinity of these antibodies for EKLF may be weaker than the high affinity of avidin/streptavidin for biotinylated EKLF. However, it has the advantage that only endogenous EKLF will be purified. The importance of post-translational modification of EKLF could be further studied in the earlier described EKLF$^{-/-}$ cell line (Chapter 2). These pro-erythroblasts can be transfected with constructs that code for EKLF with mutations at post-translational modification sites. These mutations could effect target gene expression and demonstrate the importance of particular post-translational modification.

The bio-tagged EKLF protein will also be useful in identifying EKLF partners, similar to the work performed with GATA-1 complexes.[26] While *in vitro* studies have demonstrated EKLF co-operates with other proteins to regulate gene expression.[2,7,38] the bio-tagged EKLF allows identification and characterization of such proteins *in vivo*. An interesting question is whether different EKLF containing complexes are formed at various stages of cellular differentiation (i.e. induced versus non-induced MEL cells or I/11 cells). More information with regard to EKLF partners may broaden the understanding of EKLF target gene regulation and ACH formation.

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Summary

Summary

The human body consists of a huge number of cells. At the base of all these cells is one fertilized oocyte. By cell divisions the number of cells is increased during development, and cells specialize into various types of cells, i.e. muscle, brain and blood cells. Groups of specialized cells form the various tissues. DNA contains the blueprint of all cells. By each cell division the DNA is duplicated and equally distributed over the two daughter cells. Hence, practically all the cells contain identical genetic information. The DNA in each cell contains about 25.000 genes. Each gene contains information necessary to produce a particular protein. These proteins are involved in many processes such as energy metabolism, cell composition and gene regulation.

Although all cells contain the same genetic information, they specialize into various cell types. For this, the genes are specifically activated or repressed. A tight regulation of the genes makes it possible to specialize into a particular type of cell. Transcription factors play an important role in this tight regulation of activating or repressing genes. EKLF is such a transcription factor. EKLF is mainly expressed in red blood cells. These cells give blood its red color. They carry oxygen from lungs to tissues in the body that require the oxygen for energy metabolism. Red blood cells are filled with hemoglobin that actually binds the oxygen. Each hemoglobin particle consists of two α-globin, two β-globin and four heme molecules. The genetic blueprint for the β-globin gene is called the β-globin locus. In the mouse, this locus contains four globin genes. Two of them, the embryonic β-globin genes, are active during embryonic development of the mice and the other two, the adult β-globin genes are activated later in development and stay active during the rest of the lifespan of the mouse. Apart from the four β-globin genes, the locus contains regulatory elements. These include the Locus Control Region (LCR), and a number of other hypersensitive sites. The LCR is required for a proper regulation of the globin genes; in absence of the LCR the genes are not activated, and mutations in the LCR can lead to impaired activation. The LCR is located relatively far from the genes it controls. However, when a globin gene is active in red blood cells, the LCR is physically in close proximity to this gene. In fact, the globin locus forms a spatial organization that consists not only of the active gene and the LCR, but also of the other hypersensitive sites that are located even further away on both sides of the genes. This structure is called the Active Chromatin Hub (ACH). Prior to the stage in which the genes are active, a substructure of this ACH is formed. Unlike the complete ACH, this substructure does not contain the globin genes and part of the LCR. In Chapter 4 we show that a similar substructure is found in red blood cells that are EKLF deficient. We demonstrated that EKLF is necessary for the completion of the ACH, a requirement for activating the genes.

The transcription factor EKLF is necessary for the proper development of red blood cells; apart from the regulation of the β-globin genes it regulates also other genes. However, it was not known which other genes. To study the genes EKLF activates (or represses), we used a culture method with erythroid progenitor cells. These progenitor cells are restricted to the red blood cell lineage, but have not yet developed to fully differentiated, hemoglobin-containing red blood cells. The culture method allows us to culture the cells as progenitors or induce them to start their terminal differentiation to red blood cells. We performed this

culture technique with normal (wild type) cells, and cells that do not have EKLF. Genes that become active in normal cells, but not in cells lacking EKLF apparently require the presence of EKLF to become active. In Chapter 3 we describe a number of these EKLF dependent genes. We show that EKLF is not required in the progenitor cells, but only in the last steps of erythropoiesis. The genes that require EKLF to become active are specific for red blood cells and they become active late in their development. The genes we have focused on in Chapter 3 are involved in hemoglobin metabolism and in membrane stability. Failure of activation of these genes further clarifies the problems that red blood cells have in the absence of EKLF. In addition we demonstrated that EKLF activates some genes not only in adult red blood cells (definitive cells), but also in red blood cells during embryonic development (primitive cells). Lack of activation of these genes caused by absence of EKLF disturbs the primitive cells; the hemoglobin metabolism is disorganized and the cell membrane appears less stable.

Synoptically, we have shown that EKLF is involved in the spatial organization of the β-globin locus, and in activating a number of genes that are important for the last steps in both primitive and definitive red blood cell differentiation.

Samenvatting

Samenvatting

Het menselijk lichaam bestaat uit enorm veel cellen. Aan de basis van al deze cellen staat één bevruchte eicel. Tijdens de ontwikkeling vermeerdert het aantal cellen door celdelingen, en cellen specialiseren zich tot verschillende soorten cellen, bijv. spier-, hersen- en bloedcellen. Groepen van gespecialiseerde cellen vormen de diverse weefsels. DNA bevat de blauwdruk van alle cellen. Voor iedere celdeling wordt het DNA gekopieerd en gelijkelijk verdeeld over de twee dochtercellen. Derhalve bevatten praktisch alle cellen identieke genetische informatie. Het DNA in iedere cel bevat ongeveer 25.000 genen. Elk gen bevat informatie die nodig is om een bepaald eiwit te maken. Deze eiwitten zijn betrokken bij vele processen, zoals stofwisseling, opbouw van de cel en genregulatie. Alhoewel alle cellen dezelfde genetische informatie bevatten, specialiseren ze zich tot verschillende soorten cellen. Om dit te bewerkstelligen worden genen specifiek geactiveerd of onderdrukt. Een strikte regulatie van de genen maakt het mogelijk om tot een bepaald soort cel te specialiseren. Transcriptie factoren spelen een belangrijke rol in de strikte regulatie van het activeren of onderdrukken van de genen. EKLF is zo'n transcriptie factor. EKLF komt voornamelijk voor in rode bloedcellen. Deze cellen geven bloed zijn rode kleur. Zij transporteren zuurstof van de longen naar de weefsels in het lichaam die zuurstof nodig hebben voor de stofwisseling. Rode bloedcellen zijn gevuld met hemoglobine, wat het zuurstof feitelijk bindt. Elk hemoglobine deeltje bestaat uit twee α-globine ketens, twee β-globine ketens en vier heem moleculen. De genetische blauwdruk voor het β-globine heet het β-globine locus. Het β-globine locus in muizen bevat vier globine genen. Twee daarvan, de embryonale β-globine genen, zijn actief tijdens de embryonale ontwikkeling van de muis en de andere twee, de volwassen β-globine genen worden later in de ontwikkeling actief en blijven actief gedurende de levensduur van de muis. Behalve de vier β-globine genen, bevat het locus regulerende elementen. Tot deze regulerende elementen behoren de 'Locus Control Region' (LCR) en een aantal andere 'hypersensitive sites'. De LCR is vereist voor een correcte regulatie van de globine genen. Zonder de LCR worden de genen niet geactiveerd, en mutaties in de LCR kunnen leiden tot een gebrekkige activiteit. De LCR ligt relatief ver van de genen die ze reguleert. Wanneer echter een globine gen in rode bloedcellen actief is, ligt de LCR in fysieke nabijheid van dit gen. Sterker nog, het β-globine locus neemt een ruimtelijke organisatie aan waarbij niet alleen het actieve gen en de LCR betrokken zijn, maar ook de andere hypersensitive sites, die nog verder aan beide kanten van de genen verwijderd liggen. Dit complex wordt de 'Active Chromatin Hub' (ACH) genoemd.

Voorafgaand aan de fase dat de genen actief zijn wordt er een incompleet complex van de ACH gevormd. Deze verschilt van de complete ACH door de afwezigheid van de globine genen en een gedeelte van de LCR. In hoofdstuk 4 laten we zien dat rode bloedcellen zonder EKLF een zelfde incomplete structuur hebben. We hebben aangetoond dat EKLF nodig is voor de voltooiing van de ACH, een vereiste voor het activeren van de genen.

De transcriptie factor EKLF is nodig voor een correcte ontwikkeling van rode bloed cellen; buiten het reguleren van de β-globine genen is het ook betrokken bij de regulatie van andere genen. Welke deze andere genen zijn, was echter niet bekend. Om te bestuderen welke genen EKLF activeert (of onderdrukt), hebben we een kweek methode gebruikt met voorloper cellen van rode bloedcellen. De uitgroei mogelijkheden van deze voorloper cel-

len is beperkt tot rode bloedcellen, maar ze zijn nog niet ontwikkeld tot volledig gedifferentieerde, hemoglobine bevattende rode bloedcellen. De methode maakt het mogelijk om de cellen als voorloper cellen te kweken of om ze aan te zetten tot hun definitieve differentiatie tot rode bloedcel. We hebben deze kweek methode toegepast op normale cellen en op cellen die geen EKLF hebben. Genen die actief worden in normale cellen, maar niet in cellen zonder EKLF hebben EKLF kennelijk nodig om geactiveerd te worden. In hoofdstuk 3 beschrijven we een aantal van deze EKLF-afhankelijke genen. We laten zien dat EKLF niet nodig is in de voorloper cellen, maar slechts in de laatste stappen van de rode bloedcel ontwikkeling. De genen die EKLF nodig hebben om actief te worden zijn specifiek voor rode bloedcellen en zij worden laat in de ontwikkeling van de cellen geactiveerd. De genen waarop we onze aandacht hebben gericht in hoofdstuk 3 zijn betrokken bij het hemoglobine metabolisme en de stabiliteit van het membraan. Het niet actief worden van deze genen heldert de problemen op die rode bloedcellen hebben als EKLF niet aanwezig is. Verder hebben we aangetoond dat EKLF enkele genen niet alleen in volwassen rode bloed cellen (definitieve cellen), maar ook in rode bloedcellen tijdens de embryonale ontwikkeling (primitieve cellen) activeert. Het niet actief worden van deze genen door de afwezigheid van EKLF ont-regelt de primitieve cellen; het hemoglobine metabolisme is ontregeld en de cel membraan lijkt minder stabiel.
Samengevat hebben we aangetoond dat EKLF betrokken is bij de ruimtelijke ordening van het β-globine locus en bij het activeren van genen die belangrijk zijn in de laatste stappen van de ontwikkeling van zowel primitieve als definitieve rode bloedcellen.

Abbreviations

3C	Chromosome Conformation Capture
4-OHT	4-hydroxy tamoxifen
aa	amino acid
ACH	Active Chromatin Hub
AGM	Aorta Gonad Mesonephros
BFU-E	Burst Forming Unit, Erythroid
bp	base pair
CBP	CREB-binding protein
CD34	Cluster of Differentiation 34
cDNA	complementary DNA
CFU-E	Colony Forming Unit, Erythroid
CFU-GEMM	Colony Forming unit for Granulocytes, Erythrocytes, Macrophages, and Megakaryocytes
CFU-S	Colony Forming Unit of the Spleen
ChIP	Chromatin immuno-precipitation
CHX	Cycloheximide
CKII	Casein kinase II
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CREB	cAMP response element-binding protein
CTCF	CCCTC-binding factor
Dex	Dexamethasone
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNAseI	Deoxyribonuclease I
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
E12.5	Embryonic day 12.5
EKLF	Erythroid Krüppel-like factor
Endo	Endoglin
EPO	Erythropoietin
FACS	Fluorescence activated cell sorter
GMP	Granulocyte monocyte progenitor
HAT	Histone acetyl-transferase
HDAC	Histone de-acetylase
HPC	Hematopoietic progenitor cell
HPFH	Hereditary Persistence of Fetal Hemoglobin
HS	Hypersensitive site
HSC	Hematopoietic Stem Cell
IL-3	Interleukin-3
Lin	Lineage
LCR	Locus Control Region
MEL	Murine erythroleukemia
MEP	Myeloid and erythroid progenitor
mRNA	messenger RNA
NF-E2	Nuclear Factor-Erythroid 2
NLS	Nuclear Localisation Signal
PAS	Para-Aortic Splanchnopleura
PCR	Polymerase Chain Reaction
RBC	Red Blood Cell
Rh	Rhodamine
RNA	Ribonucleic acid
SCF	Stem Cell Factor
TGF-β	Transforming Growth Factor beta
TRAP	Tagging and recovery of associated proteins

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2. **Drissen R**, Palstra R, Gillemans N, Splinter E, Grosveld F, Philipsen S, de Laat W (2004) The active spatial organisation of the β-globin locus requires the transcription factor EKLF. Genes Dev (accepted).
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