In vivo and in vitro studies of the proto-oncogene Evi1

Dominik Spensberger

In vivo and *in vitro* Studies of the Proto-oncogene *Evi1*

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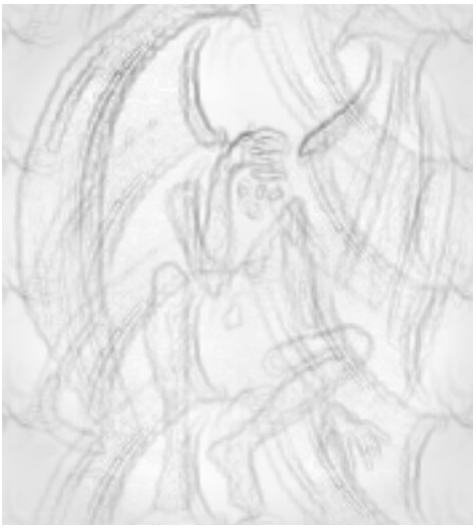

Dominik Spensberger

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Chapter 1

Introduction

1. Hematopoiesis

1.1 Origin of blood cell formation

Hematopoiesis is the process of proliferation, differentiation and maturation of all different types of blood cells (Figure 1). The primary organs involved in hematopoiesis during embryogenesis are the yolk sac and the fetal liver[1,2]. The yolk sac is responsible for the primitive erythropoiesis, which begins during the first 3 to 6 weeks of gestation in humans and 7 to 7.5 days of gestation in mice. At the early stage of embryo development, yolk sac blood islands are formed which support the differentiation of the primitive erythroblast and endothelial cells. The primitive erythroblasts enter the newly formed vascular network, where they continue to divide, differentiate and acquire hemoglobin[3]. Primitive hematopoiesis is mainly restricted to the erythroid lineage. The cells are large, nucleated, and contain early embryonic globin chains not found in later stages of development. The process continues until approximately day13 in mice when the hematopoietic stem cells (HSC) inhabit fetal liver to take over the role as the primary hematopoietic organ until birth[4,5]. Erythroid cells formed in the fetal liver are enucleated and morphologically similar to those found in adult, with the exemption to hemoglobin chains. The spleen and the thymus also are implicated in hematopoiesis during this period to support the differentiated lymphoid cells in fetal and adult period. The fetal hematopoiesis progresses towards the definitive hematopoiesis when the HSCs migrate towards newly developed long bones. In humans the bone marrow is the primary organ of hematopoiesis. The adult definitive hematopoiesis takes place around the time of birth, and is a life long lasting process.

1.2 Hematopoietic stem cells

The hematopoietic cascade of different blood lineages originates from a single common cell the hematopoietic stem cell. HSCs are characterized by unique properties of self-renewal, and the capacity to proliferate and differentiate into progenitors of each of the different blood cell lineages (pluripotency)[6]. The occurrence of HSCs in a normal adult bone marrow is approximately 1 to 10 cells in 100.000 cells[7,8]. The sustained and steady number is achieved mainly due to self-renewal characteristics. The pivotal role of HSCs is to give rise to all different blood cells, i.e. erythrocytes, platelets, basophils, neutrophils, eosinophils, macrophages, mast cells, B-lymphocytes, T-lymphocytes, natural killer cells, and dendritic cells[9]. The ability for a single cell to give rise to different types of lineage specific and functionally different types of cells is achieved via two proposed mechanisms. The first model anticipates that exogenous signals, such as ligands, interact with specific receptors to activate lineage-restricted genes in a highly

balanced manner[10,11]. In the second model the cell fate decision is made stochastically within one cell cycle transit[12,13]. However, up to date little is known about the molecular mechanism that initiates the decision process of stem cells to become a specific cell type.

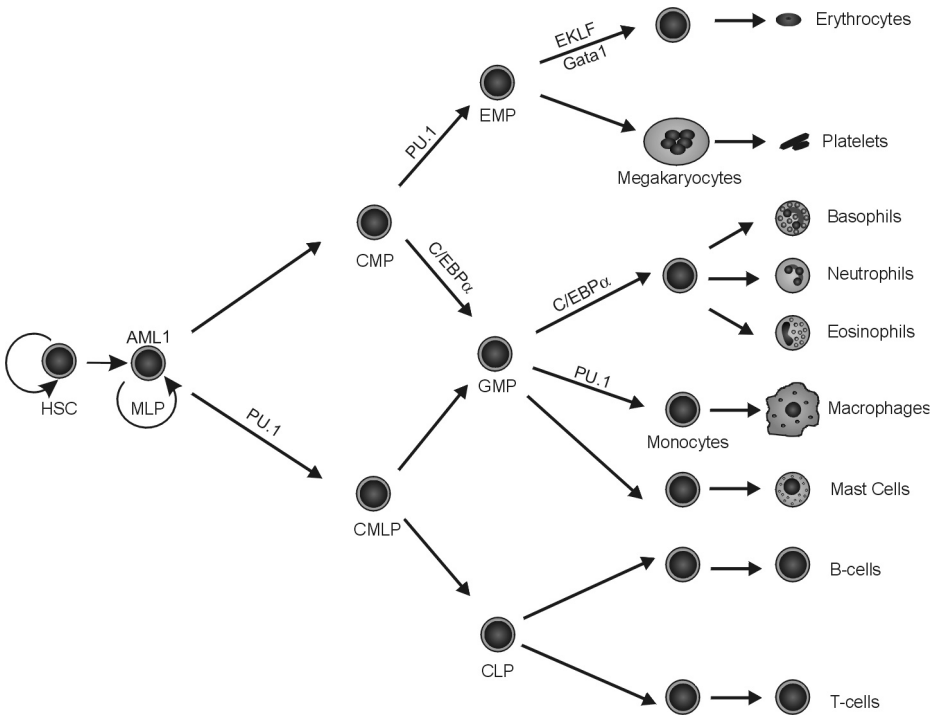

Figure 1 Schematic representation of hematopoietic cascade
The very first step in hematopoietic differentiation involves a commitment of the hematopoietic stem cell (HSC) to give rise to multilineage progenitor (MLP) cells. MLP give further rise to either the common myeloid progenitor (CMP) or common myeloid/lymphoid progenitor (CMLP). CMP and CMLP progenitors are able to advance to granulocutes/ monocyte progenitors (GMP), as well as give rise to separate lineages: erythroid/megakaryocyte progenitors (EMP) or common lymphoid progenitors (CLP), respectively. EMP continues to differentiate exclusively towards erythroid/ megakaryocytic lineage and CLP towards B-cells and T-cells. Key hematopoietic transcriptional factors that influence commitment to certain lineages are depicted.

1.3 Erythropoiesis

The process of red blood cells (erythrocytes) production from a HSC via several differentiation steps is called erythropoiesis (Figure 2). Colony forming unit-spleen (CFU-S) is one of the first experimentally defined cells that loses the ability to self-renewal but retains pluripotency. CFU-S may further commit towards the common myeloid progenitor (CMP) which gives rise to megakaryocytic/erythroid progenitor (MEP). Morphological differences between the early erythroid progenitors cells cannot be established, however, the first cell from the erythroid

progenitor line that can be identified in vitro using a cocktail of growth factors and cytokines is burst-forming unit-erythroid (BFU-E)[14]. Stimulation of an early BFU-E progenitor for 5 to 7 days in mice and for 14-16 days in humans with erythropoietin (Epo), interleukin 3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), thrombopoietin, and stem cell factor (SCF) gives hemoglobinized cell clusters of more than 500 erythroblasts. BFU-E is the early progenitor which precedes the colony-forming unit-erythroid (CFU-E), that closely associates with the proerythroblast[14,15]. In contrast to the BFU-E the CFU-E is Epo dependent but lost its dependency on other cytokines. CFU-E is capable of full erythroid differentiation in 2 days or 5 to 8 days, for mouse or human marrow respectively, and gives rise to small cell clusters containing between 8-64 well-hemoglobinized erythroid cells.

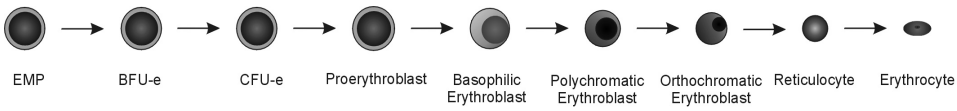

Figure 2 Erythroid lineage differentiation pathway
Erythroid/megakaryocyte progenitor (EMP) is the earliest known precursor cell that can differentiate into erythrocytes or megakaryocytes. Burst forming units-erythroid (BFU-e) and colony forming units-erythroid (CFU-e) can be identified by morphology of the colony in a functional assay. Proerythroblast is the earliest cell of the immature forms to be morphologically recognizable. The process continues until enucleation, as the cell becomes a mature erythrocyte.

The pro-erythroblast is the most primitive erythroid cell that can be identified morphologically. It is characterized by its relatively large size (14 to 19μm in diameter), large nucleus that fills 80% of the cell, and thin circumference of basophilic cytoplasm[16]. During progression to the next stage of differentiation i.e. the basophilic erythroblast, the cells become smaller as the chromatin starts to condenses, giving the nucleus a granular appearance. The nuclear chromatin continues to condense, and the nucleus becomes smaller as well as the rest of the cell when it develops into a polychromatic erythroblast. At this stage the accumulation of hemoglobin becomes detectable with histological staining procedures[17]. The orthochromatic erythroblast is a small (8-12μm and 7-8μm in diameter in humans and mice respectively) nucleated precursor cell of erythrocyte, characterized by pyknotic degeneration of the nucleus due to condensation of the chromatin. The nucleus decreases in size by pyknotic degeneration as the chromatin extensively condenses[18]. The reticulocyte is the first enucleated cell in which the shrunken nucleus is extruded. Reticulocytes are slightly larger in volume than erythrocytes, and contain some of the cytoplasmic organelles, such as ribosomes, mitochondria, and the Golgi complex. Finally, mature erythrocytes have a small size, lack organelles and have a biconcave disk shape[19].

1.4 Myelopoiesis

Pluripotent hematopoetic stem cells have the ability to commit towards different lineages of blood cells as described above. The process in which the stem cell gives rise and become a monocyte or granulocyte is termed myelopoiesis. A granulocyte is a general term for three different types of cells; basophils, neutrophils and eosinophils that are characterized by presence of cytoplasmic storage granules[20,21]. As with erythropoiesis it requires different stages of progression during the differentiation process.

Neutrophil origin follows the commitment and differentiation pattern in the very early stages as erythroid precursors, i.e., from hematopoietic stem cell towards myeloid/lymphoid progenitor (MLP) followed by common myeloid progenitor (CMP). The common myeloid progenitor cell becomes granulocyte/monocyte progenitor (GMP) followed by an additional narrowing towards terminal differentiation, as it becomes granulocyte colony-forming unit (CFU-G) (Figure 3). CFU-G is the first immature cell that is fully committed towards terminal neutrophilic differentiation and gives rise to a myeloblast. The myeloblast is the first early progenitor cell that can be morphologically recognized[21]. It has a very immature appearance, distinguishable by a large nucleus and multiple nucleoli. Myeloblasts undergo division and maturation into promyelocytes. This is the largest cell of the neutrophilic series which makes and accumulates large numbers of azurophilic granules in the cytoplasm[22]. The neutrophilic myelocyte loses many of the azurophilic granules, as peroxidase-negative granules become more prominent. This is the last stage at which the cell has mitotic capability. At the myelocyte stage the cell generates oxidation components such as the phagocyte respiratory burst oxidase (gp91phox) and cytochrome heavy chain, which can be used as detection indicator[23].

The last stages of the neutrophilic differentiation pathway are metamyelocytes, band-forms, and mature polymorphonuclear neutrophils. These cells are non-dividing, with specific morphological features such as absence of nucleoli in the nucleus, presence of tertiary granules in the cytoplasm, and presence of a segmented nucleus in the mature neutrophil. Late markers for mature neutrophils include lactoferrin (LF), neutrophil gelatinase (NG), and in mice the GR-1 receptor marker[23]. The bone marrow is the main organ that supports neutrophilic proliferation and maturation. Within the bone marrow neutrophils are found in two functionally different compartments, a proliferating compartment and a storage compartment. The actively proliferating neutrophil precursor cells (myeloblast through myelocyte) are localized in mitotic or proliferating compartment. In the maturation storage compartment more differentiated cells are found; metamyelocyte, band, and mature neutrophils[24]. The polymorphonuclear neutrophils

enter the blood circulation where they function for the next 24 to 48 hrs before death or loss[25].

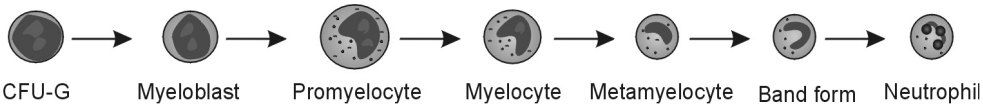

Figure 3 Schematic representation of the differentiation stages of myeloid lineage
Colony forming unit-granulocyte (CFU-G) is the first committed immature progenitor cells that give rise to mature neutrophils. During differentiation the cell undergoes morphological changes such as granulation and nucleus segmentation. The neutrophil has a lifespan of about 3 days.

2. Transcription factors in hematopoietic development

2.1 Introduction to transcription factors

Transcription factors are proteins which posses the ability to recognize and bind to specific short DNA base sequences and regulate transcription of genes[26-29]. The sites are identified via specific motifs present within the transcription factor, the DNA binding domains[30-32]. Common domains in transcription factors that are involved in DNA binding are zinc fingers (C_2H_2, C_4, or C_6)[33,34] , helix-turn-helix (two α helices and a short extended amino acid chain between them), leucine-zipper (two α helices joined together)[35], and helix-loop-helix (similar to leucine-zipper structure)[36] motifs. Although the motifs might differ between transcription factors, the role remains the same, which is specific DNA sequence recognition followed by control and regulation of gene expression. This is achieved by binding to promoter regions, frequently a few hundred basepairs upstream of the major transcription start site of a gene, to either inhibit or activate transcription.

Although physical interaction between DNA and the transcription factor is essential, this interaction is not solely responsible for its activity. Other specific functional domain(s) present within the transcription factors are required to accomplish the protein's functions[26]. Transcription factors in general are known to form complexes with other proteins, critical for their function. As a result these interacting proteins can regulate transcription factor function. These co-factors, or protein complexes may influence the fate of transcription factors from being either a repressor or an activator[37-40]. Transcription factors may undergo posttranslational modifications. Eukaryotic proteins are modified by a stable modifications, such as disulfide formation, glycosylation, and lipidation, that allows the protein to form the correct structure and proper functional state[41]. Furthermore, many other covalent modifications may occur transiently but they play important roles in regulating the protein biological function.

The most common covalent modifications are phosphorylation, acetylation, methylation,

sumoylation and ubiquitination[29,42]. These modifications may take place at a single site, but also at multiple sites[41]. The complexity of protein modifications is not only restricted to the type of modification at a given residue, but also by the fact that the same residue can be modified in many different ways[43-45]. Posttranslational modification of transcription factors appears to play an important role as it influences the expression of the target genes. The most well studied transcription factor that may undergo many different modifications, thereby affecting its function and regulation, is the tumor suppressor factor p53 [46].

The fate of uncommitted progenitor cells towards fully functional lineage specific cells has in recent years mainly been studied in the light of extrinsic (growth factors or stroma) or intrinsic (stochastic) signals[12,13,47,48]. Although both signals have a major influence on the cell's fate, the importance of transcription factors should not be overlooked, as they are key molecules that influence the expression of genes that regulate committed mature cell development.

2.2 Transcription factors in erythroid development

GATA1. The transcription factor that plays a major role in erythroid lineage development and differentiation is GATA1. GATA1 is a member of a zinc finger transcription factor family containing 6 members. GATA1 is required not only for the proper development of the erythroid, but also for the megakaryocytic and eosinophilic lineages. The identification of GATA1 was accomplished by recognizing a specific transcription factor that was able to bind the β-globin 3' enhancer sequences in chicken and human[49,50]. GATA1 recognizes and binds a specific DNA consensus sequence, (A/T)GATA(A/G), via its C_4 zinc finger domains[51-53]. The expression of GATA1 is restricted to erythrocytes, megakaryocytes, eosinophils, and mast cells, [54-57]. The importance of GATA1 as a key regulator in erythropoiesis was demonstrated both *in vitro* and *in vivo*. Embryonic stem (ES) cells deficient in GATA1 by gene disruption were capable to contribute to all different tissues, including white blood cells. However, there was no contribution towards mature red blood cells[58]. In accordance with the *in vitro* observations, GATA1 -/- mice showed embryonic lethality between day E10.5 and E11.5, due to profound anemia[54,59]. Characterization of the cell stage in the absence of GATA1 revealed a block of differentiation at the proerythroblast stage[60]. Other mouse models with partial disruption of the GATA1 gene showed that GATA1 levels played a pivotal role in control of erythroid cell development[61,62]. The finding that GATA1 is a key factor in erythroid development may indicate the direct role of its target genes in erythropoiesis. The abundance of GATA consensus sequences in the genome that can be found in the promoters of many genes does not reflect its real target

genes. The most well studied direct targets of GATA1 are the genes encoding α and β-globins, the heme biosynthesis enzymes ALAS2, ALAD, and HMBS, ABC mitochondrial erythroid (ABCme), erythropoietin (Epo), Epo receptor (EpoR), the anti apoptotic protein Bcl-$_{XL}$, and the transcription factors GATA2, EKLF, and TAL1[49-51,63-70]. In addition, interactions of GATA1 with other transcription factors or co-factors have been shown to influence the expression of GATA1 target genes. Putative target genes may either be repressed or activated. GATA1 is able to form homo-dimers via its N- or C-terminal zinc finger. The dimerization plays an important role in self-regulation, as shown in the zebrafish model [71]. A very important partner of GATA1 isolated in a yeast two-hybrid screen is Friend of GATA1 (FOG1)[72]. The interaction occurs via the N-terminal zinc finger domain of GATA1, and plays an essential role during erythroid and megakaryocytic differentiation[73]. Ablation of the GATA1 domain required to form a complex with FOG1 inhibits the progression towards erythroid terminal differentiation. Depending on the promoter context FOG1 can either stimulate or represses GATA1 activity at the promoter level. PU.1 has been demonstrated to act as a binding partner of GATA1 and inhibits GATA1 binding to DNA. In addition, it has been reported that GATA1 prevents c-Jun from forming a complex with PU.1[74]. These interactions may be of great importance in the decision making of precursors to develop either into the myeloid or into the erythroid lineage. Other proteins that have been reported to interact with GATA1 are the tumor suppressor protein retinoblastoma (Rb) erythroid Krüppel-like factor (EKLF), LIM-only protein (LMO2), and P300/CBP[75-77].

GATA2. The second hematopoietic member of the GATA family was identified by Yamamoto and colleagues based on the sequence homology with GATA1[78]. GATA2 contains a C-terminal DNA binding zinc finger domain and an N-terminal zinc finger involved in protein-protein interaction similar to the other GATA family members[79]. The expression of GATA2 is restricted to hematopoietic stem cells, hematopoietic early progenitors, and endothelial cells[79]. Constitutively active GATA2 supports proliferation and blocks terminal erythroid differentiation of erythroid precursor cells[80,81]. The expression and tight regulation of GATA2 depends on cooperation with GATA1. The level of GATA2 must decrease when the GATA1 level increase to allow erythroid terminal differentiation. The importance of GATA2 in the erythroid lineage was demonstrated by ablation studies of GATA2 *in vivo*. GATA2 -/- mice are embryonic lethal at day E10-11 due to defective primitive and definitive erythropoiesis[82,83]. *In vitro* differentiation using GATA2 null ES cells implies a key role for GATA2 in maintenance and/or proliferation of hematopoietic precursors cells[84].

EKLF. The erythroid Krüppel-like factor (EKLF) belongs to a large family of transcription factors that is characterized by a common arrangement of the zinc fingers, as previously found in the Drosophila Krüppel protein[85]. The three C_2H_2 zinc fingers of EKLF are positioned at the C-terminal part of the protein. In addition, the protein contains two transactivation domains (TD) within the proline rich region domain spanned along a major part of the protein[86,87]. EKLF was isolated from mouse a erythroleukemia cell line, and the expression is restricted to the erythroid lineage[88-90]. The mRNA transcript of EKLF is present as early as E7.5 in the developing embryo, and continues to be expressed in the adult spleen[91]. This indicates that both primitive and adult hematopoietic cells express EKLF and that its expression is required for the survival and maturation of erythroid cells. The zinc finger domain of EKLF specifically binds the DNA sequence 5'-CCACACCCT-3'[88]. The specificity of the binding and its target genes was demonstrated in β-thalassemia patients, as mutations in the EKLF DNA binding site of β-globin promoter region strongly decrease the expression of β-globin chain[92-94].
The role of EKLF *in vivo* was addressed using EKLF -/- mice[89,90]. The homozygous null mice die at E14, which indicates that primitive erythropoiesis, is not severely affected, as the embryos are able to survive until fetal liver erythropoiesis takes over. At a later stage (E12), fetal erythropoiesis is impaired resulting in severe anemia.

NF-E2. The nuclear factor E2 (NF-E2) is a heterodimeric basic-leucine zipper (b-Zip) transcription factor[95,96]. The two subunits of NF-E2 are the p45 tissue-restricted subunit present in the erythroid, megakaryocytic, granulocytic and mast cell lineages, and a wider expressed p18 small subunit, a member of the Maf protein family[96]. The p45 subunit contains a transactivating domain at the N-terminus and a cap *'n collar* (cnc) domain at the C-terminus. The p18 subunit includes the DNA-binding motif and recognizes sites containing an AP-1 motif, 5'-(T/C)GCTGA(G/C)TCA(T/C), however, function specificity is achieved with some additional basepairs upstream of the AP-1 binding motif[96]. In erythroid precursors NF-E2 associates with the promoter of the porphobilinogen deaminase (HMBS) gene [97], α and β-globin LCR enhancer element[95,96], and other genes involved in iron metabolism which suggests an important role in regulating tissue-specific gene expression[98,99]. Although NF-E2 controls many genes involved in heme and globin synthesis, the p45 subunit of NF-E2 null mice model revealed an unexpected phenotype[98]. The erythroid lineage was only mildly affected, but megakaryocytic development was impaired giving rise to a lower platelet counts. Targeted disruption of the gene encoding for

the smaller subunit, p18, did not result in an overt phenotype[100].

2.3 Transcription factors in neutrophilic development

The progression/maturation process from an immature myeloblast to an active, fully differentiated neutrophil depends on a defined expression of an array of cell specific genes. Regulation occurs via specific transcription factors that control the expression of granulocytic genes. Amongst many transcription factors involved in neutrophilopoeisis, two factors that stand out are C/EBPα and PU.1 (Spi1).

PU.1. PU.1 was first identified as the product of viral integration in the *Spi-1* locus in Friend virus induced erythroleukemia[101]. Insertion in the upstream 5' region of PU.1 leads to an aberrant constitutive expression of PU.1 in proerythroblasts causing uncontrolled proliferation and block of terminal differentiation[102,103]. In human and mouse PU.1 expression is observed in macrophages, mast cells, B lymphoids, early erythroid cells, and granulocytes but is undetectable in T cells[101,104]. Biochemical studies further delineated specific transcriptional domains of PU.1[105]. The N- and C-terminus consist of a transactivating region and a DNA specific binding region, respectively. Distal to the N-terminal transactivation region, a proline-, glutamic acid-, serine-, and threonine-rich protein-protein interaction domain called region PEST is present. A helix-turn-helix DNA binding motif in PU.1 binds a specific purine-rich DNA sequence 5'-AAAG(A/C/G)GGAAG-3', nominated as the PU box[106]. Homology base pairing revealed that PU.1 is a member of the large ETS family of transcription factors, with a common 80 amino acids DNA binding domain which characterizes the family[107]. To gain insight into the specific role of PU.1 *in vivo*, a knockout model was established[108,109]. Disruption of the PU.1 gene is embryonically lethal between day E17 and E18. The PU.1 null mouse model is characterized by impaired development in the lymphoid, myeloid and erythroid lineage. PU.1 overexpression in a transgenic model showed development and progression of erythroleukemia within few months after birth [110]. The expression level of PU.1 in early progenitor cells, ES cells or $CD34^+$ cells, is low and rises during myeloid terminal differentiation[111]. The important role of PU.1 in myeloid cell development depends on interacting proteins[112]. Granulocytic genes that have been shown to be targets of PU.1 either solely or in conjunction with other co-operative transcription factors are genes encoding for myeloperoxidase (MPO), neutrophil elastase (NE), myeloblastin (MBN), G-CSF receptor, GM-CSF receptor, lysozyme, a component of the phagocyte respiratory burst oxidase (gp91phox), tyrosine kinase c-fos, and beta 2 leukocyte integrin (CD18)[13].

C/EBPα. CCAAT/enhancer binding protein α (C/EBPα) is a transcription factor of which its expression in the hematopoietic system is restricted to the myeloid lineage[113]. The importance of C/EBPα as a key factor in granulopoiesis was demonstrated in null mice[114]. Homozygous C/EBPα null mice showed a complete absence of mature neutrophils and eosinophils. Other lineages, monocytes, macrophages, monocytes, erythrocytes, and platelets were unaffected. The deficiency takes place at an early stage of myelopoiesis, i.e., at the granulocyte/monocyte progenitor (GMP) level. The lack of neutrophils in C/EBPα null mice indicates a pivotal role of this transcriptional factor as a potential regulator of its targets genes[114,115]. One important gene that was observed to be down regulated in C/EBPα (-/-) newborn was the gene encoding for G-CSF receptor. However, lack of G-CSF receptor expression does not fully explain the observed phenotype, as G-CSF receptor (-/-) animals develop mature neutrophils, although in reduced numbers[116]. This would indicate that additional target genes of C/EBPα play a critical role in neutrophil development.

C/EBPα belongs to a family of leucine zipper (bZIP) transcription factors that bind to specific DNA sequences[117]. The domain responsible for binding DNA is located at the C-terminal part of the protein, in close proximity and upstream of the leucine zipper, a domain required for homo- or hetero-dimerization. In addition, the N-terminus consists of two regulatory and transactivating domains TAD1 and TAD2[118,119]. The expression level of C/EBPα is tightly controlled during myeloid differentiation, as shown in different myeloid cell line models[120-122].

Recently, mutations in the coding sequence of C/EBPα were found with the frequency of 9% of acute myeloid leukemia (AML) samples analyzed. The phenotype observed in the knockout C/EBPα animal model showed block in the granulocytic lineage, however, the mutations found in AML samples do not involve null mutants, but rather out-of-frame insertions/deletions or single point mutations in different functional domains of C/EBPα[123,124].

AML1. The expression of AML1 is mainly restricted to myeloid and lymphoid lineages during differentiation, but also includes early precursor cells[125-127]. AML null mice do develop primitive hematopoiesis lack definitive hematopoiesis. In vitro differentiation of null ES cells also failed to contribute towards hematopoietic lineage specific progression[128]. AML1 is a DNA binding transcription factor and binds TGT/cGGT consensus sequence via the runt N-terminus DNA binding domain[129]. The binding between AML1 and DNA occurs also indirectly, via other Ets family members, such as MEF, Ets-1, PU.1 and C/EBPα and synergistically enhance transcription[130-132].

c-Myb. The transcription factor c-myb was identified in a retroviral screen[133,134]. Structurally, c-

Myb possesses two important domains, an N-terminal DNA binding domain and a transactivating domain localized more centrally[135,136]. The expression level of c-myb in hematopoietic precursor cells is high and gradually decreases as the cell progresses towards maturation[137]. The control of c-myb expression is important especially during myeloid differentiation, as inhibition of c-myb negatively influences the proliferation, while over-expression reduces growth arrest during terminal erythroid and myeloid differentiation[138-141]. c-Myb knockout embryos die at E15.5 due to lack of lymphoid, myeloid, and erythroid lineages[142]. A more indirect role of c-Myb in myeloid development is achieved via co-operation with other factors such as members of the AML1 family and C/EBPα[143,144]. In addition, c-Myb is able to regulate the expression of c-Myc, c-kit, and Flt3[145]. These findings indicate that multiple indirect mechanisms exist by which c-myb controls myeloid development.

2.4 Transcription factors in AML

Transcription factors play a very important role in the pathogenesis of acute leukemia. Alteration or disruption of transcription factors in AML affects the normal cell cycle and differentiation of developing cells. Transcription factors are highly regulated during normal cell development, and follow a specific pattern of expression and interaction with other regulatory proteins. However, the same transcription factors are frequently involved in AML, such as Evi1/AML1 in t(3;21), AML1/ETO in t(8;21), PZLF/RARα in t(11;17) or PML/RARα in t(15;17)[146]. Deregulation of transcription factors and consequently progression towards leukemia can be due to two different mechanisms. The first model implies a quantitive change, where the inappropriate silencing or/and aberrant expression leads to loss or gain of function, respectively. The second model involves mutations where the corresponding proteins either become truncated or chimeric proteins are generated with altered functional properties. Those alterations play a crucial role in neoplastic progression[146].

Acute Myeloid Leukemia (AML) is a malignant disease of hematopoietic precursors in the bone marrow and blood. The diseases can occur in a variety of ways, perturbing the normal physiological process of different blood lineages to proliferate, differentiate, or undergo apoptosis. Due to different factors affecting development of AML, patients show heterogeneous offset of the disease. In the majority of cases the chromosomal alteration determine the outcome and progress of the disease. The disease can be simply characterized into 3 major groups: favorable [t(8,22), t(15,17) and inv16], 60-80% survival) intermediate (representing normal karyotypes 40% survival) and unfavorable (other clonal cytogenetic abnormalities, 20% survival)[147].

EVI1 (ecotropic virus integration 1) is an oncogene involved in translocation or inversion on human chromosome 3, t(3;3)(q21;26) or inv(3)(q21;q26) respectively[148]. AML with aberrant expression of EVI1 belongs to an unfavorable risk group[149].

3. Ecotropic virus integration 1 (Evi1)

3.1 EVI1, a transforming gene in AML and MDS

EVI1 was isolated and characterized from mouse retrovirally induced leukemic cell lines and codes for a nuclear protein of 1042 amino acids with particular motifs typical for transcription factors[150]. Mouse *Evi1* is located on chromosome 4, whereas in human *EVI1* gene is located on chromosome 3 band q24-q28. It consists of 12 exons with two non-coding exons, spaning over 100 Kb of genomic sequence. The transcript encodes for a full 145 kDa nuclear protein, but a shorter splice form of 88 kDa lacking zinc fingers 6 and 7 exists in human and mouse[151-153]. The sequence between the human and mouse gene is highly conserved, and is identical for 91% at the nucleotide level and 94% at the amino acid level. EVI1 has ten Krüppel-like zinc fingers (C_2H_2), of which seven are at the N-terminal part of the protein and three are more C-terminaly located (Figure 4). The C and N terminal zinc finger regions are spaced by a proline rich region. Within the proline rich region a repression region has emerged recently which has shown to be responsible for EVI1 repression activity[154,155].

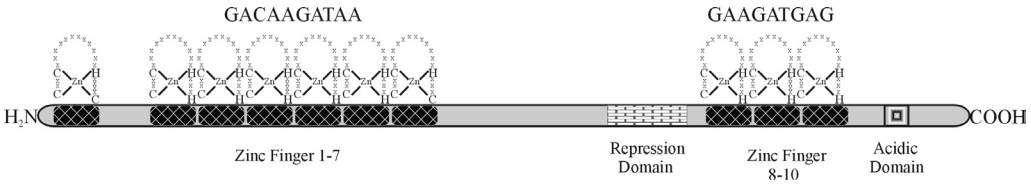

Figure 4 Schematic representation of ecotropic viral integration 1 (EVI-1) protein
The protein consists of several different motifs. EVI-1 possesses ten C_2H_2 zinc fingers of which seven are located at the N-terminus and three at the C-terminus (black). The zinc fingers structure with cysteine-histidine pairings that co-ordinate tetrahedral binding to a single zinc atom (Zn) is depicted. The N-terminal zinc finger can bind a specific DNA sequence, as does the C-terminus. In addition, EVI-1 contains a repression domain (brick area) located between both zinc finger domains. At the C-terminus a small acidic domain (white/black square) is located with unknown function.

At the C-terminus a small acidic domain is present with unknown function to date. Both zinc finger domains are able to bind to DNA. The first seven zinc fingers of EVI1 recognize the DNA nucleotide sequence GACAAGATAA[156-158] and the three C-terminal fingers bind to the

GAAGATGAG DNA sequence[159]. Although the EVI1 zinc fingers are capable of binding DNA, only one group of investigators have provided data that suggest binding to these sequences by the full length protein[160]. Zinc fingers may not solely bind DNA sequences, but may also interact with other proteins.

Spatial and temporal expression of EVI1 was demonstrated by Perkins *et al*[158]. Using targeted mutagenesis the sixth exon encoding for two zinc fingers of EVI1 was disrupted . Homozygous null mouse die in utero at embryonic day 10.5 and exhibit multiple developmental malformations i.e. widespread hypocellularity, hemorrhage, and disruption in the development of the heart, somites, and neural crest-derived cells[161]. In adult mice the expression of Evi1 was found only in the kidney and in developing oocytes[162]. However, further analysis revealed expression in other organs, including spleen, heart, muscle, brain[163]. The expression in blood cells has been controversial topic as different reports indicate either low expression or undetectable level[164]. Apart from being in mouse myeloid leukemia aberrantly expressed as a result of a proviral insertion, in humans it has been demonstrated that EVI1 is abnormally expressed as the result of chromosomal aberrations involving chromosome 3q26[165]. Functional evidence that aberrant EVI1 expression may play a role in transformation was obtained from experiments using myeloid precursor cell line 32Dcl3[166]. This cell line is capable of sustained proliferation in the presence of IL-3, but upon substitution by G-CSF 32Dcl3 cells enter a differentiation program and develop into mature neutrophils[167]. Morishita and colleagues showed that enforced overexpression of Evi1 blocked differentiation of 32Dcl3 cells towards mature neutrophils but did not affect the proliferation capacity of cells upon IL-3 exposure[168]. In contrast, other investigators found that overexpression of Evi1 increased proliferation of 32Dcl3 cells[153,169]. Other models showed that inappropriate Evi1 expression in other blood lineages might have different outcomes. Using the 32DEpo1 murine cell line which proliferates depending on growth factors IL-3 or EPO, showed that in the presence of Evi1 the cells could not be sustained in cultured upon EPO stimulation only[170]. EpoR was shown to be a target gene for GATA-1; an important regulator during erythroid differentiation[171-173]. As indicated above, Evi1 is capable of interacting with GATA-like sequences. However both transcripts, GATA1 and EpoR, were not affected by Evi1, as the steady state levels of Gata1 and EpoR mRNA were equal in the Evi1 positive and negative cells[164]. The same observation was noticed using mouse bone marrow progenitor cells, in which Evi1 overexpression caused a drastic reduction in the numbers of Epo sensitive colony forming cells[164]. The data indicate that Evi1 might potentially interfere with Gata1 binding sites, however these data did not provide sufficient evidence that this is indeed the case. Endogenous *EVI1*

mRNA expression levels were found in human undifferentiated erythroleukemic cell lines (K 562, HEL, LAMA 84) and spontaneously terminal erythroid differentiation exhibiting cell lines (KU 812, JK-1). No effect on erythroid differentiation of these cells was observed by EVI1[174]. Sitailo *et al* demonstrated oncogenic properties in megakaryocytic lineage by Evi1. While embryonic stem cells (ES) express Evi1 at low endogenous levels, overexpression of Evi1 in ES cells increased the proliferation capacity of undifferentiated ES cells[169]. Stimulation of ES cells with different cytokines bearing the Evi1 transgene resulted in a shift towards megakaryocytic cells.

3.2 Evi1 mouse models

As discussed earlier, different *in vitro* models were used to determine the role of *Evi1* in transformation. To study the role of Evi1 *in vivo* Louz et al generated the first transgenic *Evi1* animal model[175]. The *Evi1* gene was cloned under the murine stem cell antigen 1 promoter; *Sca-1* (Ly-6E.1)[176]. Sca-1 is expressed on multipotent hematopoietic stem cells (HSC) and has been used as a marker for HSC in adult bone marrow and fetal liver but also in mobilized peripheral blood and spleen in the adult animals[177-181]. Although the three different lines generated in this study did not develop signs of leukemia during the 18 months follow up period, they showed decrease numbers of erythroid colony forming units (CFU-E). Treatment of neonatal mice with Cas-Br-M MuLV virus to induce additional genetic "hit", significantly increased leukemia progression and development in transgenic animals indicating a role of *Evi1* in tumor progression.

Recently, a transplantation model with retrovirally delivered *Evi1* into lineage negative bone marrow cells showed development of myelodysplastic syndrome (MDS) in Evi1 positive animals[182]. Interestingly, the first signs of disease developed after a period of 10 months post bone marrow transplantation. In addition, transgenic animals did not develop a classical acute myeloid leukemia, but a myelodysplastic like disease characterized by pancytopenia, hypercellular bone marrow and dyserythropoesis. The effect of transformation was proposed to be due to interference and repression of genes that play an important role in differentiation of red blood cells (EpoR) and platelets formation (c-Mpl). In case of Evi1 positive animal both marker genes were down regulated. It is of interest to notice that although the mice as mentioned earlier developed syndromes of MDS after 10 months, both markers were low after 4 months without any signs of disease.

In this thesis we applied a novel approach to obtain an Evi1 transgenic mouse model by using

a specific Vav promoter restricted only to hematopoietic lineages[183,184]. LoxP sites upstream of Evi1 mouse cDNA flanked a transcriptional control sequence. Using a mouse model that expressess Cre gene under the erythroid-specific β-globin gene promoter and locus control region[185] or the promoter from the erythropoietin receptor gene[186] the transgene expression is restricted specifically to the erythroid lineage only.

3.3 Transformation and binding molecules

Evi1's structural domains, functions as transcriptinal repressor and diverse abilities to deregulate cellular processes indicate that these roles may be executed in conjunction with other proteins. It has been shown that Evi1 forms complexes with many different classes of proteins, which may play a key role in Evi1 functioning (Figure 5).

Smad3

An important pathway that may be affected by Evi1 is the one activated by transforming growth factor-β (TGF-β)[187]. The TGF-β pathway plays an important role as a negative regulator of cellular growth, differentiation and apoptosis[188-190]. The intracellular effectors of the TGF-β pathway are the members depicted as Smads. Smad3 can regulate transcriptional activity in a positive or a negative manner. It has a low affinity towards Smad-binding elements (SBE) with the CAGAC consensus sequence[191]. In addition, it binds promoter regions with GC-rich motifs[192]. Due to the low DNA binding affinity, Smad3 requires cooperation with other sequence-specific transcription factors to bind target genes[193-195]. This results in deregulation of Smad3 transcriptional activity and confers signaling specificity. The direct effect of such de-regulation was shown in the presence of Evi1. The repressive activity of Evi1 was achieved by the ability to bind via the first zinc finger domain to Smad3. Interaction between Evi1 and Smad3 inhibits cells to undergo growth arrest in presence of TGF-β. The association inhibits Smad3 DNA binding and negatively modulates transcriptional repression[196].

C-terminal Binding Protein (CtBP)

The repressive domain of Evi1 has been mapped within the proline-rich region[197]. Within that region there are two motifs, PFDLT and PLDLS, both recognized by C-terminal binding protein (CtBP)[198]. In vertebrates CtBP targets and acts as a cofactor of DNA binding transcription factors such as the Ikaros family members Eos[199], Friend of GATA1 (FOG1)[200], Ikaros[201],

Krüppel like factor 8 (KLF8)[202] and others[203]. The repression activity exhibited by CtBP suggests a critical role in a variety of different cell types and not a specific function for a certain class of transcriptional factors. The interaction between CtBP1 and Evi1 was shown to be necessary for Evi1 transcriptional repression activity[154,155]. Using mutation analysis within the Evi1 PLDLS motif the transcriptional repression was diminished. Mutation within the PFDLT region did not affect repression capability of Evi1 to such extend as the PLDLS region[154]. In addition the transforming ability of Evi1 was lost in RatFL cell line upon deletion PLDLS region of CtBP. The region of interaction between CtBP1 and Evi1 was also shown to be presumably responsible for cell cycle deregulation due to increased cellular proliferation rate of 32Dcl3 cell line[153]. The data suggests that repression activity correlates with the preferential binding recognition between those two regions and that may influence *Evi1* oncogenic activity.

Histone deacetylace family (HDACs)

Another group of co-factors that may play a role in Evi1 transcriptional repression was identified by Vinatzer *et al*[204]. A member of the histone deacetylase family, HDAC-1, was shown to physically interact with Evi1. HDAC-1 is a member of the HDAC class I family with three other members, namely HDAC-2, HDAC-3 and HDAC-8[205]. HDAC-1, as all other members of its class, is expressed in most cell types and localizes in the nucleus[206-208]. HDAC-1 is a member of three different protein complexes: Sin3, NuRD and Co-REST[209]. Binding of HDAC-1 is not restricted to multi-protein complexes, as it can bind directly to DNA binding proteins, for instance YY1 or Rb binding protein-1[210,211].

HDAC-1 was shown to bind Evi1 at multiple sites, i.e. via the first seven zinc fingers of domain 1, domain 2 via zinc finger eight to ten, and via the repression domain[204]. Chakraborty *et al*[153] showed interaction between Evi1 and HDAC4 and confirmed findings of Vinatzer *et al* in a limited analysis of the first domain of zinc finger 1-7. However, the specificity and role of the interaction with regard to the transformation ability of Evi1 remains elusive.

JNK1

Evi1 has a protective role in stress induced cell death by interfering with the mitogen-activated protein (MAP) kinase pathway[212]. The first domain of Evi1 with zinc fingers 1-7 interacts with JNK1, and thereby inhibits activity of c-Jun. Inhibition of JNK1 activity by Evi1 prevents stress-induced cell death, and inhibits cell apoptosis. The proposed model is more suitable for Evi1's

role in organogenesis and morphogenesis during normal development, as no data up to date supports apoptosis inhibition in Evi1 mediated oncogenic progression.

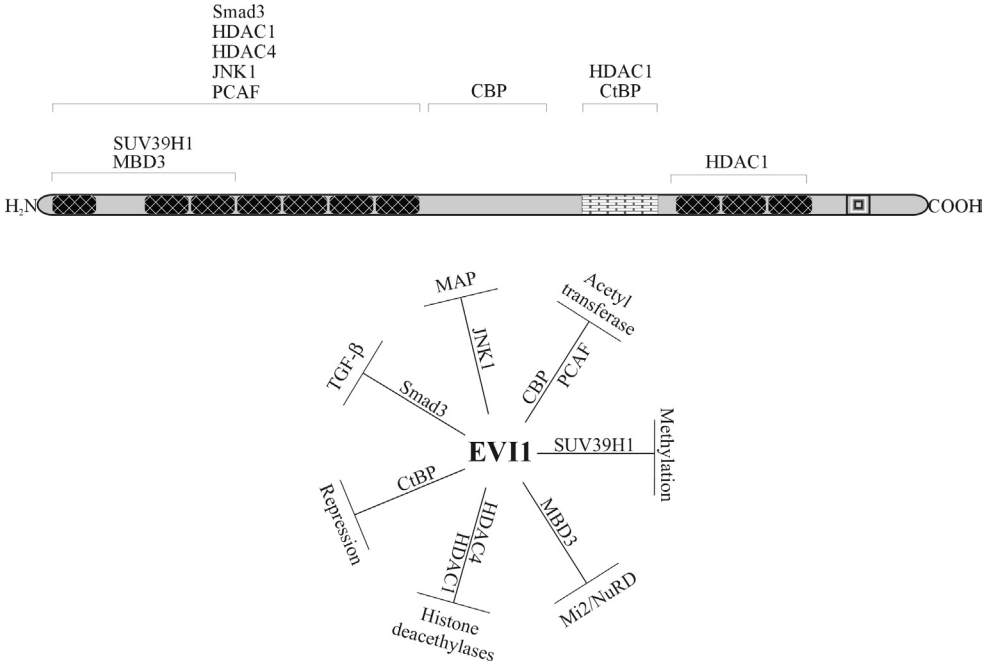

Figure 5 Schematic representation of EVI-1 and its co-regulators binding partners
Different EVI-1 regions have been found to be involved in interaction with several proteins. The N-terminus zinc fingers of EVI-1 interact with histone deacethylases 1 and 4(HDAC1 and HDAC4), p300/CBP-associated factor (P/CAF), methyl binding domain 3 (MBD3), c-Jun N-terminal kinase (JNK1), mothers against decapentaplegic homolog 3 (Smad3) and the mammalian ortholog of suppressor of position-effect-variegation, SU(VAR)3-9, SUV39H1. The three zinc fingers at the C-terminus are found to bind to HDAC1. Within the repression domain two binding motifs are found that interact with C-terminal binding protein (CtBP1) and also with HDAC1. The more central part of EVI-1 binds the CREB-binding protein (CBP). Interaction between EVI-1 and different functional proteins play a role in affecting several diverse pathways.

Lysine Acetyltransferases (CBP and PCAF)

Two other proteins that have been shown to bind Evi1 via the proximal zinc finger domain are CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF). These proteins belong to a family of lysine acetyltransferases, which play an important role in leukemia development. Both proteins have been shown to act as transcriptional co-activators and contain intrinsic histone acetyl transferase (HAT) activity[213,214]. The substrates of CBP in mammals are histones, transcription factors, and the early region 1A (E1A) protein of adenoviruses. PCAF has similar substrates namely histone 3 and 4 (H3 and H4), transcription factors, the early region 1A (E1A)

of adenoviruses, and in addition the HIV transcriptional activator, TAT. In addition, CBP and PCAF also exhibit autoacetylation properties.

Among transcription factors that are acetylated either via CBP and/or PCAF are c-myb GATA1, p53, EKLF, NF-κB and others[215-220]. Evi1 binds CBP and PCAF via its proximal zinc finger domain, however the binding site of CBP is more distal than the PCAF site[153]. Acetylation of Evi1 is one of the first post-translation modifications reported for this protein. This modification was shown to interfere with Evi1 transcriptional repression activity and nuclear organization. Because the modification occurs within the DNA binding motif it would be of interest to investigate if the modification influences the DNA binding properties of Evi1.

Methyl Binding Domain (MBD3)

In this thesis I report on the identification of a novel binding partner of Evi1, i.e. methyl-binding domain 3b (MBD3b). MBD3 gene encodes for a nuclear protein with an N-terminal methyl-binding domain. It is the only member of methyl binding domain family (MBD1, MBD2, MBD4, and MeCP2) that has no affinity for methylated CpG regions in mammals[221,222]. MBD3 gene encodes the full-length protein MBD3a and a splice variant lacking the first 54 amino acids of the MBD motif, MBD3b. MBD3a and b are core proteins of the histone repressor complex Mi2/NuRD[216].

3.4 EVI1 defective expression and fusion transcripts in human disease

Inappropriate EVI1 expression in hematopoietic cells results in or at least contributes to cellular transformation and leukemia progression. The expression in human AML or MDS is due to inversion or translocations involving the chromosome band 3q21q26 where *EVI1* is located. The rearrangement within 3q21 caused by t(3;3) or inv(3) involves constitutively enhanced transcription from an enhancer of the Ribophorin I (RPN1) gene located in close proximity to the breakpoint[223]. Other genomic rearrangements involve 3q26 locus and other chromosomal regions and consequently other genes. These translocations result in production of EVI1 containing fusion proteins. Those fusions involve AML1, MDS1, ETV6, and RPN1 genes and will be discussed shortly (Figure 6).

AML1/EVI1. The translocation (3;21)(q26;q22) was identified a decade ago in the SKH1 chronic myelocytic leukemia (CML) cell line in addition to the already present translocation (9;22)(q34;q11). The fusion involves the AML1 gene located on chromosome 21q22 and the

EVI1 gene on chromosome 3q26[224]. Detailed analysis of the AML1/EVI1 fusion protein revealed the presence of a truncated N-terminal part of AML1 containing the runt domain and a full length EVI1 protein. Interestingly, although it was first postulated that a 5' non-coding region of *EVI1* is involved in this translocation, it turned out to be a novel gene called MDS1 located upstream of *EVI1*[225,226]. AML1 contains two distinct well-defined domains: a runt domain at the N-terminus, which is highly homologous to the Drosophila segmentation gene *runt* and a C-terminal proline-serine-threonine (PST) domain involved in trans-activation activity[227,228]. The runt domain of AML1 plays an important role in heterodimerization with PEBP2β protein required to bind the DNA. The PST domain is required for transcriptional activation upon complex formation with p300, CBP, and PCAF[228].

The role of the chimeric fusion protein was elucidated using the myeloid precursor cell line 32Dcl3. Overexpression of AML1-MDS1/EVI1 showed a block of differentiation upon stimulation with G-CSF and decreased expression of myeloperoxidase (MPO), a marker for neutrophilic differentiation[229,230]. Although AML1 on its own is a transcriptional activator, the chimeric protein becomes a repressor due to the presence of EVI1. This observation suggests that the fusion of AML1-MDS1/EVI1 might interfere with AML1 binding sites. Using DNA-binding affinity approaches the AML1-MDS1/EVI1 chimeric protein showed not only stronger DNA binding versus AML1 protein alone but also demonstrated that it contains a dominant transactivation suppressive activity.

MDS1/EVI1. MDS1 was identified as a part of *AML1-MDS1/EVI1* fusion transcript in a 3;21 translocation. It encodes for a small protein with high homology to the positive regulator (PR) domain of the retinoblastoma-interacting zinc finger protein (RIZ)[231,232], the transcriptional repressor BLIMP1 required for B cell terminal differentiation[233,234], and the *Caenorhabditis elegans* transcription factor, Egl-43[235]. The gene is located on chromosome band 3q26.2 in close upstream proximity, around 300kb, to *EVI1*[236,237]. MDS1 splices into the second exon of *EVI1* resulting in an addition of 188 amino acids to the 5' end of *EVI1*. The *MDS1/EVI1* and *EVI1* expression pattern in normal man and mouse tissue[163,232] and in myeloid leukemia is closely related[238]. However, the functional role in early hematopoietic precursor cells during proliferation and differentiation is unclear. MDS1/EVI1 appears to have an opposite effect as compared to EVI1. EVI1 is a repressor, as shown in many different models. On the other hand the MDS1/EVI1 protein does not enhance proliferation, nor does it induce a block of differentiation. Furthermore, MDS1/EVI1 was also shown to posses a positive growth-inhibitory effect on TGF-β signaling pathway[169,239].

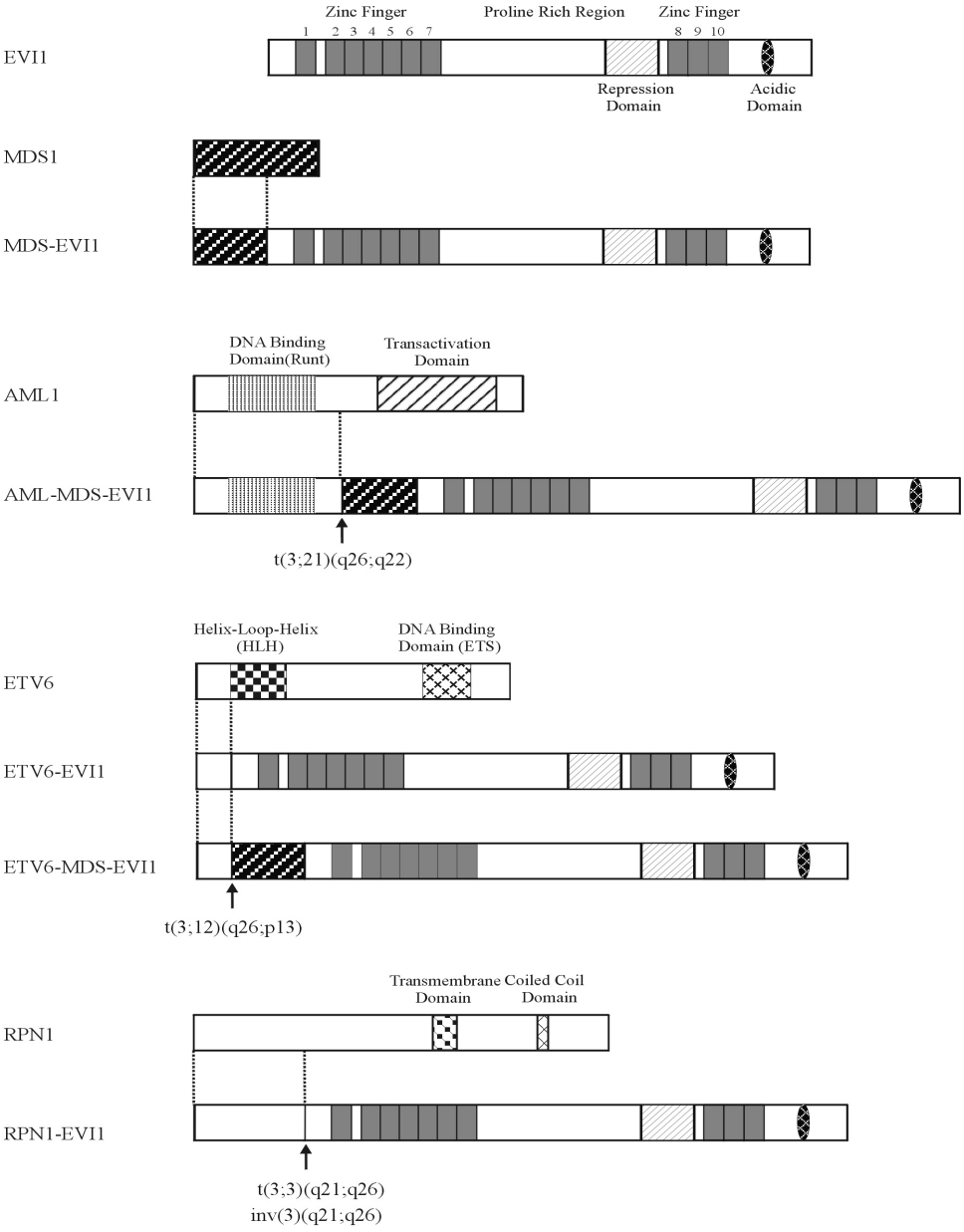

Figure 6 Diagram of proteins involved in rearrangement with EVI-1 and generation of fusion proteins
Diagram of normal proteins, acute myeloid leukemia 1 (AML1), myelodysplastic syndrome 1 (MDS1), *ets* variant 6 (ETV6), Ribophorin 1 (RPN1), and EVI-1. The functional domains of each protein are depicted. The vertical doted lines represent graphically break-point fusion junction between EVI-1 and a corresponding protein. The arrow indicates chromosomal breakpoints for each fusion protein as found in AML patients.

In addition, MDS1/EVI1 has an antagonistic effect on EVI1, and functions as a positive transcriptional regulator[237].

RPN1/EVI1. A small cohort of patients (n=9) with either inv(3)(q21q26) or t(3;3)(q21;q26) was analyzed for the presence of the *RPN1* gene as a fusion partner of *EVI1*, as the gene is located in a close proximity of the breakpoint 3q21q26[240]. The analysis showed that in all but one case the *RPN1/EVI1* fusion gene was detected and driven by the *RPN1* promoter. The *RPN1* first exon was fused in frame to the second exon of *EVI1*, with the exception of one case where the second exon was also absent and the *RPN1* first exon was fused to the third exon of EVI1[241].

ETV6/EVI1. A less frequent fusion that is found with chromosome 3 aberrations is *ETV6* (TEL1) gene[242]. ETV6 is a member of *ets* family transcription factors that share a conserved DNA binding domain[243]. Up to date four cases were reported where *ETV6* gene was spliced and fused with *EVI1* or *MDS1/EVI1*[244-246]. The translocation (3;12)(q26;p13) generates either *ETV6-MDS1/EVI1* or *ETV6/EVI1* fusion gene, which is inappropriately expressed by constitutive activation from the *ETV6* promoter. Molecular analysis of the fusion gene revealed that no functional domains of ETV6 were involved[227,244,247]. However, the oncogenic role of the fusion transcripts has not been addressed any further.

4. Regulation of gene expression and the "histone code"

The nucleus of eukaryotic cells contains the genetic information encoded by the DNA polymer. The DNA associate with nuclear proteins and it is stored in the form of chromatin. The basic repeating unit of chromatin is the nucleosome, which is composed of 146 base pairs of DNA wrapped around an octamer of the core histones. The core histone octamer is composed of two copies of each histone proteins, H2A, H2B, H3, and H4[248]. Histones consist of globular domain that mediates histone-histone interaction and amino acids NH_2-terminus (tail).

A short, 10-60bp DNA 'linker' separates each nucleosome from each other resembling an arrangement as 'beads-on-a-string', and forms a chromatin fiber of 10nm in diameter. The folding of chromatin undergoes further condensation into 30nm thick fibers achieved by linker histone H1, which interacts with the nucleosome core[249]. The last level in chromatin condensation is formation of 100-400nm interphase fibers, or the highly compressed chromosome structures during methaphase[250]. This high compaction of chromatin fibers prevents accessibility for

transcription machinery to read the nucleotide sequence, and therefore it must be dynamic and flexible to be able to allow unfolding and folding transition[251].

Two different forms of chromatin were established using cytological studies, transcriptionally active euchromatin, less dense and appearing as a relaxed structure, and transcriptionally silent heterochromatin, more dense and usually located at the periphery of the nucleus[252]. The mechanisms behind the transition from euchromatin to heterochromatin and vice versa are poorly understood. However, histone N terminal tails, which protrude from the nucleosome octamer, are subject to a variety of posttranslational modifications, such as acetylation and methylation of lysines (K) and arginines (R), phosphorylation of serines (S) and threonines (T), ubiquitylation and sumoylation of lysines (K) as well as ADP-ribosylation[253,254]. The complexity of modification goes further, as lysine residue can be mono-, di-, or tri- methylated, and arginine residue can be either mono- or di-methylated[255-260].

Modification of a specific amino acid at the histone level correlates with either repressive or active state. It is well established, for example, that acetylation of histone tails at H3-K4, H3-K36 and H3-K79 is associated with transcriptional activation, and methylation at H3-K9, H3-K27 and H4-K20 prevent transcription to occur[253,261-264].

The array of different histone amino termini modifications has led to the idea of a "histone code". It postulates that specific covalent modifications, alone or in combination, at a particular amino acid of the N-terminal of histone tails confer a cooperative or adverse environment for chromatin-associates proteins, which in turn impose distinct chromatin states from active to silent and influence its functional properties. In recent years those specific histone modification patterns were shown to be associated with particular enzymes that can covalently modified histone at specific site(s). A number of mediators and histone modifiers have been identified. Histone acetyltransferases (HATs), are associated with acetylation of histones by transferring an acetyl group to lysine residues, and thereby attract chromatin remodeling complexes[253,265]. The process is reversible by a group of enzymes that removes the acetyl group and inhibits transcription, the histone deacetylases (HDACs)[266]. The same patterns of modification apply to a group of enzymes that methylate specific residue (arginine, lysine, glutamine), which dictates either active or silent transcription[267,268]. The modification has been recently shown to be also reversible as the first Lysine-specific histone demethylase 1 (LSD1) was isolated[269]. The number of enzymes that play a distinct role in histone modifications has grown rapidly, for review see Peterson *et al*[251].

However, the first enzyme that was identified to carry out histone modification at H3 lysine 9 came from studies of suppressor of position effect variegation (PEV) in *Drosophila*[270]. Characterization of the PEV suppressors, such as Su(var)3-9, the *trithorax*-group protein Trithorax (TRX), and the *Polycomb*-group protein Enhancer of zeste E(Z) revealed a common motif designated the SET (Suppressor of Variegation, Enhancer of Zeste and Trithorax) domain[271-273]. The human and mouse ortholog was identified by Rea and colleagues and shown to specifically methylate H3-K9 and to associate with repression of transcription[274]. The catalytic domain responsible for the modification was mapped in mutagenesis studies. This showed that not only the evolutionary conserved SET motif is required but that in addition two small cysteine-rich flanking regions which enclos the SET domain, named pre- and post-SET, play an important functional role.

In addition to SuV39H1 several other methyltransferases were identified which showed specificity for H3K9 methylation. SuV39H2 was isolated by O'Carroll and colleagues and showed high homology to SuV39H1 with the exception of a presence of highly basic N-terminus[275]. Analysis of proteins with motifs comparable to SuV39H1 and SuV39H2 resulted in the isolation of a gene encoding for the G9a protein, with methylation specificity for H3-K9 in addition to methylation specificity towards lysine 27 (K27) at histone 3 (H3-K27)[276]. Generation of null mice for either SuV39H1 or SuV39H2 did not show any phenotype. SuV39H1/SuV39H2 double null mice develop normally until embryonic day 12.5, but at later stage of development the embryos show growth retardation, high reabsorption rates, and prenatal lethality, due to chromosomal segregation defects as the majority of cells exhibit increased numbers of chromosomes (tetraploid and octaploid)[256]. On the contrary, G9a null mice do not survive beyond day 8.5 due to profound levels of apoptosis in combination with developmental defects[277]. The global pattern of H3-K9 methylation also differs between these two models. Although there is lack of methylation observed at the pericentric heterochromatic regions in the SuV39H1/2 double null mice the broad pattern of H3-K9 methylation is still retained as compare to G9a null mice where chromatin wide methylation is abolished completely[256,277].

5. Scope of the thesis

The Ecotropic integration site 1 (*EVI1*) gene is implicated as a factor in development and progression of acute myeloid leukemia (AML). The *EVI1* locus rearrangement at chromosome 3 in patients causes inappropriate expression of EVI1 and development of myelodysplastic syndrome (MDS) and AML. However, the molecular mechanisms behind *EVI1* oncogenic role are still poorly understood up to date.

Chapter 1 gives an overview of the role of transcriptional factors in erythropoiesis and myelopoiesis, regulation of transcription and what is known about the role and mechanism of Evi1 in leukemia progression.

To mimic the aberrant expression of *EVI1* as observed in AML patients we generated a novel transgenic mouse model for the *Evi1* gene. In **Chapter 2** and **Chapter 3** we describe the analysis and phenotype of inappropriate activation and expression of *Evi1* during fetal erythroid development.

To gain more insight into the possible role of EVI1 in leukemogenesis we performed a protein-binding screen to determine which factor(s) may cooperate with EVI1 in leukemia. In **Chapter 4** we identified a novel binding partner of Evi1, methyl binding domain 3b (Mbd3b) that is a member of the histone deacetylase complex NuRD. **Chapter 5** describes another group of proteins that were found to interact with Evi1, and belongs to the histone methyltransferase family. Chapter 4 and 5 provide evidence that Evi1 may have a role in epigenetic control of the gene expression.

Chapter 6 will summarize the results presented in this thesis, draw a conclusion and discuss the possible mechanism of Evi1 in leukemogenesis.

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Chapter 2

Evi1 interferes with fetal erythroid development in a conditional transgenic mouse model.

Submitted for publication

Evi1 interferes with fetal erythroid development in a conditional transgenic mouse model.

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Abstract

Patients with myelodysplastic syndrome (MDS) carrying 3q26 aberrations are characterized by refractory anemia and high expression of the proto-oncogene *EVI1*, suggesting direct interference of *EVI1* with erythropoiesis. Although forced expression of *Evi1* in non-committed murine hematopoietic stem cells has been shown to induce multi-lineage cytopenia, the specific *in vivo* effects of *Evi1* overexpression within the erythroid lineage have not been determined. We established novel *Vav-loxp-stop-loxp-Evi1* transgenic mouse lines that conditionally express *Evi1* in the hematopoietic system, and crossed these with the erythroid-lineage specific *pEV-Cre* line to specifically induce *Evi1* expression in erythroid cells *in vivo*. Erythroid-specific *Evi1* overexpression induced fetal anemia, with major defects in definitive erythropoiesis. E12.5 fetal livers showed an arrest in erythroid differentiation, a high percentage of apoptotic and dysplastic cells, and a reduced number of erythroid progenitors. Molecular analysis of *Evi1* expressing erythroid cells revealed a significant upregulation of *Pu.1* and *c-myb* expression. Our data demonstrate that the specific enforcement of *Evi1* expression in the erythroid lineage interferes with fetal erythropoiesis.

Introduction

Evi1 (ecotropic virus integration site 1) is a myeloid transforming gene that was originally identified as a common retroviral target in virally induced murine leukemia.[1,2] *Evi1* encodes for a nuclear DNA binding protein with two zinc finger domains, i.e. an N-terminal domain containing zinc fingers 1-7 and a more C-terminal domain containing zinc fingers 8-10. Zinc fingers 1-7 recognize the sequence GA(C/T)AAGA(T/C)AAGATAA that shows overlap with the consensus sequence bound by GATA family transcription factors.[3-5]

In humans, *EVI1* that is located at chromosome 3q26 is expressed at very low levels in normal blood or BM.[6,7] Patients with acute myeloid leukemia (AML) or myelodysplastic syndrome

(MDS), harboring 3q26 translocations, frequently show aberrant expression of *EVI1* (reviewed by Buonamici et al.[8]). Moreover, *EVI1* overexpression is observed in 10% of AML patients and 30% of MDS patients without abnormalities in 3q26,[7,9] as a result of currently unknown mechanisms. Applying gene expression profiling, we recently found that *EVI1* expressing cases constitute a specific subset of AML samples that are severely resistant to anti-leukemic therapy.[9,10]

Various *in vitro* studies have provided support for a direct involvement of *Evi1* in the development of MDS and AML. Evi1 blocked G-CSF induced differentiation of 32Dcl3 cells,[11] interfered with proliferation of the erythropoietin (Epo-) responsive cell line 32D-Epo and decreased Epo-induced colony formation from BM progenitors.[12]

The available *in vivo* studies have mainly addressed the effects of Evi1 on non-committed progenitors. We previously used the *Sca1* promoter to drive pan-hematopoietic expression of *Evi1* in transgenic mice.[13] Mice of two *Sca1-Evi1* transgenic lines developed normally, but virally induced leukemia development was greatly enhanced in these mice compared to non-transgenic littermates, providing evidence for a cooperative potential of *Evi1* in tumor progression. Severely impaired hematopoiesis with major defects in BM, spleen and peripheral blood was observed specifically in males of a third line in which the transgene had integrated on the X-chromosome. However, it remains unclear whether these defects were due to a position effect of the transgene or to overexpression of *Evi1*.[13] Recently, Nucifora and her colleagues[14] expressed Evi1 in mouse BM progenitors using a retroviral expression vector. Following transplantation of the transduced BM, the recipient mice developed an MDS-like syndrome, characterized by an early phase of BM hyperproliferation and delayed differentiation with no peripheral abnormalities, eventually culminating in BM apoptosis, peripheral pancytopenia and death.[14]

Together, the *in vitro* and *in vivo* data indicate that the effects of *Evi1* on hematopoiesis may be diverse and dependent on the developmental stage of the hematopoietic cells. The fact that we did not obtain high *Evi1* expression in the *Sca-Evi1* model, and the observation that *Evi1* coupled to strong promoters yielded no transgenic lines at all or lines in which *Evi1* expression could not be detected (D.S and R.D, unpublished data), suggest that high *Evi1* expression in the hematopoietic system is embryonic lethal.

We have generated *Evi1* transgenic mouse lines with Cre recombinase-inducible *Evi1* expression in the hematopoietic system. These novel lines allow us to study the effects of Evi1 in separate hematopoietic lineages. We examined in this study the effects of *Evi1* activation on survival, proliferation and differentiation of erythroid progenitors, by breeding the *Evi1* transgenic lines

with the erythroid lineage specific *pEV-Cre* transgenic line. We show that erythroid specific *Evi1* overexpression interferes with fetal erythroid development.

Results

Generation of transgenic mouse lines with conditional *Evi1* expression

To establish an *Evi1* transgenic mouse model that allows strict control over the spatio-temporal expression of *Evi1*, we made use of a *loxP*-flanked transcriptional stop box (*LSL*) that represses *Evi1* transcription unless the stop box is deleted by the Cre enzyme. Upon transfection of Phoenix cells with a *PLNCX2-LSL-GFPEvi1* construct in the absence or presence of a *Cre* expression construct, we exclusively observed nuclear GFP-tagged Evi1 expression in presence of Cre, demonstrating that this approach results in low background expression and efficient induction of *Evi1* expression after Cre-mediated recombination *in vitro* (Figure 1A).

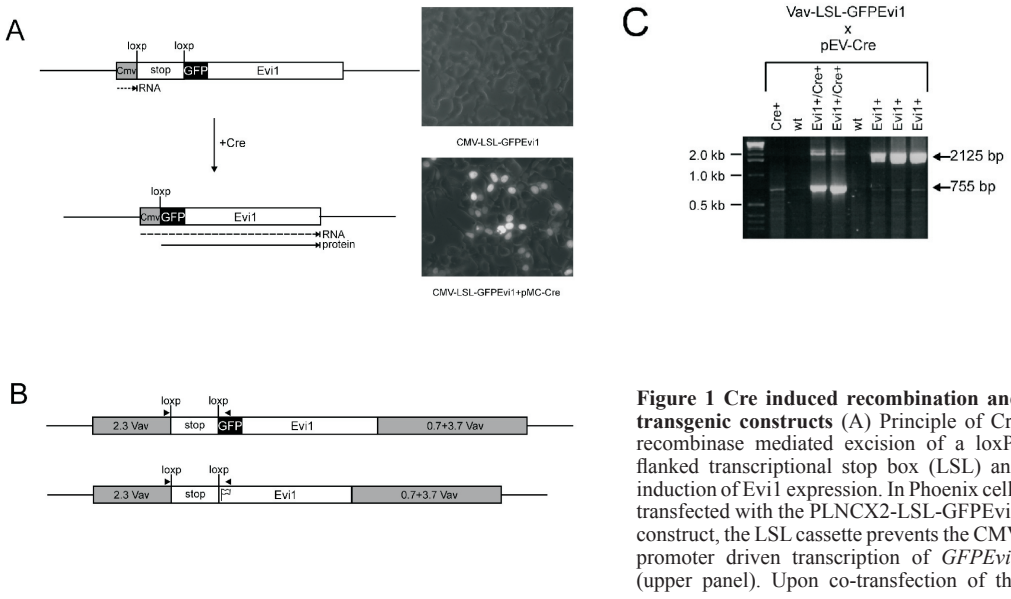

Figure 1 Cre induced recombination and transgenic constructs (A) Principle of Cre recombinase mediated excision of a loxP-flanked transcriptional stop box (LSL) and induction of Evi1 expression. In Phoenix cells transfected with the PLNCX2-LSL-GFPEvi1 construct, the LSL cassette prevents the CMV promoter driven transcription of *GFPEvi1* (upper panel). Upon co-transfection of the Cre expression plasmid *pMC-Cre*, recombination takes place between the *loxP* sites, leading to excision of the stop sequence, transcription and nuclear expression of GFP-tagged Evi1 protein that is visible in the nucleus (lower panel). (B) Maps of the *Vav-LSL-GFPEvi1* and the *Vav-LSL-FlagEvi1* transgenic constructs. Upstream and downstream *Vav* regulatory sequences are indicated.
Arrowheads indicate the position of PCR primers used for the detection of recombination. (C) Recombination PCR. In *Vav-LSL-GFPEvi1*$^{+/-}$ livers (Evi1+) a 2.1 kb PCR product is detected representing the non-recombined transgene. In *Vav-LSLEvi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ transgenic livers (Evi1+/Cre+) a 0.75 kb PCR product is amplified, that corresponds to the recombined transgene.

To generate the transgenic mice we used constructs that contain regulatory sequences of the *Vav* gene, the *LSL* cassette and either *GFP*-tagged or *Flag*-tagged versions of the *Evi1* gene (Fig 1B). The *Vav* regulatory sequences drive transgene expression in all fetal and adult hematopoietic cell lineages, irrespective of the stage of development, and in few non-hematopoietic tissues.[16,20-22] We generated four *Vav-LSL-GFPEvi1* transgenic lines (*GFPEvi1* lines A, B, C and D) and three *Vav-LSL-FlagEvi1* transgenic lines (*FlagEvi1* lines A, B and C). In the absence of Cre protein, we could not detect background recombination or any obvious phenotypic defects in any of these lines (data not shown).

Specific induction of *Evi1* expression in the erythroid lineage

The transgenic lines were used to investigate whether erythroid lineage-specific *Evi1* overexpression would directly affect erythropoiesis. Crossings were initiated between heterozygous mice from all *Vav-LSL-GFPEvi1* and *Vav-LSL-FlagEvi1* transgenic lines and *pEV-Cre*$^{+/-}$ transgenic mice. In the *pEV-Cre* line, Cre expression starts at the pro-erythroblast stage and recapitulates the expression pattern of *β-like globin* genes, thereby efficiently driving recombination of floxed loci within the erythroid lineage with very low recombination activity in other lineages.[18]

PCR confirmed recombination between *loxP* sites and deletion of the stop box in the genomic DNA of fetal livers isolated from E12.5 *Vav-LSL-GFPEvi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ (Figure 1C) and *Vav-LSL-FlagEvi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ (not shown) double transgenic embryos as opposed to single transgenic littermates. Depending on the number of copies, deletion of the stop sequence could have taken place in two ways; between *loxP* sites flanking the same stop sequence(s) or between *loxP sites* flanking separate stop sequences in case of a multi-copy transgene.

By consequence, in *Vav-LSL-GFPEvi1* lines A and B and *Vav-LSL-FlagEvi1* line C (Figure 2A) deletion of the stop sequence was accompanied by a significant induction of *Evi1* mRNA expression in the fetal liver as shown by real-time quantitative (RQ)-PCR. Although FlagEvi1 or GFPEvi1 protein were undetectable by western blotting, we could demonstrate a low but consistent induction of GFPEvi1 protein by flowcytometry in double transgenics of lines *Vav-LSL-GFPEvi1* A, B (data not shown) and C (Figure 2B) compared to controls.

Embryos double transgenic for *pEV-Cre* and *Vav-LSL-GFPEvi1* lines A, B and C or *Vav-LSL-FlagEvi1* line C displayed a clear and very similar phenotype (see below), therefore data of these four lines were combined and from this point on will be referred to as *Vav-LSL-Evi1*. In *Vav-LSL-GFPEvi1* line D and *Vav-LSL-Flag* lines A and B expression of *Evi1* was very low

or undetectable by RQ-PCR or flow cytometry, and accordingly, these lines did not show any phenotypic defects (data not shown).

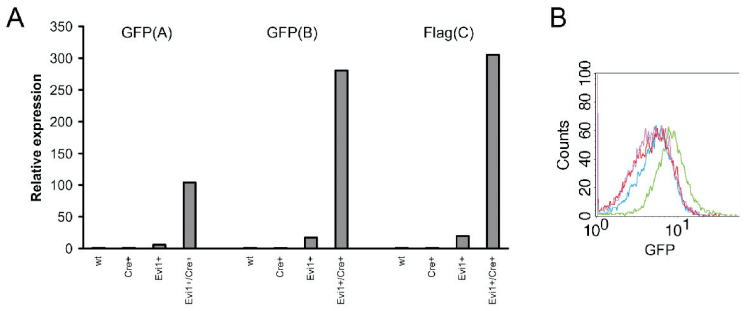

Figure 2 *Evi1* transgene expression in E12.5 fetal livers (A) RQ-PCR showing *Evi1* transgene mRNA expression in double transgenic and control livers of *Vav-LSL-GFPEvi1* line A and B and *Vav-LSL-FlagEvi1* line C. Data are expressed as the mean relative *Evi1* expression of littermates with the same genotype. (B) Flowcytometric analysis of a *Vav-LSL-GFPEvi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ liver (green) and WT (red), *pEV-Cre*$^{+/-}$ (pink) and *Vav-LSL-GFPEvi1*$^{+/-}$ (blue) controls showing increased GFP fluorescence in the double transgenic liver. Dead cells were excluded from the analysis.

Enforced expression of Evi1 in the erythroid lineage induces fetal anemia

We determined the genotype distribution of offspring from crossings between *Vav-LSL-Evi1*$^{+/-}$ and *pEV-Cre*$^{+/-}$ mice at E12.5 and in neonates. Whereas the genotype distribution followed a Mendelian ratio at E12.5, the percentage of *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ offspring was reduced to 15% in neonates (Table 1). Surviving *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ neonates had normal blood counts (unpublished data). This suggested that part of the double transgenic animals died between E12.5 and birth, and indeed we observed presence of dead double transgenic embryos in E13.5 litters (data not shown).

	E12.5			neonate		
genotype	n	%	(expected %)	n	%	(expected %)
Wt	45	29.4	(25)	39	33.9	(25)
Cre+	40	26.1	(25)	26	22.6	(25)
Evi1+	34	22.2	(25)	33	28.7	(25)
Evi1+/Cre+	34	22.2	(25)	17	14.8	(25)
Total	153			115		

Table 1 Genotype distribution of offspring from *Vav-LSL-Evi*$^{+/-}$ x *pEV-Cre*$^{+/-}$ crossings at E12.5 and in neonates

We focused on the analysis of the E12.5 embryos. Double heterozygous embryos from the four *Vav-LSL-Evi1* lines could be readily distinguished from littermates at this stage because of the pale appearance of their visceral yolk sacs (Figure 3A). Moreover, *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ embryos were paler than their littermates and showed a clear reduction in the size of the fetal liver (Figure 3A) that contained significantly less cells compared to controls (Figure 3B). Although these embryos were clearly anemic they were alive as assessed by heart beating.

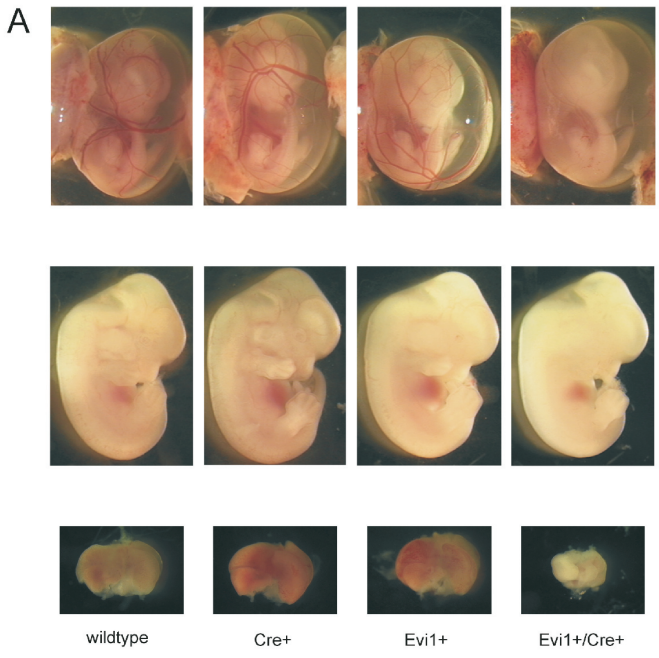

Figure 3 Evi1 induces fetal anemia (A) Photographs showing E12.5 embryos within their yolk sacs (upper panels), the same embryos without yolk sac (middle panels) and fetal livers isolated from these embryos (lower panels). *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ embryos show compared to control littermates a clear pallor of the yolk sac (far right, upper panel). The embryo itself is also paler than the controls (far right, middle panel) and has a very pale liver that is reduced in size (far right, lower panel). (B) *Vav-LSLEvi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ E12.5 transgenic livers contain significantly less cells than control livers by Bonferroni statistic (*$p<0.001$, $n\geq28$). Data are expressed as mean ± SD.

While the blood of control embryos mainly consisted of a uniform population of primitive erythrocytes at E12.5 (Figure 4A), fetal blood from *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ embryos contained abnormal immature cells, and mature primitive erythroid cells of which many were larger than normal and showed signs of dysplasia (Figure 4A). Flowcytometric analysis of fetal

blood also demonstrated the increased average size of *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal blood cells compared to controls (Fig 4B) and confirmed the expression of GFPEvi1 protein in double transgenic blood in *Vav-LSL-GFPEvi1* lines A, B (not shown) and C (Figure 4B).

The reduced fetal liver cellularity clearly indicated that definitive erythropoiesis was disturbed in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/}$ embryos. Cytological analysis showed an overrepresentation of immature erythroid cells in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal livers compared to controls (Figure 5A-B). Flowcytometric analysis demonstrated a clear reduction of the TER119^{+}/CD71^{-} cell population in double transgenic fetal livers confirming the maturation arrest within the erythroid lineage (Figure 5C). This arrest was accompanied by an increased percentage of dysplastic cells that were characterized by asynchronous maturation of the nucleus and cytoplasm (Figure 5D-E). Moreover, a clear increase in fetal liver apoptosis was observed since the percentage of Annexin V^{+}, propidium iodide^{-} (PI^{-}) cells had doubled in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ compared to control livers (Figure 5F). As a result of increased apoptosis, the percentage of dead cells (Annexin V^{+}, PI^{+}) was also increased in *Vav-LSL-Evi1*$^{+/-}$*/ pEV-Cre*$^{+/-}$ compared to control livers (Figure 5F).

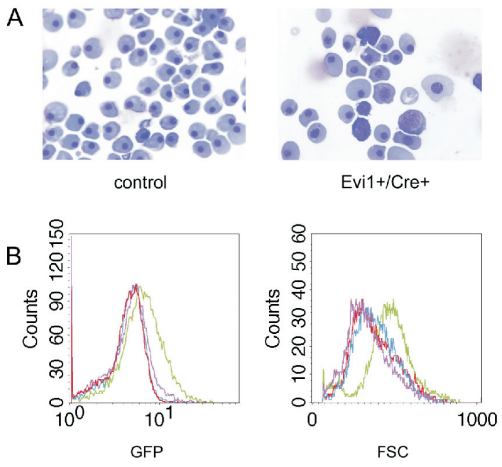

Figure 4 E12.5 fetal blood in *Vav-LSLEvi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ embryos and controls (A). Control blood (left) contains a uniform population of mostly nucleated primitive erythrocytes with few enucleated definitive erythrocytes. In *Vav-LSLEvi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal blood a less uniform population of cells is observed with presence of immature cells, and cells that shown signs of dysplasia (large size, abnormal ratio between cytoplasm and nucleus). (B). Flowcytometric analysis of *Vav-LSL-GFPEvi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal blood (green) and WT (red), *pEV-Cre*$^{+/-}$ (pink) and *Vav-LSL-GFPEvi1*$^{+/-}$ (blue) control fetal blood. Dead cells were excluded from the analysis. The *Vav-LSL-GFPEvi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ sample displays increased GFP fluorescence (left panel) and has a higher forward scatter (FSC) compared to the controls indicating larger cell size (right panel).

E12.5 Vav-LSL-Evi1$^{+/-}$/pEV-Cre$^{+/-}$ fetal livers contain a reduced number of CFU-E

To determine the number of BFU-E (burst forming unit-erythroid) and CFU-E (colony forming unit-erythroid) progenitors in E12.5 double transgenic livers, we performed colony assays. Fetal liver cells were grown in semi-solid medium in presence of Epo, or Epo and SCF, to determine the number of CFU-E and BFU-E, respectively, per liver. E12.5 *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal livers consistently contained less CFU-E than single transgenic and WT littermates. In contrast,

no difference in the number of BFU-E was observed (Figure 6A).

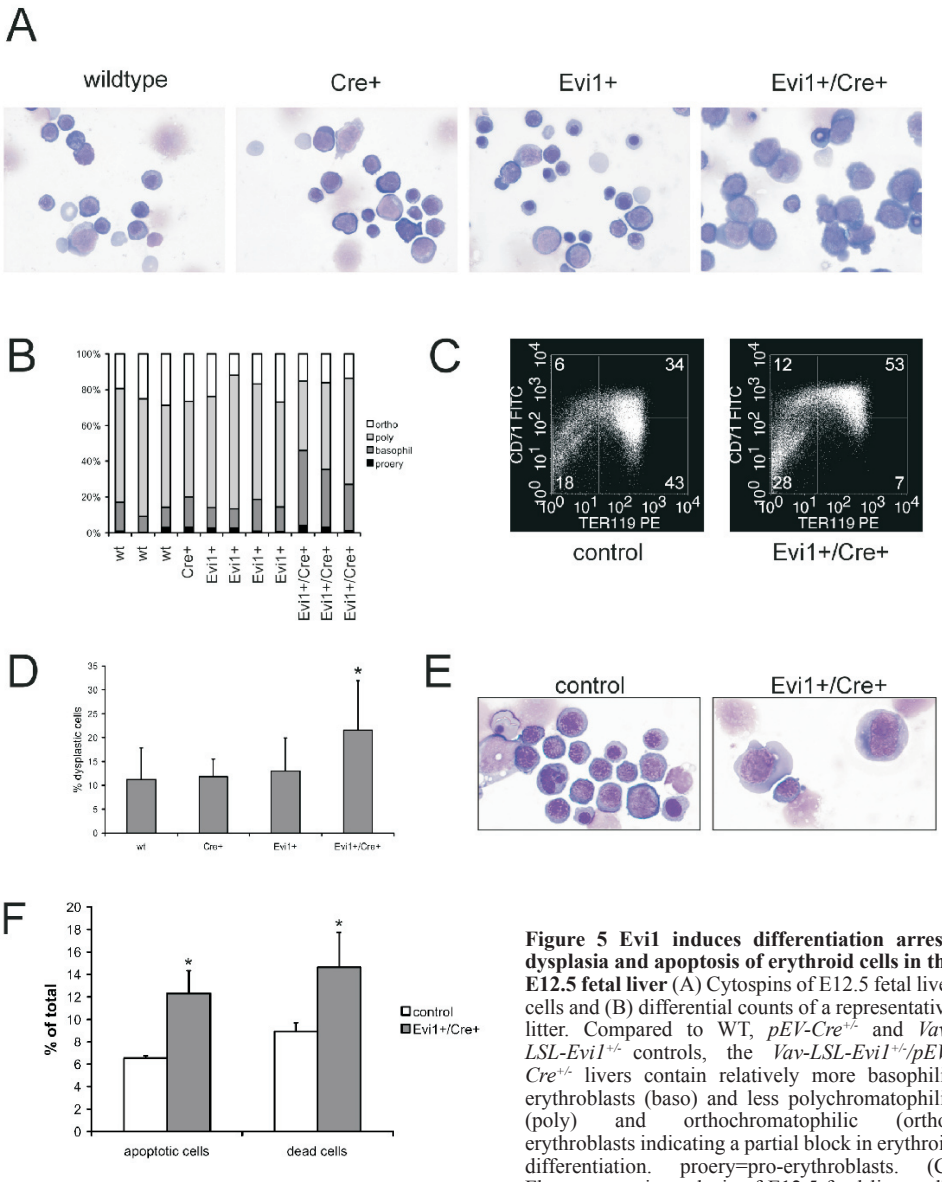

Figure 5 Evi1 induces differentiation arrest, dysplasia and apoptosis of erythroid cells in the E12.5 fetal liver (A) Cytospins of E12.5 fetal liver cells and (B) differential counts of a representative litter. Compared to WT, *pEV-Cre*$^{+/-}$ and *Vav-LSL-Evi1*$^{+/-}$ controls, the *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ livers contain relatively more basophilic erythroblasts (baso) and less polychromatophilic (poly) and orthochromatophilic (ortho) erythroblasts indicating a partial block in erythroid differentiation. proery=pro-erythroblasts. (C) Flowcytometric analysis of E12.5 fetal liver cells labeled with anti-TER119-PE and anti-CD71-FITC. Dead cells (7AAD^{+}) were excluded from the analysis. The ratio of cells in each quadrant is shown. The TER119^{+}/CD71^{-} population that contains the more mature erythroid cells is decreased in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ livers, while more immature populations are increased. (D) *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal livers contain significantly more dysplastic cells than all control groups by Tamhane's T2 statistic (*p≤0.031, n≥18). (E) Normal cells in a control and dysplastic cells in a *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ liver. The dysplastic cells are larger than normal, have an abnormal ratio between cytoplasm/nucleus, and show asynchronous maturation of cytoplasm and nucleus, i.e. an immature non-condensated nucleus in combination with a more mature acidophilic cytoplasm. (F) *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal livers (n=4) contain significantly more apoptotic and dead cells (*p=0.006, Mann Whitney U Test) than controls (n=7). E12.5 fetal liver cells were labeled with AnnexinV and PI and analyzed by flowcytometry to determine the percentage of apoptotic (Annexin V^{+}/PI^{-}) and dead cells (Annexin V^{+}/PI^{-})

per fetal liver. For the analysis of this experiment, WT, *Vav-LSL-Evi1*$^{+/-}$ and *pEV-Cre*$^{+/-}$ livers were joined in one control group. Data are expressed as mean ± SD.

Expansion of *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal liver progenitors in liquid culture

Fetal liver progenitors can be expanded in a liquid culture system that exploits the vigorous self-renewal capacity of erythroid progenitors.[23,24] When we cultured fetal liver cells of double transgenic and controls in the presence of Epo, SCF and glucocorticoids to stimulate the self-renewal of erythroid progenitors, only in the most severely affected double transgenic fetal livers (containing less than 10^6 cells per liver), cells failed to expand normally.

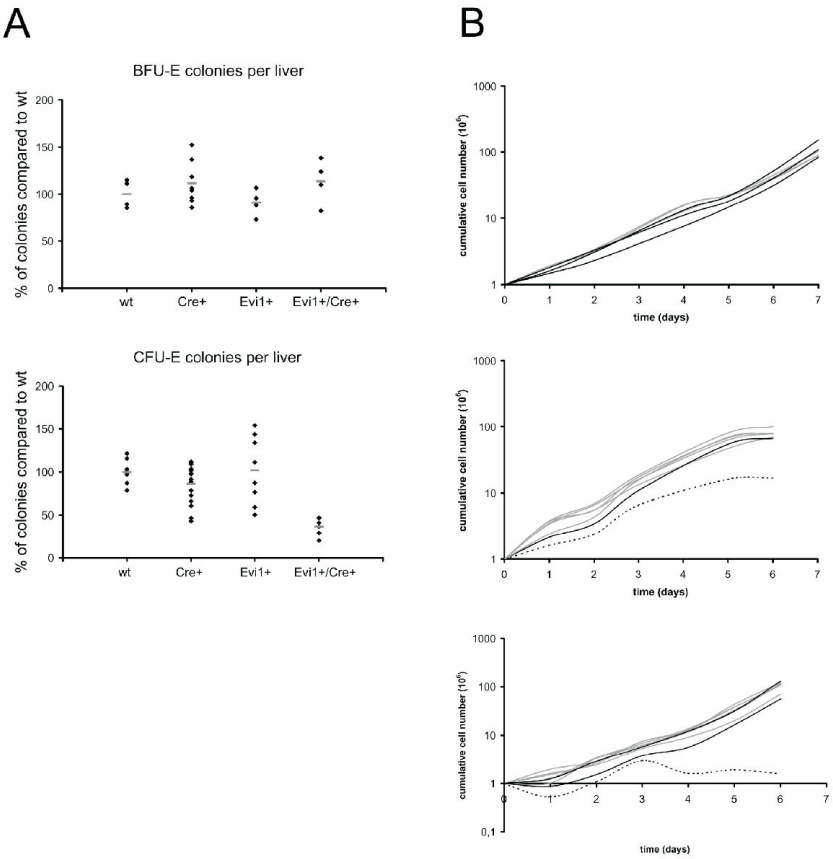

Figure 6 ***In vitro*** **culture of** ***Vav-LSL-Evi1***$^{+/-}$***/pEV-Cre***$^{+/-}$ **fetal livers** (A) *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal livers contain a reduced number of CFU-E. Fetal liver cells from *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ and control livers were seeded in duplicate in methylcellulose and counted as described in Methods. Values are expressed as the total number of BFU-E and CFU-E colonies per liver relative (in %) to the average number of colonies of fetal livers from wild type littermates. This was done to correct for differences between experiments with respect to assay conditions and the stage of the used embryos. Grey bars indicate the mean of all livers for each genotype. While no significant difference in the number of BFU-E was found (upper panel), the number of CFU-E (lower panel) was significantly lower in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ compared

to control livers (*p≤0.001, Mann Whitney U Test). (B) Expansion of fetal liver progenitors in liquid medium containing Epo, SCF and dexamethasone. Three independent experiments are shown representing three different litters. *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ cultures are shown in black, all control livers are in grey. Dashed black lines indicate the growth curves of cultures obtained from double transgenic livers that were severely reduced in size (containing less than 10^6 cells per liver). These cultures show reduced expansion compared to control livers and less affected double transgenic livers.

For most *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal livers, expansion of double transgenic fetal liver cells was comparable to control littermates, showing that erythroid progenitors in these livers were still responsive to growth factors (Figure 6B). Moreover, progenitors from these cultures differentiated normally upon transfer to medium containing Epo and transferrin (data not shown).

Pu.1 and c-myb expression is up regulated in Vav-LSL-Evi1$^{+/-}$/pEV-Cre$^{+/-}$ livers

RQ-PCR was applied to compare the expression of a number of important regulators and markers of erythroid development between double transgenic and control livers (Figure 7). Pu.1 is a transcription factor that promotes the self-renewal of erythroid progenitors, thereby preventing differentiation.[25] In mouse erythroleukemia (MEL) cells, virally induced Pu.1 blocks differentiation by acting as a functional antagonist of Gata1.[26,27] Interestingly, expression of *Pu.1* was upregulated in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ livers compared to controls, and this was also the case for *c-myb* that acts downstream of Pu.1 in MEL cells.[28]

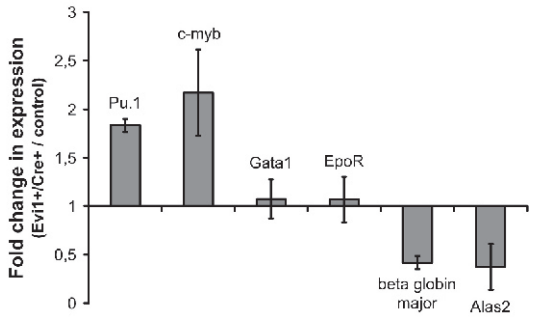

Figure 7 Upregulation of Pu.1 and c-myb expression in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal livers Fold change in RNA expression of genes involved in erythroid development in compared to controls from the same litter as determined by RQ-PCR. Mean and SD of three independent experiments are shown.

The transcription factor Gata1 plays a critical role in erythroid development, since both loss and gain of Gata1 function severely affect growth and survival of erythroid cells, and binding sites for Gata1 are found in the regulatory sequences of many erythroid specific genes (reviewed by Ferreira et al.[29]). *Gata1* expression levels were comparable between *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$

and control livers (Figure 7). Epo is the major growth factor for erythroid cells. Although it was previously reported that expression of the *Epo Receptor* (*EpoR*) was down regulated in Evi1 overexpressing BM cells[14], *EpoR* expression was unchanged in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ livers compared to controls (Figure 7). In contrast, transcription of the genes encoding for the major β-globin chain (a subunit of hemoglobin) and aminolevulinic acid synthase 2 (Alas2, a heme biosynthesis enzyme) was lower in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ livers compared to controls. This confirmed the observed maturation arrest since these Gata1 target genes are normally up regulated during terminal erythroid differentiation.[30]

Discussion

We established novel *Evi1* transgenic mouse lines with conditional expression in the hematopoietic system. In this report we have demonstrated the use of these *Vav-LSL-Evi1* lines to study the specific *in vivo* effects of Evi1 in the erythroid lineage. Although aberrant *Evi1* expression is associated with MDS/AML with severe anemia, previous *in vivo* studies did not resolve whether Evi1 interferes with erythroid development directly, by affecting committed erythroid progenitors, or indirectly by deregulating non-committed stem cells. The experiments presented here show that Evi1 directly interferes with erythroid development *in vivo*. Enforced erythroid lineage specific *Evi1* overexpression induced fetal anemia that was characterized by arrested erythroid maturation, increased apoptosis and dysplasia.

Fetal anemia induced by erythroid lineage specific Evi1 *overexpression is transient*

Our data show that the Evi1-induced fetal anemia causes embryonic lethality in 40% of cases. In the other cases the anemia appears to be transient since surviving *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ mice have normal blood counts showing that steady-state erythropoiesis is unaffected in these adults. The restriction of the anemic phenotype in embryonic stages is not unique to our model. For instance, *ATF4* and *Stat5a/5b* null mutants,[31,32] and mutant mice expressing a truncated Gata1 protein[33] display a similar transient embryonic erythroid phenotype. In the first two studies[31,32] this phenomenon was explained by the considerably lower erythropoietic reserve capacity of early embryos compared to adults; this capacity is thought to be lower because the rapid growth of the embryo must be matched by a rapid concomitant increase in the amount of red cells. Since erythropoiesis during early development is in many aspects similar to stress erythropoiesis in the

adult, mild erythroid defects in the adult *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ mice may be revealed under stress conditions, like in the *Stat5a/5b* null mutants.[31]

An alternative explanation for the transient fetal anemia may be that Evi1 specifically affects the development of erythroid cells in response to interactions with fetal liver supportive cells, since it is striking that erythroid progenitors from most *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal livers proliferated and differentiated normally in a liquid culture system containing saturating amounts of growth factors. There is evidence that during early stages of fetal liver erythropoiesis higher levels of Epo are necessary to sustain survival and differentiation than at later stages.[34] It is possible that during this critical period the survival factor levels in *Evi1* overexpressing livers are insufficient to compensate for the effects of Evi1. When sufficient Epo is present, like in liquid culture or in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ embryos at a later stage, stimulation of Epo-dependent survival pathways may rescue the remaining progenitors. The fact that *EpoR* expression in E12.5 *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ livers did not differ from controls, and the observation that in surviving E13.5 *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ embryos fetal liver cellularity had returned to normal (unpublished data) support this notion. Only in the most severely affected E12.5 *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ livers, that contain the lowest total amount of cells and the highest percentage of apoptotic and dead cells, and that show decreased proliferation *in vitro*, the rescue may come too late, leading to embryonic lethality.

We observed a reduction in the number of CFU-E erythroid progenitors in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ fetal livers. Although this suggests a direct interference of Evi1 with commitment of progenitors to the CFU-E stage, a more plausible explanation may be that the reduction is secondary to the observed maturation arrest, since *pEV-Cre* driven recombination in the erythroid lineage is temporally restricted, recapitulating the expression pattern of *β-like globin* genes.[18] To determine the effect of Evi1 at the more immature stages of erythroid development it would be applicable to use transgenic mouse lines that express Cre at earlier stages of erythropoiesis.

Possible mechanisms involved in Evi1 induced anemia

The mRNA expression levels of a number of key factors involved in erythroid development were examined to learn more about targets and pathways that could be affected by *Evi1* overexpression. Epo is the main growth factor for erythroid cells. Interaction with its receptor, EpoR, stimulates signaling cascades involved in survival, proliferation and differentiation of erythroid progenitors (reviewed by Lacombe and Mayeux[35]). The transcription factor Gata1 is part of a large and highly dynamic protein complex that changes in abundance during hematopoietic development

and that is involved in development of the erythroid and megakaryocytic lineages (reviewed by Cantor and Orkin[36] and by Ferreira et al.[29]). Previous studies in BM and 32D-Epo cells have provided evidence for interference of Evi1 with Gata1 function and/or Epo signaling, possibly by downregulation of *EpoR* expression.[12,14] We however did not detect any difference in either *EpoR* or *Gata1* expression between *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ and control fetal livers.

Strikingly, we did observe increased expression of the transcription factors *Pu.1* and *c-myb*. In MEL cells, virally induced Pu.1 causes a block in terminal differentiation by functionally interacting with Gata1 and thereby repressing Gata1 target genes.[26,27] C-myb has been shown to act downstream of Pu.1 in MEL cells.[28] Although we cannot exclude that the changes in *Pu.1* and *c-myb* expression are (partly) due to the observed arrest in maturation, the direct or indirect induction of *Pu.1* expression by Evi1 is an interesting option that deserves further investigation.

Materials and Methods

Generation of expression constructs

A *loxP*-flanked transcriptional stop (*LSL*) cassette[15] was inserted into *Xho*I / *Not*I digested *PLNCX2* to generate *PLNCX2-LSL*. Blunt-ended *GFPEvi1* and *FlagEvi1* fusions, containing the full length 4.5 kb Evi1 cDNA, were inserted into the *Stu*I site of *PLNCX2-LSL* resulting in *PLNCX2-LSL-GFPEvi1* and *PLNCX2-LSL-FlagEvi1*. The *Vav-LSL-GFPEvi1* and *Vav-LSL-FlagEvi1* transgenic constructs were generated by digestion of a *Vav-hCD4* construct[16] with *Sfi*I and *Not*I (resulting in removal of *hCD4* sequences), blunting of the overhangs, and insertion of blunt-ended *LSL-GFPEvi1* and *LSL-FlagEvi1* fragments after excision from the *PLNCX2-LSL-GFPEvi1* and *PLNCX2-LSL-FlagEvi1* constructs by *Eco*RI and partial *Cla*I digestion, respectively. The integrity of the constructs was confirmed by restriction enzyme analysis.

Transfection and immunofluorescence

Phoenix cells were transfected using the calcium phosphate transfection method with 10µg of the *PLNCX2-LSL-GFPEvi1* plasmid in presence or absence of 0.5 µg of the *pMC-Cre* plasmid[17]. Two days after transfection expression was visualized using a Leica DMIRB fluorescence microscope (Leica, Rijswijk, The Netherlands).

Generation of transgenic mice

Fragments for microinjection were prepared by linearization of the *Vav-LSL-GFPEvi1* and *Vav-LSL-FlagEvi1* transgenic constructs with *Pvu*I (which partially removes the vector backbone), gel-purification of the desired fragments with the Jetsorb kit (Genomed, Löhne, Germany), and an additional purification step using Elutip-d columns (Schleicher and Schuell, Dassel, Germany). Fragments were microinjected into the pronucleus of fertilized FVB mouse eggs and transferred into pseudo-pregnant mothers. Transgenic founders were bred with FVB mice to establish transgenic lines. *Vav-LSL-GFPEvi1* and *Vav-LSL-FlagEvi1* transgenic mice were crossed with the *pEV-Cre* transgenic line[18] to induce specific excision of the transcriptional stop sequence in the erythroid lineage of double transgenic offspring. All mice were maintained under standard conditions and treated according to institutional animal health care and use guidelines.

Collection of embryos and fetal livers

Vav-LSL-FlagEvi1$^{+/-}$ or *Vav-LSL-GFPEvi1*$^{+/-}$ mice were crossed with *pEV-Cre*$^{+/-}$ mice and embryos were dissected at E12.5. Fetal blood was collected in tubes containing PBS / 5mM EDTA. Single cell suspensions of fetal livers were made by pipetting in PBS / 0.5% BSA.

Genotype analysis

DNA isolated from embryonic heads or livers and adult tails was used for genotype analysis. Genotyping was done by PCR using the following primer sets: *Vav-LSL-GFPEvi1*: 5'-GATCACATGGTCCTGCT-3' and 5'-CAGTTTTCACACTCATAGTGC-3', *Vav-LSL-FlagEvi1*: 5'-GGACGACGATGACAAAGAT-3' and 5'-CAGTTTTCACACTCATAGTGC-3', *pEV-Cre*: 5'-GCACGTTCACCGGCATCAAC-3' and 5'-CGATGCAACGAGTGATGAGGTTC-3'. PCR conditions for all reactions were 1' 94°, 1' 55°, 1' 72°C (36 cycles).

Detection of in vivo recombination

PCR on genomic DNA isolated from fetal livers was applied to detect the deletion of the transcriptional stop sequence. The following primer sets were used for this PCR: 5'-GGAAGTGGTGTTGTAGTTGTC-3' and 5'-CCTTGAAGAAGATGGTGCG-3' for *Vav-LSL-GFPEvi1* mice, 5'- GGAAGTGGTGTTGTAGTTGTC-3' and 5'-CAGTTTTCACACTCATAGTGC-3' for *Vav-LSL-FlagEvi1* mice. PCR was performed under the following conditions: 1' 94°, 1' 55°, 3' 72°C (38 cycles).

RQ-PCR

Total RNA was extracted from single cell suspensions of fetal livers with guanidium thiocyanate and purified by centrifugation in cesium chloride solution. RNA was transcribed into cDNA using Superscript (Invitrogen, Breda, The Netherlands) and random hexamers according to the recommendations of the manufacturer. RQ-PCR amplification was performed in an ABI PRISM 7900 HT Sequence Detector (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), using 12.5 µl SYBR Green PCR Master Mix (Applied Biosystems), 2 µl (1/4 diluted or undiluted) cDNA, 0.5 µl of each primer (10 pmol/µl) and 9.5 µl water. Sequences of primer pairs used for RQ-PCR are available on request. The PCR conditions were 2' 50°C (1 cycle), 10' 95°C (1 cycle), 15'' 95°C followed by 1' 60°C (45 cycles). Average Ct (threshold cycle) values from duplicate readings were normalized using the endogenous reference (ΔCt = Ct target RNA - Ct *Hprt*). To quantify the relative expression of *GFPEvi1* and *FlagEvi1* RNAs, the ΔCt values of transgenic fetal livers were compared with the mean ΔCt value of samples from WT littermates as a calibrator, using the "deltadelta Ct" method (ΔΔCt=ΔCt sample - ΔCt WT). To quantify the relative expression of erythroid RNAs the mean ΔCt of all (WT and single transgenic) samples from control littermates was used as the calibrator. Relative expression was calculated by the formula $2^{-\Delta\Delta Ct}$.

Liquid culture of fetal liver cells

Following disaggregation of the fetal liver, the total amount of cells was determined by counting cell suspensions with a CASY1 electronic cell counter (Schärfe-System, Reutlingen, Germany). Fetal liver cells were expanded in StemPro-34™ medium (Invitrogen) supplemented with 0.36 units/ml human recombinant erythropoietin (Epo; Eprex, Janssen-Cilag, Tilburg, The Netherlands), 100 ng/ml murine recombinant SCF (R&D Systems, Minneapolis, MN, USA) and 10^{-6} M dexamethasone (Sigma-Aldrich, Zwijndrecht, The Netherlands) with daily adjustment of the cell density to $1x10^6$ cells/ml. To measure differentiation, fetal liver cultures were washed twice with 50 ml PBS and spun at 700 rpm for 7' to enrich for erythroid progenitors that were subsequently cultured for 48h in StemPro-34™ medium supplemented with 5 units/ml Epo and 1 mg/ml iron saturated human transferrin (Intergene, Purchase, NY, USA).

Colony assays

Fetal liver cells were assayed for their content of CFU-E and BFU-E by in vitro colony formation in semisolid methylcellulose culture medium. Cells were plated in duplicate in 35-

mm Petri dishes (BD, Aalst, Belgium) at a concentration of 60,000 to 100,000 cells per dish. The components included in the methylcellulose cultures are described in de Jong et al.[19] Cultures were maintained in a humidified atmosphere of 5% CO_2 in air. Colonies were counted at day 2 (CFU-E) and day 8 (BFU-E).

Flow cytometry

Single cell suspensions of fetal liver cells were incubated with PE-conjugated anti-mouse TER119 and FITC-conjugated CD71 (BD) to determine the differentiation status. Dead cells were excluded from analysis by staining with 7-aminoactinomycin-D (7AAD; Invitrogen). To identify apoptotic cells, single cell suspensions were stained with AnnexinV-FITC and Propidium iodide (PI, BD). A total of $5x10^4$ events were counted per sample.

Cytology and differential counting

Cytospins of freshly isolated fetal liver, bone marrow or spleen cells were stained with May-Grünwald Giemsa and double-blindly analyzed. A differential count was performed on 200 or 300 nucleated cells per slide and the percentage of dysplastic erythroblasts was obtained. Dysplasia was characterized as deformation of the nucleus, megaloblastic changes or asynchronous maturation of the cytoplasm and the nucleus.

Statistics

Data sets were analyzed using SPSS statistical analysis software (SPSS, Chicago, IL). Small datasets (with group sizes smaller than n=10) were analyzed by pair wise comparisons between groups using the Mann Whitney U test. For larger data sets, first the normal distribution was confirmed by determining the Kolmogorov-Smirnov statistic. Significant differences among groups were subsequently determined using ANOVA. When significance was detected, pair wise comparisons were made between groups using either Tamhane's T2 or Bonferroni post hoc tests in case of respectively unequal and equal variation of the dependent variable across the groups. Statistical significance was defined as $p<0.05$.

Acknowledgements

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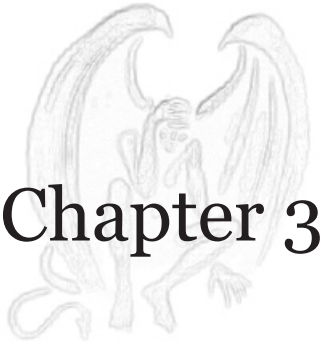

Chapter 3

Early expression of Evi1 in fetal erythroid development causes considerable increase of cell death in a conditional transgenic mouse model.

Ongoing work

Early expression of *Evi1* in fetal erythroid development causes considerable increase of cell death in a conditional transgenic mouse model.

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Abstract

Aberrant expression of *EVI1* has been observed in patients with myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) carrying 3q26 aberrations. In MDS patients expressing this aberration, refractory anemia is frequently observed suggesting direct interference of EVI1 with erythropoiesis. We recently established novel *Vav-loxp-stop-loxp-Evi1* transgenic mouse lines that conditionally express *Evi1* in the hematopoietic system.

Here we crossed these animals with mice expressing the Cre-enzyme in early erythroid-progenitors under the control of the Epo-receptor promoter (ErGFPCr). The effects of *Evi1* expression on early erythroid progenitors differentiation revealed a strong correlation between inappropriate expression of *Evi1* and cell death. The fetal liver from E12.5 embryos showed significant decrease in size and cell number. Furthermore, a significant decrease of early progenitors (CFU-e and BFU-e) was found and forced in vitro differentiation of these cells showed inability of progression towards mature erythrocytes. In addition, fetal blood cells showed anisocytosis, which is a characteristic feature of anemia.

These findings support the notion that inappropriate expression of *EVI1* plays a role in development and progression of MDS or AML in humans. Moreover, we have found that earlier expression of Evi1 during erythroid differentiation has a more severe outcome as compared with previously analysed β-globin specific erythroid Cre lines.

Introduction

Evi1 (ecotropic virus integration site 1), which was originally identified as a myeloid transforming gene by means of retroviral insertional mutagenesis in mouse leukemias, encodes a nuclear DNA binding zinc finger protein.[1,2] Patients with acute myeloid leukemia (AML) or myelodysplastic

syndrome (MDS) harboring 3q26 translocations, the region where *EVI1* is located, frequently show aberrant expression of *EVI1*.[3-5] Moreover, in 10% of AML patients and 30% of MDS patients without abnormalities in 3q26,[4,6] *EVI1* overexpression has been observed as a result of unknown mechanisms. Evi1 protein contains two zinc finger domains, i.e. an N-terminal domain of seven zinc fingers and a more C-terminal domain containing three zinc fingers. Although the exact mechanism of transformation by Evi1 is unknown, the fact that EVI1 is capable of binding DNA through the two zinc finger domains, each recognizing a specific and unique nucleotide sequence, suggests that it is involved in transcriptional control .[7-9]

Several *in vivo* and *in vitro* studies have provided support for an involvement of *Evi1* in hematopoietic differentiation and consequently in the development of MDS and AML. Evi1 has been shown to interfere with cytokine induced neutrophilic or erythroid differentiation using 32D cell line models or bone marrow progenitor cells *in vitro*. [10,11] Moreover, evidence for interference with differentiation by Evi1 *in vivo* has been obtained from studies using *Sca-Evi1* transgenic lines showing mild erythroid defects. [12] Severe erythroid defects were reported from studies in which *Evi1* transduced BM progenitors were transplantated into sublethally irradiated mice.[13] Our previous observation that only low or undetectable levels of *Evi1* expression could be achieved in the *Sca-Evi1* model and the findings showing that *Evi1* coupled to strong promoters (e.g. H-2k) yielded no transgenic lines at all (D.S and R.D, unpublished data), prompted us to generate *Evi1* transgenic mouse lines with Cre recombinase-inducible *Evi1* expression in the hematopoietic system. [14] We examined the effects of *Evi1* activation on survival, proliferation and differentiation of erythroid progenitors, by crossing the *Evi1* transgenic lines with the erythroid lineage specific *pEV-Cre* (Cre under the control of the beta-globin locus control region) transgenic line. Although this combination of mice allowed expression of Evi1 at a relatively late stage of erythroid development, significant interference by Evi1 with fetal erythroid development was observed. To investigate the effects of Evi1 in more immature erythroid precursors we bred the Cre recombinase-inducible *Evi1* lines with mice expressing the Cre enzyme under control of the Epo-receptor promoter, *ErGFPCre*. We show that Evi1 expression in the double transgenic animals results in a more severe phenotype that observed in *Evi-1* mice crossed with pEV-Cre. We observed substantial cell death of fetal liver cells, suggesting that inappropriate expression of Evi1 is detrimental for erythroid precursors and may reflect the erythroid malformations frequently observed in MDS patients showing high EVI1 expression as the result of 3q26 aberrations.

Results

V*av-LSL-Evi1 and ErGFPCre* double transgenic animals: macroscopic defects of fetal erythroid development.

The transgenic lines *Vav-LSL-Evi1* previously demonstrated to express Evi1 following pEV-Cre breeding were used for this study and crossed with *ErGFPCre* transgenic mice. In the *ErGFPCre* line, Cre expression is under the control of the Epo-receptor gene, allowing expression of the enzyme in the erythroid lineage starting at early stage, thereby efficiently driving recombination of floxed loci within the entire erythroid lineage (Figure 1A)[17]. Although only one litter of neonates has been analyzed, no double transgenic *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCr*$^{+/-}$ offspring was observed (Table 1). However, Southern blotting (Figure 1B) revealed that 9/50 (19%) E12.5 embryos were double heterozygous for *ErGFPCre* and *Evi1* (Table 1).

	N	Evi1+/Cre+	WT	Evi1+	Cre+
E12.5	50	9	13	12	16
New Born	7	0	1	3	3

Table 1 Genotypic distribution of E12.5 and newborn animals from ErGFPCr$^{+/-}$/Vav-LSL-Evi1$^{+/-}$ crossings
ErGFPCr$^{+/-}$/Vav-LSL-Evi1$^{+/-}$ double heterozygous animals follow normal Mendelian distribution. Analysis of neonates did not reveal presence of ErGFPCr$^{+/-}$/Vav-LSL-Evi1$^{+/-}$ animals.

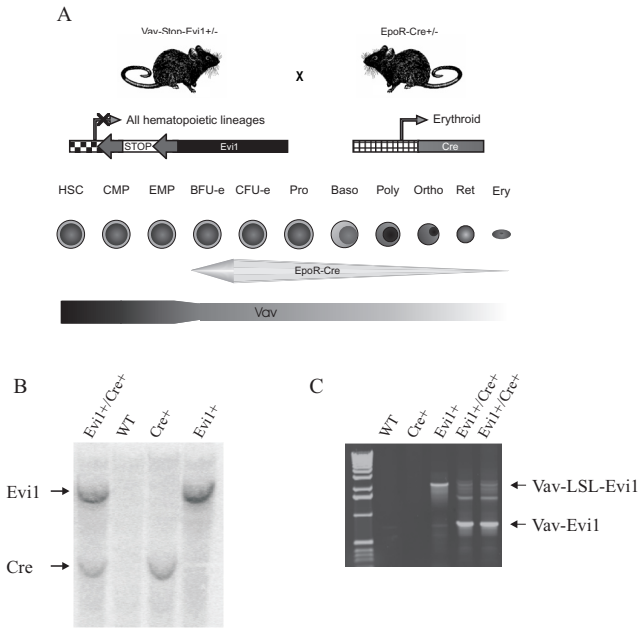

Figure 1 Expression pattern of ErGFPCr$^{+/-}$ and Vav-LSL-Evi1$^{+/-}$ mice (A) Evi1 driven by the Vav promoter targets all hematopoietic lineages. The short LSL sequence is flanked by two LoxP sites (grey arrows), which upon deletion by the Cre enzyme removes the LoxP sites and allows transcription of Evi1. The ErGFPCr$^{+/-}$mouse expresses the Cre recombinase enzyme under the control of the Epo-Receptor gene and allows expression starting from the BFU-e stage. (B) Southern blot using genomic DNA of E12.5 littermates. DNA was digested with *EcoRV* and probed with EGFP probe. Heterozygous embryos showed presence of both constructs, wild type embryo has absence of both constructs, and in homozygous embryos single product was detected. (C) Recombination PCR on genomic DNA obtained from fetal liver cells. Vav-LSL-Evi1 band indicates non-recombined product, whereas Vav-Evi1 shows recombination upon Cre presence.

PCR confirmed recombination between *loxP* sites and deletion of the stop box in the genomic DNA of fetal livers isolated from E12.5 *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCr*$^{+/-}$ double transgenic embryos as opposed to single transgenic littermates (Figure 1C).

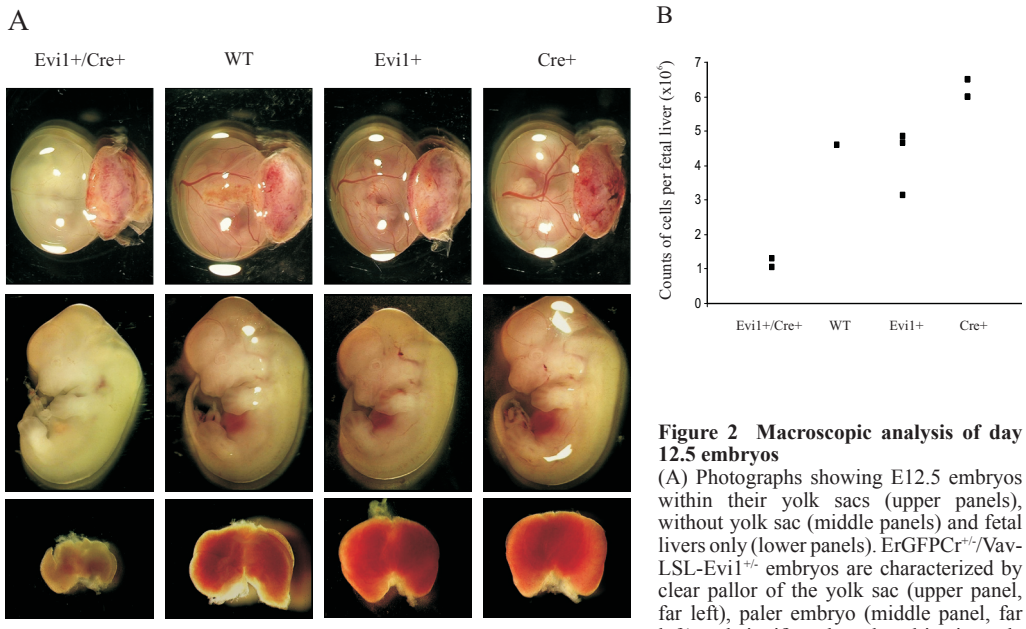

Figure 2 Macroscopic analysis of day 12.5 embryos
(A) Photographs showing E12.5 embryos within their yolk sacs (upper panels), without yolk sac (middle panels) and fetal livers only (lower panels). ErGFPCr$^{+/-}$/Vav-LSL-Evi1$^{+/-}$ embryos are characterized by clear pallor of the yolk sac (upper panel, far left), paler embryo (middle panel, far left) and significantly reduced in size pale liver (lower panel, far left). (B) ErGFPCr$^{+/-}$/Vav-LSL-Evi1$^{+/-}$ E12.5 transgenic livers showed greatly reduced number of cells as compared to control livers.

Double heterozygous embryos could be readily distinguished from littermates at this stage because of the exceptionally pale appearance of the visceral yolk sacs (Figure 2A). In addition, *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCre*$^{+/-}$ embryos were paler than their littermates and showed a clear reduction in the size of the fetal liver (Figure 2A). Moreover, double transgenic fetal livers contained significantly less cells compared to control livers (Figure 2B).

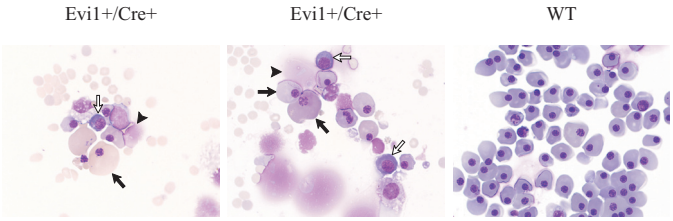

Figure 3 Cellular morphology of fetal blood cells at E12.5
Cytospins of fetal blood samples of two independent ErGFPCr$^{+/-}$/Vav-LSL-Evi1$^{+/-}$ shows heterogeneous populations of cells. The composition of blood cells range from immature cells (white arrows), dissimilar size of cells (anisocytosis) (black arrows) and dead cells (arrow heads). Control blood of wild type (WT) littermate showed a uniform population of mature erythroblasts.

Severe hypocellularity and anisocytosis of fetal red blood cells in double heterozygous embryos

Since macroscopic analysis of the visceral yolk sacs suggested inappropriate fetal erythropoiesis we analysed blood samples by May-Grunwald Giemsa staining (Figure 3). We observed hypocellularity of fetal blood samples from *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCr*$^{+/-}$ embryos as compared to control fetal blood samples. In fact, a significant number of dead cells were observed in samples from double heterozygous blood samples. Moreover, we also observed immature erythroblasts in the latter samples and not in control samples (White arrows in Figure 3). In contrast to blood samples from wt or single transgenic embryos, yolk sac blood samples from *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCr*$^{+/-}$ animals did not show a uniform population of mature erythroblasts, but demonstrated severe forms of anisocytosis (dark arrows in Figure 3), characteristically associated with severe anemia.

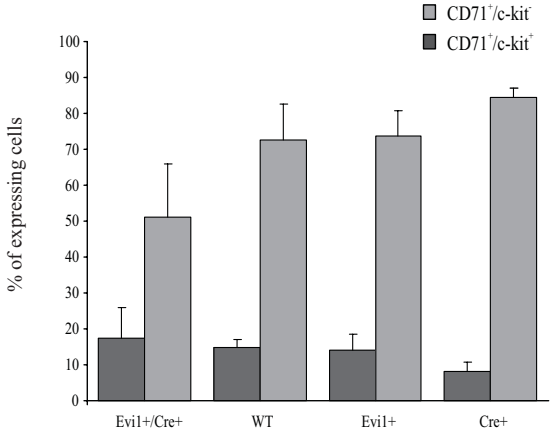

Figure 4 Percentage of immature cell population in E12.5 fetal liver
Flow cytometric analysis of CD71^{+}/c-kit^{+} and CD71^{+}/c-kit^{-} cells from fetal liver

Dysplasia, block of differentiation, and increased cell death of fetal liver erythropoiesis in double heterozygous embryos

Dysplasia is characterized by asynchronous maturation of the nucleus and cytoplasm and has previously also been described for *Vav-LSL-Evi1*$^{+/-}$*/ pEV-Cre*$^{+/-}$ animals. [14] Analysis of the preparations revealed a significant increase of the percentage of dysplastic cells in double heterozygous fetal livers (data not shown). However, immunostaining of the fetal liver cells using c-kit/CD71 did not revealed a significant increase of immature c-kit^{+}/CD71^{-} or c-kit^{+}/CD71^{+} cells (Figure 4). These data suggest that there is not a severe block and accumulation of very immature erythroid precursor cells.

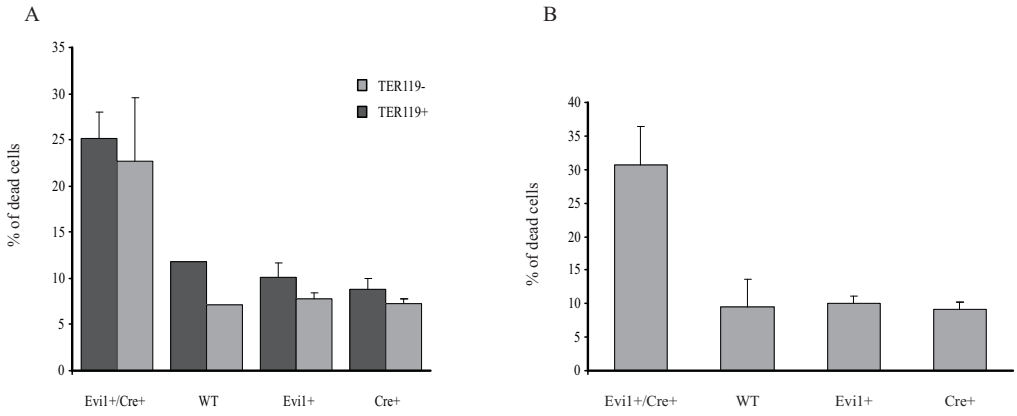

Figure 5 Significant increase of dead cells in ErGFPCr$^{+/-}$/Vav-LSL-Evi1$^{+/-}$ embryos
(A) Flowcytometric analysis of TER119^{+} or TER119^{-} cell population stained with 7Amino-Actinomycin D (7AAD). (B) Total cell death analyzed using Annexin V and PI

Since morphological and immunological analysis did not reveal a severe block or accumulation of earlier precursors, whereas macroscopic analysis showed clear interference of erythroid development (Figure 2), we decided to study survival of fetal liver cells. Fetal liver cells in one experiment were stained Ter119 and exposed to 7AAD. We observed extremely high percentages of dead cells within Vav-LSL-Evi1$^{+/-}$/ErGFPCr$^{+/-}$ fetal livers as compared to any of the control samples (Figure 5A). This increase was independent of the presence of TER119, suggesting that cell death was not only observed in the mature fraction. In a separate experiment we also analyzed whether cell death was due to apoptosis, by performing Annexin V and 7AAD staining (Figure 5B). Again a significant increase of 7AAD stained cells was observed. However, no increase in Anexin V^{+}/7AAD^{-} cells was found, suggesting that cells might not die as result of apoptosis.

Evi1 interferes and blocks terminal differentiation of erythrocytes

To analyze in more detail the ability of fetal liver cells to differentiate *in vitro*, we induced terminal erythroid differentiation in hanging drop cultures. Cells were analyzed by flow cytrometry, using the CD71 and Ter119 markers, prior and after two days of culture. In control cultures at day 0 the majority of the cells (approximately 80%) express both CD71 and TER119 markers and very few CD71^{-} TER119^{+} cells are present. After two days in culture about 30-40% of the control cells were CD71^{-} TER119^{+} , indicating that terminal erythroid differentiation has occurred (Figure 6). It is clear already by day 0 that double transgenic erythroid progenitors

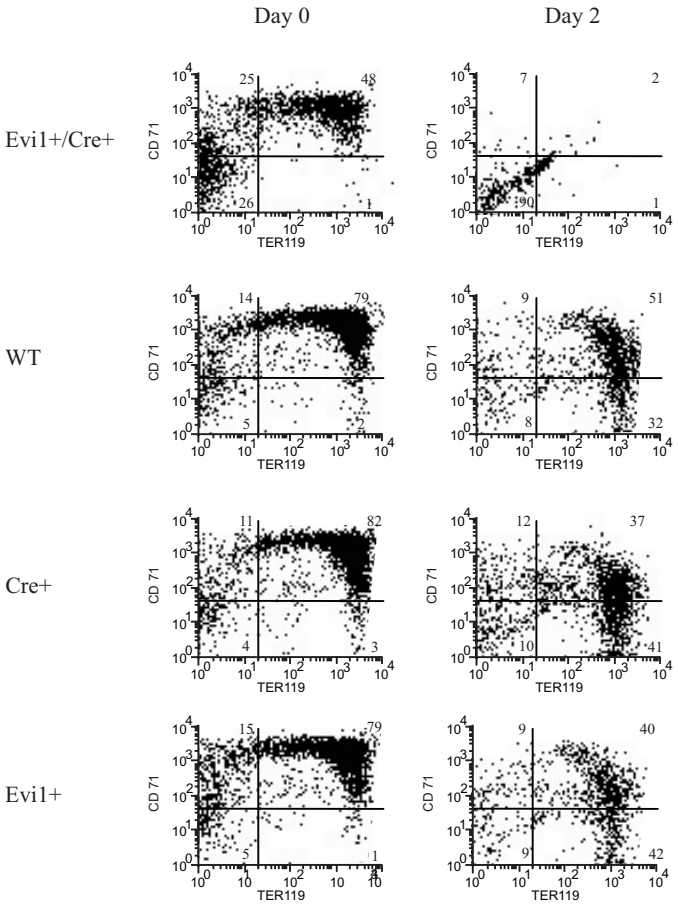

Figure 6 Decrease of mature erythroid cells in ErGFPCr$^{+/-}$/Vav-LSL-Evi1$^{+/-}$ fetal livers
Fetal liver cells were double stained with CD71^{+} and TER119^{+} at day 0 and after two days in hanging drop culture. Cells were analyzed by flow cytometry and the numbers depict the percentage of each population.

showed an increased block in differentiation since the CD71^{+} Ter119^{+} cell population is reduced.

Thus the normal down regulation of CD71 expression, reflecting normal differentiation, is not evident. After two days of differentiation the vast majority of these cells in double transgenic animals were dead and the remaining ones that survived were most likely not erythroid since they did not express CD71 or Ter119 (Figure 6). Taken together these results suggest that cells expressing Evi1 at early stages of erythroid differentiation can survive but expression in later stages is lethal.

E12.5 *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCr* fetal livers contain reduced number of BFU-E and CFU-E

To determine the number of BFU-E (burst forming unit-erythroid) and CFU-E (colony forming unit-erythroid) progenitors in E12.5 double transgenic livers, we performed colony assays. Fetal liver cells were grown in semi-solid medium in presence of Epo, or Epo and SCF, to determine the number of CFU-E and BFU-E, respectively, per liver. E12.5 *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCr*$^{+/-}$ fetal livers contained significantly lower numbers of BFU-E and CFU-E than livers obtained from single transgenic or WT littermates (Figure 7).

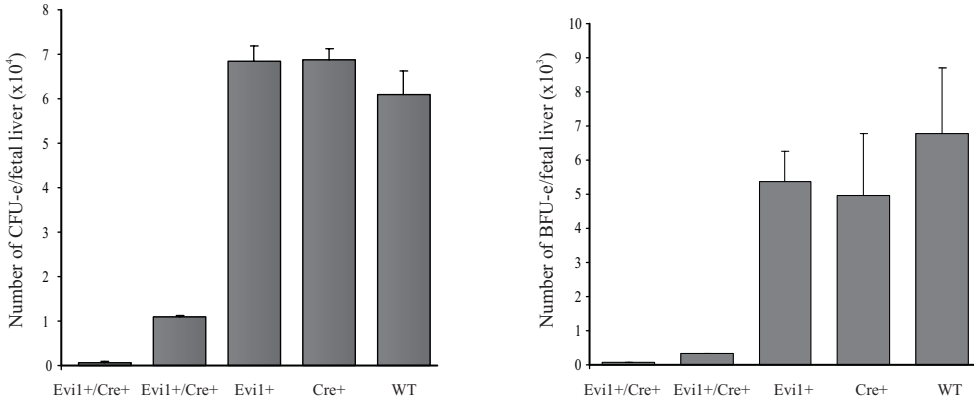

Figure 7 BFU-E and CFU-E colony assay on E12.5 fetal liver cells
$5x10^4$ single cell suspension cells isolated from fetal livers were plated in BFU-e or CFU-e methylcellulose medium. Colonies were counted on day 2 for CFU-e and day 8 for BFU-e. All experiments were performed in duplicate.

Discussion

We previously established novel *Evi1* transgenic mouse lines with conditional expression in the hematopoietic system and showed that Evi1 interfered with fetal erythroid development when the mice were bred with pEV-Cre animals, in which the Cre gene was under the control of the β-globin–LCR. Here we demonstrate that *Vav-LSL-Evi1* mice when crossed with animals expressing Cre under the control of the Epo-R promoter display a more severe erythroid dysplasia in E12.5 embryos, which is most likely explained by an earlier onset of the Evi1 gene in those double transgenic embryos. We observed a reduction in the number of CFU-E as well as BFU-E erythroid progenitors in *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCr*$^{+/-}$ fetal livers. This suggests a direct interference of Evi1 with more primitive erythroid progenitors than we previously reported in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ embryos, in which an effect on CFU-E but

not BFU-E progenitors was observed. These data together with the severe effects observed on primitive hematopoieisis in the yolk sac (Figures 3), which appeared to be less pronounced in *Vav-LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$, suggest that the animals reported here have a much more severe phenotype. Since only few neonates have been analyzed so far, it is currently unclear whether *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCr*$^{+/}$mice will be born alive. Given the fact that the phenotype of the *Vav-LSL-Evi1*$^{+/-}$*/ErGFPCr*$^{+/-}$embryos appears more severe than those of Vav-*LSL-Evi1*$^{+/-}$*/pEV-Cre*$^{+/-}$ embryos, double transgenic animals may not be born. In case newborn double transgenic animals will be born, they would provide an interesting model to study the effects of Evi1 on adult erythropoieisis in the context of dysplasia, which is frequently found in AML or MDS with high EVI1 expression.

Our studies suggest that the effects of Evi1 are not simply explained by interference of a differentiation program. In fact it appears that high Evi1 expression is detrimental for the erythroid cells, independent of the stage of differentiation. In a way this effect is very similar to what Buonamici et al.[13] have described following transduction of Evi1 into bone marrow precursors and subsequent transplantation. These data have been interpreted as an erythroid defect corresponding to the erythroid defects observed in myelodysplastic syndrome. Here we show, based on the Annexin V/7AAD staining, that the massive death of erythroid cells may not be explained by apoptosis. Although this might be true, we assume that it is still possible that the timing of the analysis may affect this conclusion and that in case we analyze the data at an earlier time, e.g. E11.5 or E12, we may find that apoptosis is the major cause of cell death.

Most data available from *in vivo* studies have revealed that aberrant expression of Evi1 has unfavorable effects on the cells it is expressed in. This would mean that to become a transforming gene for hematopoietic cells additional transforming events are required, which prohibit the "death promoting" effects of Evi1. The development of strategies to directly target oncoproteins has been one of the most attractive and promising approaches of cancer research in the last decade. The fact that EVI1, which may be highly expressed in certain AML patients, can be so damaging to cells suggests that to eradicate EVI1 expressing tumor cells, one should not target the function of Evi1, but rather interfere with pathways that allow Evi1 to be present in those cells. Targeting genes or products thereof that cooperate with Evi1 in transformation, may release the "suicidal" effects of Evi1 and thereby lead to destruction of tumor cells by aberrant expression of EVI1.

Materials and Methods

Collection of embryos and fetal livers from mutant mice

The generation of *Vav-LSL-FlagEvi1*$^{+/-}$ or *Vav-LSL-GFPEvi1*$^{+/-}$ mice has previously been described [14]. Both transgenic line showed similar phenotype and have been used in this study and will further be designated *Vav-LSL-Evi1*. The animals were crossed with *ErGFPCre* knock in mice [15] (kindly donated by Dr. Klingmuller, Freiburg, Germany) and embryos were dissected at E12.5. Fetal blood was collected with PBS / 5mM EDTA. Single cell suspensions of fetal livers were made by pipetting in PBS / 0.5% BSA. Following disaggregation of the fetal liver, the total amount of cells was determined by counting cell suspensions with a CASY1 electronic cell counter (Schärfe-System, Reutlingen, Germany).

Genotype analysis

DNA isolated from embryonic heads or livers and adult tails was used for genotype analysis. Genotyping was done by PCR using the following primer sets: *Vav-LSL-GFPEvi1*: 5'-GATCACATGGTCCTGCT-3' and 5'-CAGTTTTCACACTCATAGTGC-3', *ErGFPCre*: 5'-CAGCACCTGGGCCAGCTCAAC-3' and 5'- TGGCAGAAGGGGCAGCCACAC-3'. PCR conditions for all reactions were 1' 94°, 1' 55°, 1' 72°C (36 cycles).

Southern blot was performed by digesting genomic DNA with *Eco*RV and hybridizing with EGFP fragment derived from C_3EGFP (Clontech, Palo Alto, CA) using *Eco*47III and *Xho*I restriction enzymes.

Detection of in vivo recombination

PCR on genomic DNA isolated from fetal livers was applied to detect the deletion of the transcriptional stop sequence. The following primer sets were used for this PCR: 5'-GGAAGTGGTGTTGTAGTTGTC-3' and 5'-CCTTGAAGAAGATGGTGCG-3' for *Vav-LSL-GFPEvi1* mice. PCR was performed under the following conditions: 1' 94°, 1' 55°, 3' 72°C (38 cycles).

Colony assays

Fetal liver cells were assayed for their content of CFU-E and BFU-E by in vitro colony formation in semisolid methylcellulose culture medium. Cells were plated in duplicate in 35-mm Petri dishes (BD, Aalst, Belgium) at a concentration of $5x10^4$cells per dish. The components included

in the methylcellulose cultures are described in de Jong et al.[16] Cultures were maintained in a humidified atmosphere of 5% CO_2 in air. Colonies were counted at day 2 (CFU-E) and day 8 (BFU-E).

Hanging drop cultures.

Hanging drop cultures were performed as described previously. [17] In short, cells were spun down and resuspended in HD medium (DMEM supplemented with 20% FCS, 0.1% β-mercaptoethanol, $2x10^{-4}$ M Hemin, 1% penicillin/streptomycin, 2U/ml Epo, 5μg/mL insulin). 20 μL drops containing approximately $2.5x10^4$ cells each were pipetted on the inner side of the lid of a tissue culture Petri dish. After distribution of the drops, the lid was inverted and placed on the Petri dish, which contained PBS to humidify the culture chamber. Cultures were harvested after 2 days by washing the drops with PBS supplemented with 0.5% heat inactivated FCS.

Flow cytometry

Single cell suspensions of fetal liver cells were incubated with PE-conjugated anti-mouse TER119, FITC-conjugated CD71 or PE conjugated c-kit (BD) to determine the differentiation status. Dead cells were excluded from analysis by staining with 7-aminoactinomycin-D (7AAD; Invitrogen). Identification of apoptotic cells was performed using single cell suspensions and stained with AnnexinV-FITC and Propidium iodide (PI, BD).

Cytology and differential counting

Cytospins of freshly isolated fetal blood or fetal liver cells were stained with May-Grünwald Giemsa and double-blindly analyzed. A differential count was performed on 200 or 300 nucleated cells per slide and the percentage of dysplastic erythroblasts was obtained. Dysplasia was characterized as deformation of the nucleus, megaloblastic changes or asynchronous maturation of the cytoplasm and the nucleus.

Acknowledgements

We thank Dr. J.M. Adams (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) for the *Vav-hCD4* vector, and Dr U. Klingmuller (Max-Planck-Institute, Freiburg, Germany) for the ErGFPCr animals. Supported by the Dutch Organization for Scientific Research (NWO), the Dutch Cancer Society (KWF), and fellowship 2002/06 awarded to E.A. by the José Carreras foundation and the European Hematology Association.

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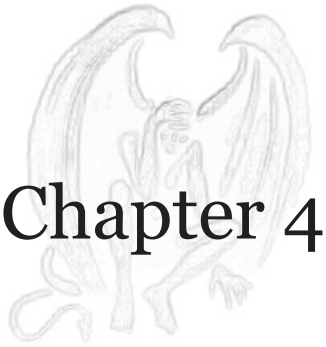

Chapter 4

The myeloid transforming protein Evi1 represses transcription via interaction with Methyl-CpG Binding Domain protein 3b, a member of the NuRD repressor complex.

Submitted for publication

The myeloid transforming protein Evi1 represses transcription via interaction with Methyl-CpG Binding Domain protein 3b, a member of the NuRD repressor complex.

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Abstract

Ecotropic virus integration site 1 (*EVI1*) gene is expressed in acute myeloid leukemias (AML) or myelodysplastic syndrome (MDS) patients with chromosomal abnormalities involving 3q26 locus, where the gene resides. In this study we investigated by means of protein interaction screens, which proteins interact with Evi1 and are critical for transcriptional repression by Evi1. Using yeast two-hybrid screen we have identified a novel binding partner of Evi1, the methyl binding domain 3b (Mbd3b) protein, a member of the histone deacetylase complex NuRD. Mbd3b binds Evi1 through the methyl binding domain (MBD) and the adjacent downstream 40 amino acids. Biochemical analysis demonstrated that both proteins interact *in vitro* and *in vivo*. Evi1 and Mbd3b co-localized in subcellular speckles in the nucleus. Immunoprecipitation experiments and GST pull down assays showed that the first three zinc fingers of Evi1 are required for interaction with Mbd3b. Moreover, we demonstrate that Evi1 was capable of interacting with endogenously expressed Mbd3b. Using a reporter assay we demonstrated that Evi1 enhances the transcriptional repression of Mbd3b, in a dose dependent manner. The interaction between Evi1 with Mbd3b may be important for epigenetic chromatin modification and leukemic transformation.

Introduction

The ecotropic integration site 1 (*EVI1*) oncogene has been well recognized to be associated with human and murine myeloid tumors. In humans, *EVI1* is found to be overexpressed in AML patients with translocations or inversions involving chromosome 3, e.g. t(3;3)(q21;26) or inv(3)(q21;q26), the locus where the gene resides [1,2]. Recently, it has been shown that aberrant *EVI1* mRNA expression also occurs in patients without chromosome 3 karyotypic abnormalities [3]. The mechanism of transformation as a result of high *EVI1* expression in both cases is currently

unknown. *Evi1* was initially identied in the mouse as a common proviral integration site in virus-induced leukemias, [4] and overexpression of *Evi1* in mouse bone marrow revealed a block of differentiation in myeloid precursors cells [5]. Enforced expression of *Evi1* in the red blood cell lineage impairs erythroid differentiation [6], whereas in megakaryocytic cells the gene appears to enhance differentiation [7]. An *in vivo* model of *Evi1* expressed from the murine *Sca-1* promoter showed a reduced number of colony forming units in the erythroid lineage (CFU-E) in the bone marrow [8]. More recently, bone marrow cells transduced with *EVI1* and transplanted into recipient mice showed development of fatal myelodysplastic syndromes after a latency of 10 months [9]. Although these *in vitro* and *in vivo* findings are in agreement with the clinical manifestations of malignant disease with high *EVI1* expression, the mechanisms by which EVI1 evokes these distinct effects are largely unknown.

Evi1 encodes a nuclear protein of 145kDa with domains characteristic of transcriptional regulatory proteins. It has 10 Cys_2His_2 type zinc fingers of which 7 are at the N-terminal and 3 at the C-terminal part of the protein. A proline rich region separates the two zinc finger domains and a small acidic domain is located C-terminal of the second zinc finger domain [10]. Both zinc finger domains are able to bind specific DNA sequences in vitro: the N-terminal zinc finger domain recognizes the nucleotide sequence (GACAA) GACAAGATAA [11-13] and the C-terminal domain is capable of binding to GAAGATGAG [14]. Although the recognition sequence of the N-terminal zinc finger domain exhibits similarity to GATA1 binding sequences and it has been proposed that Evi1 may interfere with GATA1 binding sites to deregulate its targets [6], binding of Evi1 to GATA1 specific promoter sequences has not been demonstrated.

In recent years it has been demonstrated that Evi1 can bind to other proteins, and may thereby execute its capacity to transform cells. For instance, Smad3 interaction with the first zinc finger domain of Evi1 disturbs the TGFβ pathway, which is known to be a negative regulator for cellular growth and differentiation [15]. Others reported that the repression activity of Evi1 may be due to interaction with the co-repressor carboxyl-terminal binding protein (CtBP) [16,17], and with histone deacetylases, HDAC1 and HDAC2 [18]. These latter data suggest that the transformation by Evi1 is associated with transcriptional repression of crucial target genes, for which interaction with transcriptional repression complexes is essential. We identified a novel Evi1 interacting protein, methyl-CpG-binding domain protein Mbd3b, a member of the Mi2/NuRD transcriptional repressor complex. We investigated the interaction of Evi1 and Mbd3b and the role of Evi1 within this complex. Evi1 interaction appears highly specific for Mbd3b, as other members of MBD motif containing family do not interact with Evi1. We show that the interaction recruits

Mi2/NuRD into the transcriptional repression complex, via Mbd3b, by which Evi1 may exert, its function in transcriptional repression.

Results

Evi1 frequently interacts with Methyl-CpG Binding Domain Protein 3b (Mbd3b) in a yeast-two-hybrid protein interaction assay

To identify potential novel binding partner(s) of Evi1, we carried out a Gal4 based yeast-two hybrid protein interaction screen. PJ69-4A yeast cells containing the Gal4-BD-*Evi1* fusion-gene were transformed with a mouse 17-day embryo cDNA library, in which the cDNA transcripts were cloned in frame with the GAL-4 transactivation domain. Of the approximately $1x10^7$ transformed cells, 124 clones survived selection on medium lacking Trp, Leu, His, and Ade, which were further analyzed for β-galactosidase activity using the X-gal plate assay. This analysis revealed 25 positive clones, which were further confirmed in the β-galactosidase liquid culture assay (data not shown). These positive clones were sequenced and 6 encoded for a Methyl-CpG Binding Domain Protein 3 isoform b (Mbd3b), a member of the Mi-2/NuRD transcriptional repressor complex. Interaction between Evi1 and Mbd3b was confirmed on plates lacking Trp, Leu, His, and Ade (Fig. 1A). β-galactosidase reporter gene activation by Mbd3b in the presence of Evi1 appeared more than 5 fold higher as compared to what observed with Mbd3b alone (Fig. 1B).

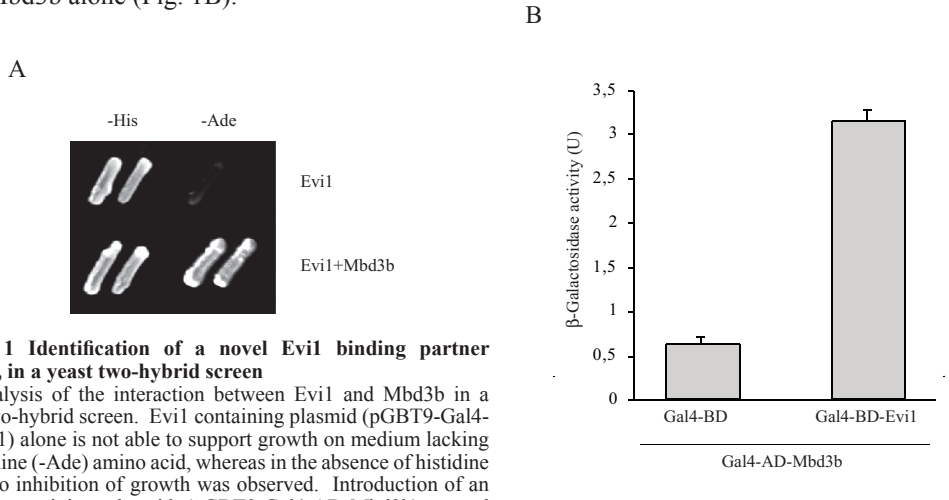


Figure 1 Identification of a novel Evi1 binding partner Mbd3b, in a yeast two-hybrid screen
(A) Analysis of the interaction between Evi1 and Mbd3b in a yeast two-hybrid screen. Evi1 containing plasmid (pGBT9-Gal4-BD-Evi1) alone is not able to support growth on medium lacking the adenine (-Ade) amino acid, whereas in the absence of histidine (-His) no inhibition of growth was observed. Introduction of an Mbd3b containing plasmid (pGBT9-Gal4-AD-Mbd3b) caused Ade transactivation due to the interaction between Evi1 and Mbd3b and allows yeast to grow. (B) pGBT9-Gal4-AD-Mbd3b was assayed in liquid culture to examine β-galactosidase activity either in the presence of pGBT9-Gal4-BD-Evi1 or an empty vector pGBT9-Gal4-BD. Increased β-galactosidase activity demonstrates the interaction between Mbd3b and Evi1.

A 40 amino acid region adjacent to the methyl CpG binding domain of Mbd3b is required for Evi1 interaction

Mbd3b has a methyl CpG binding domain (MBD) at the N-terminus, and a coiled-coil (C-C) as well as a glutamic acid repeat (EE) domain at the C-terminal part of the protein (Fig. 2A). To determine the critical regions for Evi1 interaction, we performed yeast-two-hybrid analysis using *Mbd3b* mutants and full length *Evi1*.

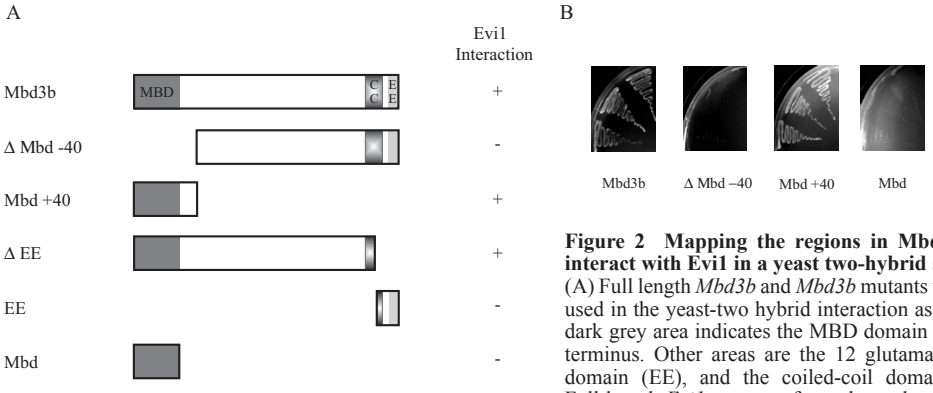


Figure 2 Mapping the regions in Mbd3b that interact with Evi1 in a yeast two-hybrid analysis (A) Full length *Mbd3b* and *Mbd3b* mutants that were used in the yeast-two hybrid interaction assay. The dark grey area indicates the MBD domain at the N-terminus. Other areas are the 12 glutamate repeat domain (EE), and the coiled-coil domain (CC). Full length *Evi1* was transformed together with full length *Mbd3b* or its mutants. (B) Yeast growth on selective plates lacking tryptophan (Trp), leucine (Leu), histidine (His), and adenine (Ade) shows selective growth of Mbd3b and MBD+40.

The EE domain was neither required nor sufficient to bind Evi1. In contrast, deletion of the MBD+40 amino acids domain abolishes Evi1 association (ΔMBD+40), while the MBD+40 domain alone is able to associate with Evi1. Notably, the 40 amino acids adjacent to the MBD domain are required to bind Evi1 (Fig. 2B).

Evi1 and Mbd3b colocalize and interact in mammalian cells

To study whether Evi1 and Mbd3b colocalize, Phoenix cells (φE) were transiently transfected with Flag-*Evi1* and HA-*Mbd3b*. Cells were stained with specific anti-Flag and anti-HA antibodies followed by secondary FITC and TRITC staining respectively. By means of confocal microscopy we observed that Evi1 was expressed in a speckled pattern in the nucleus, as described previously [26]. Moreover, HA-Mbd3b showed a similar staining pattern and merging of both images revealed

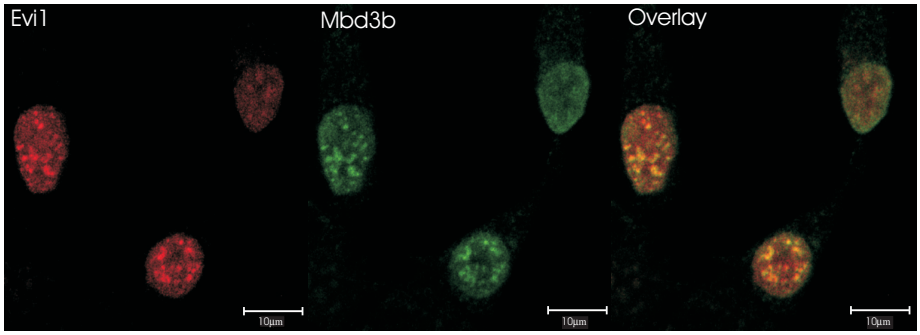


Figure 3 Evi1 and Mbd3b colocalize in the nucleus
Confocal microscopy of φE cells transiently transfected with Flag-*Evi* and HA-*Mbd3b*. Flag-Evi was stained with anti-Flag antibody followed by secondary TRITC staining (Red). HA-Mbd3 was stained with anti-HA antibody followed by secondary FITC staining (Green). Both proteins form nuclear speckles and the merged slide shows co-localization between the two proteins (Yellow).

the presence of Evi1 within several of the Mbd3b containing subnuclear speckles (Fig. 3). Immunoprecipitation of HA-Mbd3b using anti-HA in lysates from HA-*Mbd3b*/Flag-*Evi1* co-infected φE cells revealed the co-precipitation of Flag-Evi1 as determined by Western blot analysis using an anti-Flag antibody (Fig. 4A). In addition, HA-Mbd3b could be co-precipitated from the same lysates when an anti-Flag antibody was used to immuno-precipitate Flag-Evi1 (Fig. 4A). These results demonstrate the interaction between Mbd3b and Evi1 in mammalian cells.

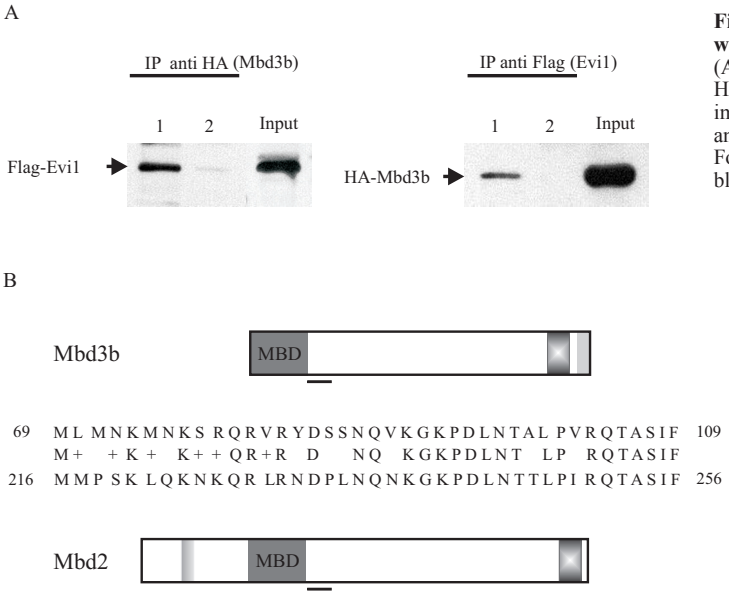
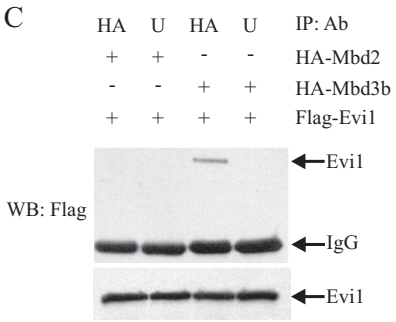


Figure 4 Evi1 physically interacts with Mbd3b in mammalian cells
(A) Co-expressed Flag-Evi1 and HA-Mbd3b in φE cells were immunoprecipitated with either anti-Flag or anti-HA antibody. Following PAGE and Western blotting, the immunoprecipitate with HA antibody was stained with anti-Flag antibody to analyze the presence of Evi1 (*left blot*). In the parallel experiment anti-HA antibody was used to study the presence of Mbd3b following anti-Flag immunoprecipitation (*right blot*). As a control (lane 2 in both experiments) lysates were exposed to unspecific antibody and beads. (B) Mbd3b binding region alignment with Mbd2 amino acid sequence. The interacting 40 amino acids of Mbd3b (position 69



to 109) show 65% identity with Mbd2 sequence (position 216 to 256) and 82% similarity (underlined region). (C) Immunoprecipitation of Evi1 from φE cells that were transiently co-transfected with Flag-*Evi1* and either HA-*Mbd2* or HA-*Mbd3*. Proteins were immunoprecipitated with anti-HA antibody or unspecific antibody (U). Western blot detection was performed using anti-Flag antibody. Bottom figure shows Western blot to demostrate Evi1 expression in all samples.

Evi1 does not interact with other MBD family members or with Mbd3a

The high homology within the MBD family members provoked us to investigate the possibility of Evi1 interaction with other members. Whereas, the homology between Mbd3b and Mbd1, Mbd4 or MeCP2 is restricted to the MBD domains only, Mbd2 shows homology with Mbd3b outside this region as well [27].

Particularly, the 40 amino acids region required for interaction between Mbd3b and Evi1 shows high homology (65%) with a comparable region in Mbd2 (Fig. 4B). Although co-precipitated with HA-Mbd3b using an anti-HA antibody, no Evi1 was observed in a co-immunoprecipitation experiment in Flag-*Evi1*/HA-*Mbd2* transfected cells (Fig. 4C). Moreover, no interaction has been observed between Evi1 and Mbd1, Mbd4 or MeCP2 using transfected φE cells (data not shown). Two splice forms of Mbd3 have been identified, i.e. Mbd3a and Mbd3b. The Mbd3a splice variant contains an additional 30 amino acids within the MBD domain. Interestingly, no interaction was observed between Mbd3a and Evi1 in a immunoprecipitation experiment using co-transfected φE cells (data not shown), indicating that the Mbd3b-specific MBD domain is required for Evi1 interaction.

The first three zinc fingers of the N-terminal zinc finger domain of Evi1 are required for Mbd3b interaction

We next investigated which domains of Evi1 are required for interaction with Mbd3b. HA-*Mbd3b* was co-transfected with Flag-*Evi1* or Flag-*Evi1* mutants lacking either zinc finger domain 1 (ΔZF1-7) or the second zinc finger domain (ΔZF8-10) (Fig. 5A). Immunoprecipitation was performed with an anti-HA antibody against HA-Mbd3b and the Western blot analysis was carried out with anti-Flag epitope antibody to detect Evi1. Full length Evi1 and the Evi1

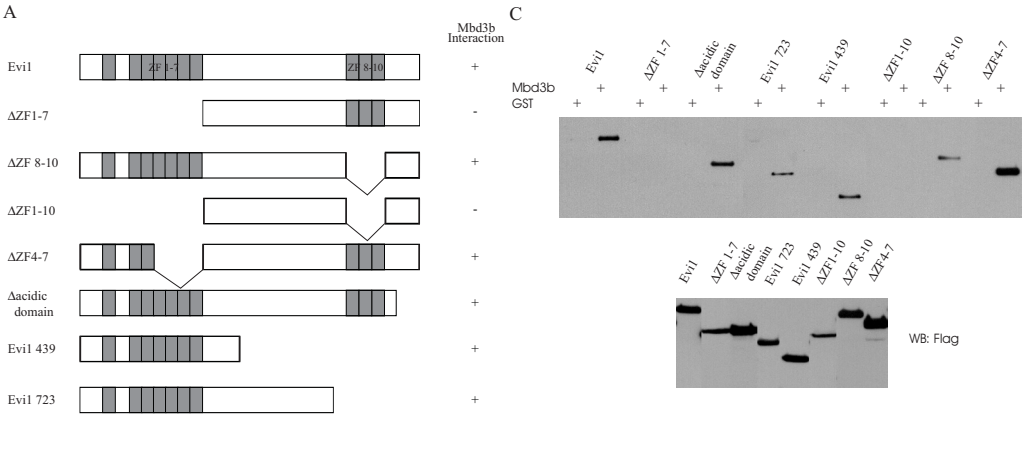


Figure 5 N-terminal zinc finger domain of Evi1 interacts with Mbd3b
(A) Diagram of *Evi1* full coding sequence and its mutants. All *Evi1* constructs were fused in frame with single Flag epitope. Interaction with Mbd3b is indicated (+). (B) φE cells were transiently transfected with HA-*Mbd3b* and constructs of full length or mutant *Evi* (Flag-tagged). Total cell lysates were immunoprecipitated (IP) with anti-HA antibody and the proteins were analyzed by Western blot with anti-Flag antibody to detect Flag-Evi1. (C) Mapping of the interaction domain of Evi1 using GST-Mbd3 in a pull-down assay. The *Evi1* or mutants were transiently expressed in φE cells. Cells were lysed and whole protein extracts were incubated with purified GST-Mbd3b or GST only coupled to sepharose beads. Protein complexes were separated by electrophoresis, and stained with Flag antibody to detect Flag-Evi1. Input levels of wt or mutants of Flag-Evi1 are shown in the lower panel of Figure 5C.

mutant lacking the second zinc finger domain did bind to HA-Mbd3b, however, the Evi1 mutant lacking the first seven zinc fingers (ΔZF1-7) did not interact with HA-Mbd3b (Fig. 5B). These results were confirmed using GST-Mbd3b pull-down experiments. Flag-*Evi1* mutants (Fig. 5A) were expressed in mammalian φE cells and lysates were exposed to purified GST-Mbd3b protein bound to glutathion-beads. Western blot analysis of captured proteins from the lysates revealed that Flag-*Evi1* mutants lacking the first 7 zinc fingers (mutants ΔZF1-7 and ΔZF1-10) did not bind to Mbd3b. Mutants of Flag-*Evi1* containing the ZF1-7 domain were all capable of binding to Mbd3b. A mutant that lacked four zinc fingers in the first domain (ΔZF4-7) showed strong Mbd3b interaction, indicating that the first three zinc fingers are sufficient for protein interaction.

Interaction between Evi1 and endogenous Mbd3b

Next, we studied whether transiently overexpressed HA-Evi1 in φE cells could interact with endogenous Mbd3b. HA-Evi1 immunoprecipitation with anti-HA antibody and subsequent staining with a specific antibody against Mbd3 showed a specific band of Mbd3b (Fig. 6A).

Figure 6 Evi1 interacts with endogenous Mbd3b
(A) φE cells transiently transfected with HA-*Evi1* were lysed and incubated with anti-HA antibody (HA) or sepharose G-protein beads (Control sample). Immunoprecipitates (IP) were separated by electrophoresis and stained with anti-MBD3 antibody. Endogenous MBD3b band was specifically detected in the presence of HA-Evi1 but not in the control sample. Western blot (WB) stained with anti-Mbd3 confirms the presence of Mbd3b protein in both lysates. (B) Immunoprecipitation of Evi1 in lysates from the myeloid leukemia cell line NFS 56, with Evi1 specific antibody. *Upper panel*: Protein extract from NFS 56 cell line was incubated with Evi1 antiserum (+) or sepharose G-protein beads (-) and stained with anti-MBD3 antibody. Omission of Evi1 antiserum results in absence of Mbd3b band. Presence of endogenous Mbd3b in both lysates is confirmed by a Western blot (WB) analysis. *Lower panel*: The same Western blot was stained with Evi1 antiserum. Presence of Evi1 protein was confirmed in the sample with Evi1 antiserum. Total cell lysate was used to detect the presence of endogenous Evi1 in NFS 56 cell line.

No Mbd3b band was detected when the same sample was incubated with protein G-sepharose beads only. To study Evi1/Mbd3b binding under more physiological conditions, we decided to study this interaction in the retrovirally-induced myeloid leukemia cell line (NFS 56) [28], which harbours an Evi1 proviral integration. Due to this proviral integration within the *Evi1* locus the gene is overexpressed and the protein is detectable using Evi1-specific antibodies (Fig. 6B). Immunoprecipitation using specific Evi1 antibody and subsequent Western blot analysis using Mbd3-specific antibodies revealed the presence of low but significant levels of a protein with a size corresponding to that of Mbd3b (Fig. 6B).

Evi1 interacts with the Mi2/NuRD complex

Mbd3b is present in Mi2/NuRD, a transcriptional repressor complex with chromatin remodelling and histone deacetylase activity. The complex contains several components, of which RbAp46/p48, HDAC1 and HDAC2 form the core complex that is mutual between Mi-2/NuRD and the Sin3-histone deacetylase complex. Factors that are restricted to Mi-2/NuRD only are: MTA2, Mi-2, Mbd3a and Mbd3b [29]. To gain more insight in the involvement of Evi1 in the Mi-2/NuRD complex, we introduced Evi1 in 293T cells expressing stably TAP-tagged-Mbd3a. It has been demonstrated that the entire Mi-2/NuRD complex can be isolated from these cells using purification steps to isolate the TAP-tagged Mbd3a protein. We wondered whether Evi1, when coexpressed in TAP-tagged Mbd3a-expressing 293T cells, could be co-purified with the Mi-2/NuRD complex using the TAP-Mbd3a purification procedure. The cells were transiently

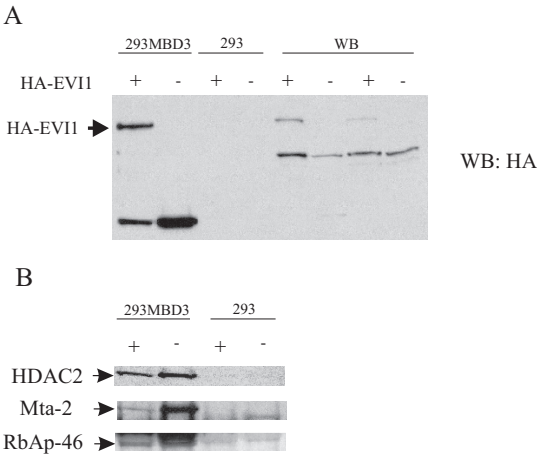


Figure 7 In vivo association of Mi2/ NuRD complex with Evi1 in 293T cell line
(A) 293T cells with or without stably expressed TAP-MBD3a were transfected with HA-*Evi1* or an empty vector. TAP-MBD3a was immunoprecipitated from the total cell lysate using sepharose IgG beads. Imunopreciptation complexes were stained for the presence of Evi1 (anti HA staining, upper blot). The arrow indicates HA-Evi1. For Western blot (WB) 5% from the total cell lysate was used as input. (B) Presence of Mi-2/NuRD proteins in IgG immunoprecipitation experiment was confirmed using specific antibodies against different components of the complex. The original blot was re-stained with anti-HDAC2, anti-MTA-2, and anti-RbAp-46 antibody.

transfected with HA-*Evi1* or with a mock expressing vector. As a control, wild type 293T cells were used using the same procedures. After purification we observed presence of Evi1 in TAP-Mbd3a samples but not in the control samples (Fig. 7a). The positive staining using antibodies directed against HDAC2, MTA-2 and Rbp-46 confirmed that we indeed purified the Mi-2/NuRD complex using this procedure (Fig. 7b). Since Evi1 does not directly associate with Mbd3a but with Mbd3b, we conclude that Evi1 physically interacts with Mbd3b in the context of Mi-2/ NuRD complex.

Evi1 enhances transcriptional repression by Mbd3b in luciferase assay

To investigate whether Evi1 is involved in transcriptional repression through Mbd3b interaction, we performed a luciferase reporter assays. The DNA binding domain of Gal4 was fused in frame to full length *Mbd3b* (Fig. 8A) and co-expressed with either full length *Evi1* or mutants of *Evi1* in φE cells. Expression of full length Evi1 decreases the luciferase activity in the presence of Gal4-Mbd3b in a dose dependent manner but not in the control experiments without Gal4-Mbd3b (Fig. 8B). Similar results were obtained using a deletion mutant lacking zinc finger 4 to 7 (ΔZF4-7). However, this repression was lost when all first seven zinc fingers of Evi1 (ΔZF1-7) were deleted. Interestingly, mutant Evi1 partially lacking the C-terminal binding protein 1 (CtBP1) interacting proline rich region (Δpro) lost its ability to repress luciferase activity. This is in agreement with previous reports showing that the interaction with the transcriptional repressor CtBP1 is also critical for transcriptional repression by Evi1. The observed effects were not due to squelching, since comparable levels of protein were detected in all samples by a Western blot (Fig. 8B).

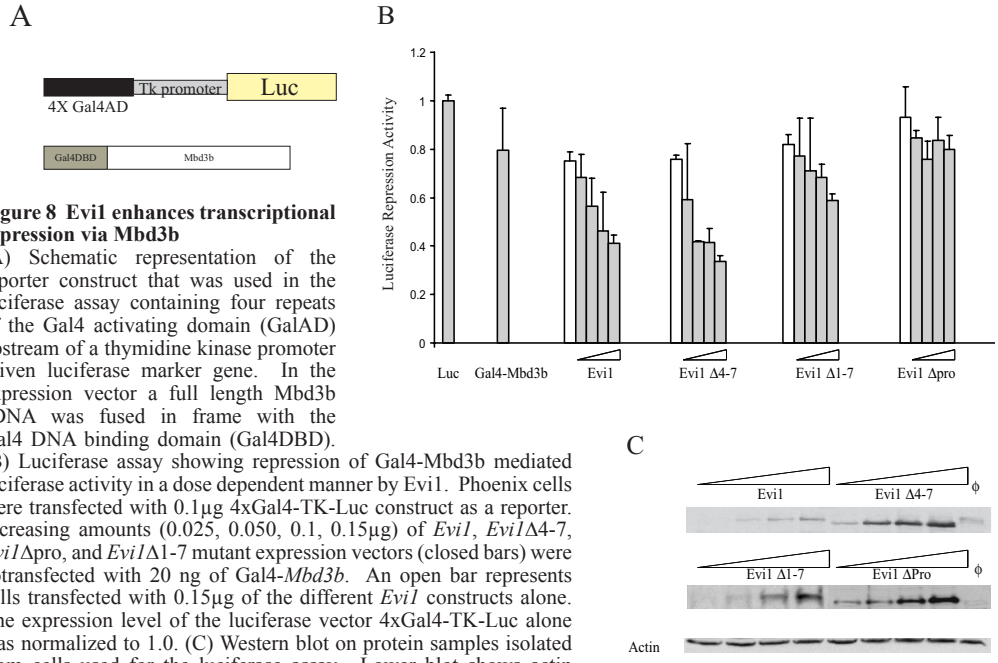


Figure 8 Evi1 enhances transcriptional repression via Mbd3b
(A) Schematic representation of the reporter construct that was used in the luciferase assay containing four repeats of the Gal4 activating domain (GalAD) upstream of a thymidine kinase promoter driven luciferase marker gene. In the expression vector a full length Mbd3b cDNA was fused in frame with the Gal4 DNA binding domain (Gal4DBD). (B) Luciferase assay showing repression of Gal4-Mbd3b mediated luciferase activity in a dose dependent manner by Evi1. Phoenix cells were transfected with 0.1µg 4xGal4-TK-Luc construct as a reporter. Increasing amounts (0.025, 0.050, 0.1, 0.15µg) of *Evi1*, *Evi1*Δ4-7, *Evi1*Δpro, and *Evi1*Δ1-7 mutant expression vectors (closed bars) were cotransfected with 20 ng of Gal4-*Mbd3b*. An open bar represents cells transfected with 0.15µg of the different *Evi1* constructs alone. The expression level of the luciferase vector 4xGal4-TK-Luc alone was normalized to 1.0. (C) Western blot on protein samples isolated from cells used for the luciferase assay. Lower blot shows actin staining for equal loading.

Discussion

Evi1 is a nuclear zinc finger protein involved in myeloid leukemia development, and acts as a transcriptional repressor [30-33]. Here we demonstrates a direct interaction between Evi1 and the methyl-binding domain 3 protein-b (Mbd3b), a member of the histone deacethylase transcriptional repressor complex Mi-2/NuRD [34,35]. Six independent Mbd3b expressing clones were identified as Evi1 binding partner in a yeast-two hybrid protein interaction screen. These observations and the fact that the interaction also occurred in mammalian cells suggest that our findings are relevant and that the Mi-2/NuRD repressor complex may be involved in the transcriptional repression frequently observed with Evi1. The specificity of the interaction between Evi1 and Mbd3b is demonstrated by the fact that Evi1 did not interact with Mbd3a or any of the other Mbd family members. The experiments showing that Mbd3b and Evi1 repress transcription in a luciferase reporter assay indicate that this interaction may occur *in vivo*. This conclusion is substantiated by the fact that transcriptional repression is dependent on domains that are also required for the interaction with Mbd3b. Since Evi1 interacts with endogenous

Mbd3b in φE cells and Evi1 transformed NFS56 cells, and is found to be associated with the Mi-2/NuRD complex via Mbd3b, we conclude that this complex may be of importance in Evi1 mediated leukemic transformation.

Mbd3 is a member of a larger family of proteins, which possesses a conserved methyl binding domain (MBD) [27]. This motif has been shown to recognize methylated CpG islands, however, Mbd3 appears to have lost this function during evolution [36,37]. Mbd3b is required for early development since Mbd3 null mice are embryonically lethal [38]. In contrast to Mbd3, other family members such as Mbd1, Mbd2 and MeCP2 repress transcription through direct interaction with methylated cytosines in CpG islands [39]. These proteins recruit repressor complexes towards particular sites in the genome and subsequently interfere with transcription. Since Mbd3 does not interact with methylated DNA through its MBD domain, it is currently unclear how Mbd3 may be involved in the transcriptional repression [36]. We demonstrate by confocal microscopy that Mbd3b colocalized with Evi1 within certain subnuclear foci. Although the exact nature of these speckles is not clear yet, it is possible that they represent loci where Evi1 interacts with DNA, as Evi1 recognizes and binds to specific nucleotide sequences via the zinc fingers 4-7 of domain 1 or the zinc fingers 8-10 of domain 2 [12,40,41] . The first three zinc fingers do not interact with DNA. Since we show here that particularly these fingers are critical for the interaction with Mbd3b, we may have identified a specific function of zinc fingers 1-3, which are highly conserved between mouse and man.

It is also possible that Mbd3b recruits Evi1 and thereby allows it to act as transcriptional repressor, possibly via the Mi-2/NuRD repression complex. In fact, the luciferase reporter experiments demonstrate recruitment of Evi1 to a site where Gal4-Mbd3b interacts with DNA, causing causes repression of transcription of the Tk-promoter driven luciferase gene. The repression activity was achieved in a dose-dependent manner as the Evi1 deletion mutant (ΔZF1-7) lost this activity, due to the inability to form a complex with Mbd3b. Although these experiments may be explained by a synergistic effect between Evi1 and Mbd3b, it is also possible that in this experiment Mbd3b mainly recruits Evi1 to the promoter sites and allows it to repress transcription. The repression potency of Evi1 was mapped earlier in the region between the two zinc finger domains, containing a specific sequence which interacts with CtBP, a repressor of transcription [31,32,42,43]. CtBP was shown to be a part of a multisubunit repressor complex that contains all the necessary proteins required for histone modifications [44]. Clearly, the repression by Evi1 was lost with the mutant Δpro (Figure 8), lacking this CtBP interacting region. Thus, recruitment of Evi1 to the Mi-2/NuRD complex through Mbd3b does not necessarily mean

that Evi1 should interact with DNA. It is of interest to note that MTA2 and RbAp46/48, other members of the Mi2/NuRD complex, contain DNA binding zinc fingers (GATA-like), presumably capable of interacting with specific nucleotide sequences or histones [34]. Moreover, several transcription factors with well-defined nucleotide recognition sites, such as Ikaros, Rorγ, and SATB1 have been demonstrated to interact with the Mi-2/NuRD [45-47]. Recently, it has been demonstrated that Gata1 interacts in erythroid cells via Fog1 with the NuRD complex [48,49]. Since aberrant Evi1 expression has been demonstrated to cause erythroid abnormalities [9,50], it will be of great interest to investigate whether the Evi1/NuRD interaction affects Gata1 or Fog1 function in erythroid precursors. Thus, although it is possible that Evi1 binds DNA and recruits repressors such as CtBP, HDACs or Mi-2/NuRD, it is also possible that Evi1 acts as an adaptor that attracts repressors such as CtBP or HDAC.

The role of Evi1 in malignant transformation and leukemia progression is well recognized and different *in vitro* and *in vivo* models have indicated a dominant role of Evi1 in leukemogenesis. One of the indisputable functions of Evi1 is its capability to repress transcription, by interaction with distinct factors [51]. In recent years it has become evident that transcriptional repressors are able to associate with multi-protein complexes that contain repressor subunits [52]. The interaction is not always direct, but frequently requires adaptor proteins [53] . Although the exact mechanism is not clear yet, our results add more evidence to the notion that Evi1 plays an important role in transcription inhibition by interacting with transcription repression complexes. For instance, it has been demonstrated that Evi1 may bind to Smad3 and block transcription initiated by this transcription factor [54] . Additional evidence showing a major function of Evi1 in repression of transcription comes from recent experiments demonstrating that Evi1 also interacts with several histone methyltransferases (unpublished observation D.S and R.D.). This would suggest, that Evi1 also interacts with proteins involved in modifications at the histone level, i.e. either histone deacetylation or histone methylation, thereby causing the creation of a repressive chromatin environment. We are currently analyzing the modifications that may take place at the histone level in the presence of Evi1. Insight into the exact interactions between Evi1 and these distinct repression complexes as well as the effects thereof will provide invaluable information required for the development of molecules that may interfere with these complexes.

Materials and Methods

Yeast Two-Hybrid protein interaction screen

Mouse *Evi1* cDNA was subcloned into the pGBT9 (Clontech, Palo Alto, CA) expression vector in frame with the Gal4-DNA binding domain (BD) to produce a fusion transcript. The construct was introduced by a standard polyethylene glycol/lithium acetate (PEG/LiAc) transformation method [19] into the yeast PJ69-4A strain (Kind gift from Dr. P.James, University of Wisconsin, Madison, WI). An expression library from mouse 17-day total embryo cDNA (Clontech, Palo Alto, CA) was then introduced into yeast and screened by selection on plates lacking tryptophan (Trp), leucine (Leu), histidine (His), and adenine (Ade), but containing 4mM of 3-aminotriazole [20]. Selected clones were further analyzed in liquid culture assay for β-galactosidase expression according to the manufacture's protocol (Clontech, Palo Alto, CA). Isolation of plasmid DNA from PJ69-4A strain was performed using DNA mini columns (Qiagen, Westburg B.V, NL). Positive clones were analyzed by restriction enzyme analysis and nucleotide sequencing (Applied Biosystems, Nieuwekerk aan den IJsel, NL) to determine the correct open reading frame with regard to the Gal4-activating domain (AD). The identity of the candidate Evi1 binding partners was determined by using public databases (NCBI).

Expression construct

Evi1 cDNA was fused in frame with FLAG (FLAG-*Evi1*) or HA (HA-*Evi1*) nucleotide sequences, inserted into the pCMV mammalian expression vector (Clontech, Palo Alto, CA) under the early cytomegalovirus promoter (CMV). All deletion mutants were generated from the original cDNA of FLAG-*Evi1* using available restriction sites (available upon request). Full length *Mbd3b* was subcloned from the pACT2 vector using *Bgl*II restriction sites and inserted into the *BamH*I site of the pCMV vector. *Mbd2* was amplified from mouse day 17 total embryonic cDNA library using *Pfu polymerase* (Promega, Madison, WI) and primers containing specific restriction sites. All inserts were cloned in frame with the HA epitope using pBluescript (Stratagene, Amsterdam, NL) followed by subcloning into the pCMV vector. All constructs were sequenced for correct in frame fusion and the expression of all proteins was confirmed by Western blot analysis.

Yeast two-hybrid plasmid

The *Mbd3b* mutant constructs were generated using available restriction sites within the pACT2 cloning vector. Deletion constructs of *Mbd3b* were generated using *Age*I and *Xho*I to generate a

Mbd3b mutant lacking the MBD domain and adjacent 40 amino acids (ΔMBD+40), *Bgl*II and *Age*I to generate a construct expressing the *Mbd3b* MBD domain and adjacent 40 amino acids (MBD+40), *Nhe*I and *Xho*I to generate a construct expressing the *Mbd3b* EE domain (EE) and *Bgl*II and *Nhe*I for a *Mbd3b* mutant lacking the EE domain (ΔEE). The *Mbd3b* MBD domain only was amplified using *Pfu polymerase* (Promega, Madison, WI) and cloned using *Eco*RI and *Xho*I sites.

The entire open reading frame of *Mbd2* was amplified using *Pfu polymerase* (Promega, Madison, WI) with primers containing either an *Eco*RI or an *Xho*I restriction site. Amplified fragments were cloned in frame with GAL4 AD (pACT2 vector) and fully sequenced.

GST fusion protein - For the preparation of a Mbd3b glutathione S-transferase (GST) fusion protein, *Mbd3b* was subcloned into the *Eco*RI site of pGEX-2Tk (Amersham Pharmacia Biotech).

Luciferase assay plasmids: For the luciferase assay experiment, we cloned full length *Mbd3b* into the pcDNA3.1 GAL4 vector (kindly donated by Dr. R. Deplus, Free University of Brussels, Belgium). In this vector the expression of the Gal4 DNA-binding domain (DBD) is driven by the CMV promoter. The reporter construct 4xGal4-TK-Luc (kindly donated by Dr. R.Deplus, Free University of Brussels, Belgium) contains four GAL4 duplicate binding sites upstream of the thymidine kinase promoter driving expression of the luciferase gene[21,22].

Immunoprecipitation and Western Blot analysis

Phoenix (φE) cells were maintained in Dubelco's modified Eagle medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% FCS. $2x10^6$ cells were seeded in $100mm^2$ dishes (Becton Dickinson, Franklin Lakes, NJ) and transfected with 20μg DNA using calcium phosphate co-precipitation [23].

Cells were harvested 48 hours post transfection, washed once with cold PBS and lyzed in Carin lysis buffer (20mM Tris pH 8.0, 137mM NaCl, 10mM EDTA, 100mM NaF, 1% NP-40, 10% Glycerol) on ice for 10min containing complete protease inhibitor mix and Pefablock (Roche, Zwijndrecht, NL). Cell debris was removed by centrifugation for 15 minutes at 13000rpm at 4^0C. The supernatant was incubated at 4^0C with an appropriate antibody (1μg) for 2hr, followed by overnight incubation with sepharose G-protein beads (Sigma-Aldrich, St.Louis, MO). Beads containing the bound proteins were collected, washed 4 times with cold lysis buffer and resuspended in 1X SDS Laemmli buffer [24]. Purified complexes were denatured, and separated on 8-10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel (SDS-PAGE), transferred

to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), blocked in 4% blocking milk (Bio-Rad, Hercules, CA) and incubated with the appropriate antibodies. Detection was performed using a secondary horseradish peroxidase-conjugated antibody (Dako Diagnostic BV, Denmark) and visualize by enhanced chemiluminescence according to the manufacturers protocol (ECL, Boston, MA). The following antibodies were used in this study: anti-HA and anti-actin (I-19)(Santa Cruz Biotechnology, Santa Cruz, CA), anti-Flag (Sigma-Aldrich, Zwijndrecht, NL), anti-EVI1 (a kind gift of Dr. J.N. Ihle, Memphis, USA), and anti-MBD3 (IBL, Naka, Japan).

GST pull down - Mbd3b was fused in frame with GST recombinant protein in pGEX-2TK vector and expressed in DH12 *Escherichia coli*. Cells were grown overnight at 37^0C, diluted to OD_{600} 0.2 and grown to OD_{600} 0.6 before induction with 1mM of IPTG for 3 hours at 37^0C. They were harvested at 4^0C. Cells were resuspended in TEND lysis buffer (1X TBS, 0.5% NP-40, 1mM EDTA, 1mM DTT) containing complete protease inhibitor mix and Pefablock, sonicated and centrifuged for 10 minutes at 13000rpm. Supernatant was incubated at 4^0C with Glutathione beads (Amersham Pharmacia Biotech), for 2hr at 4^0C, and washed extensively with TEND buffer. Purified GST-Mbd3 recombinant protein was incubated overnight at 4^0C with total cell lysates from different FLAG-EVI1 mutants expressed in φE cells. Glutathione beads were pelleted, washed 4 times in cold Carin lysis buffer and resupended in 1X SDS Laemmli buffer. Bound proteins were analyzed by SDS-PAGE.

Immunofluorescence microscopy

φE cells grown on glass cover slips were transfected with FLAG-*Evi1* and HA-*Mbd3b* using calcium phosphate coprecipitation method (Graham FL et al) and harvested 48 hours post transfection. The cells were washed once with ice cold PBS, and subjected to immunofluorescence staining as described previously [25]. In brief, cells were fixed for 15 min in 4% paraformaldehyde in PBS, and permeabilized with 0.2%Triton X-100 in PBS for 60 min at room temperature. Cells were washed once with 0.05% Tween-20 and blocked o/n at 4^0C in 1% BSA with 0.05% Tween-20 in PBS and stained with anti-FLAG and anti-HA MAbs for 2 hours at room temperature, washed extensively with 0.05% Tween-20 in PBS and detected with tetramethylrhodamine isothiocyanate (TRITC) and isothiocyanate (FITC) conjugated secondary antibodies (Dako Diagnostic BV, Denmark) for 1 hour at room temperature. After washing with 0.05% Tween-20 in PBS, cells were embedded in Vectashield (Vector Laboratories, Burlingame, CA). Stained cells were analyzed using a Zeiss confocal laser scanning microscope (LSM510).

Luciferase reporter assays

φE cells were plated on 24-well plates at $0.1x10^6$ cells/well in 1ml DMEM+10%FCS and cultured overnight, followed by transfection with 300ng 4xGAL-TK-Luc, 20ng pcDNA3-DBD-*Mbd3b*, 300ng pRSVLacZ and various concentration of the indicated expression plasmid. Final concentrations of 1µg plasmid were normalized using an empty pCMV expression vector. A volume of 100µl precipitate was added to each well. 48 hours post transfection cells were washed in PBS and lysed in 100µl lysis buffer (25mM Tris phosphate pH 7.8, 15% glycerol, 1% Triton X-100, 1mM DTT, 8mM $MgCl_2$). 25µl of cell lysates were transferred to 96-well flat bottom plates (Costar, Corning Inc, Corning, NY) and 25µl of a 16mg/ml luciferase substrate-containing buffer (Steady-Glo luciferase assay System, Promega) was added to each well. Light emission intensity was measured using a TopCount luminometer (Packard, Meriden, CT). In parallel, 25µl of cell lysate were incubated with 75µl β-galactosidase buffer containing 0.56mg/ml o-Nitrophenyl β-D-galacto pyranoside (ONPG, Sigma) for 5 minutes at 37^0C. Absorption was measured at 450nm in a microplate reader (BioRad 450, Veenendaal, NL). All assays were carried out in triplicates.

Purification of the NuRD complex using TAP-MBD3a

293T cell line stably expressing TAP (Tandem Affinity Purification)-tagged MBD3a. The cells were transiently transfected with HA-*Evi1* in pCMV mammalian expression vector. Cells were lysed in cold lysis buffer (20mM Tris pH 8.0, 137mM NaCl, 10mM EDTA, 100mM NaF, 1% NP-40, 10% Glycerol) and incubated with sepharose IgG beads for 2 hours at 4^0C. Sepharose beads were collected by short centrifugation and washed extensively in lysis buffer with increased salt concentration (20mM Tris pH 8.0, 400mM NaCl, 10mM EDTA, 100mM NaF, 1% NP-40, 10% Glycerol). Members of the complex were visualized using HDAC2 (Santa Cruz Biotechnology, Santa Cruz, CA), RbAp46 and MTA2 (Abcam, Cambridge, UK) antibodies.

Acknowledgements

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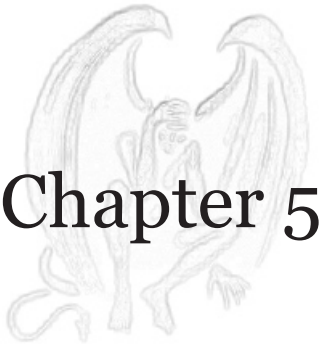

Chapter 5

Involvement of histone methyltransferase SUV39H1 in Evi1 proto-oncogene transcriptional repression.

Submitted for Publication

Involvement of histone methyltransferase SUV39H1 in Evi1 proto-oncogene transcriptional repression.

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Abstract

Deregulated expression of the zinc finger transcription factor ecotropic viral integration site 1 (EVI1) is associated with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) in patients due to chromosomal aberration of chromosome 3. However, its role in transcriptional regulation has remained elusive. Here, we show that Evi1 interacts with the histone methyltransferase SUV39H1. The direct interaction requires the N-terminal part of Evi1 and the H3-specific histone methyltransferase domain, SET, of SUV39H1. Although Evi1 does not affect SUV39H1 activity it may direct its activity to specific targets, as we show that the Evi1-SUV39H1 complex exhibits methyltransferase activity. Presence of SUV39H1 enhances Evi1 transcriptional repression in a dose dependent manner and ablation of the interacting SET domain of SUV39H1 abrogates this effect. Furthermore, we show histone H3/SUV39H1/Evi1 complex formation. In addition, we demonstrate that Evi1 also interacts with another histone methyltransferase G9a but not with SET9. These results establish Evi1 as a transcriptional regulator that is able to form higher order complexes with histone methyltransferases and may cause transcriptional repression of putative target genes during tumorgenesis.

Introduction

Ecotropic viral integration site 1 (Evi1) encodes for a zinc finger transcriptional regulator which has been identified as a common virus integration site in murine leukemia [1]. Moreover, aberrant expression of EVI1 has been shown in myeloproliferative disorders including myelodysplatic syndrome (MDS) and acute myeloid leukemia (AML), particularly in patients with translocations or inversions involving chromosome 3q26 [2]. AML patients with inappropriate expression of EVI1 belong to the high-risk group of AML patients, and current therapies are not adequate to treat the disease in those cases. In addition, *in vitro* and *in vivo* studies support the

notion that inappropriate expression of EVI1 plays a critical role in development of MDS and AML. Enforced overexpression of EVI1 blocks differentiation of myeloid progenitor (32Dcl3) cells towards mature neutrophils [3]. Furthermore, in the early erythroid lineage (32DEpo1) overepression of EVI1 diminishes erythropoietin sensitivity [4]. However, how EVI1 is involved in the process of transformation at the molecular level remains unclear.

EVI1 contains two DNA binding motifs. The N-terminal part of the protein possesses a domain containing 7 zinc fingers, which recognizes GACAAGATAA nucleotide sequences, whereas a region of 3 zinc fingers at C-terminus binds GAAGATGAG sequence [5-7]. One of the possible mechanisms to explain the oncogenic effect of Evi1 is the direct binding and interaction with putative target genes. However, up to date no EVI1 specific target genes have been identified. Another possible mechanism by which transcriptional regulators may affect target genes is through complex formation with other proteins, critical for transcriptional regulation. In fact, EVI1 has been shown to interact with several transcriptional regulators, e.g. Smad3, HDAC's, and CtBP and exhibits transcriptional repression activity [8-12].

In recent years the analysis of post-translational modifications of histone tails such as methylation, acetylation, phosphorylation, and ubiquitination has unveiled the important role of chromatin remodeling in transcriptional regulation [13-17]. Modifications at specific histone residues represent important biological effects on transcription. The so-called "histone code" affects the regulatory status of a gene, as it causes specific proteins to recognize modified histones and translate the code into either hetero- or euchromatin [18-20]. Methylation has been one of the first modifications shown to play an important role in chromatin regulation. Histone H3 methylation at different lysine residues by specific methyltransferases has been shown to either activate or silence transcription [21-23]. The first protein that showed methyltransferase specificity towards histone H3 lysine 9 (H3-K9) was the mammalian SUV39H1 enzyme, a homologous of *S.pombe* Clr4 and *Drosophila* Su(Var)3.9 [24]. This preferential methylation of H3 depends on its SET domain, a 130 amino-acid domain that is conserved among different species. In addition to SUV39H1 several other methyltransferases were identified, e.g. SET9 or G9a, with specific methylation activities with respect to histone H3-lysine 4, lysine 9 and lysine 27 [21-23].

Evi1 belongs to the PRDM family of genes. The features of the PRDM3 (MDS-Evi1) gene showed high analogue to the PRDM2 (RIZ) and PRDM1 (Blimp-1) genes, as each express a shortened protein missing the PR domain. Recently it has been demonstrated that another member of this family of proteins PRDM1-BF1 (BLIMP-1) interacts with the methyltransferase G9a, suggesting that PRDM proteins may have a function in histone methylation [25].

Here we investigated whether Evi1 may form a complex with histone methyltransferases and show that it physically interacts with SUV39H1. This interaction involves the SET domain of SUV39H1 and the N-terminus of Evi1. Our data establish an epigenetic role of Evi1 in cell transformation by recruiting higher order chromatin remodeling complexes.

Results

Evi1 and SUV39H1 interact in vivo

To establish whether Evi1 is able to interact with SUV39H1, φE cells were transiently transfected with Myc-*SUV39H1* and HA-*Evi1*. SUV39H1 and Evi1 complexes were immunoprecipitated using anti-Myc or anti-HA antibodies. Analysis of these complexes on Western blot showed that Myc-SUV39H1 is present in HA-Evi1 immunoprecipitates, while, in the reverse experiments, HA-Evi1 could be detected in Myc-SUV39H1 immunoprecipitates (Figure 1A). The interaction is specific, as only very low background levels of HA-EVI1 or Myc-SUV39H1 was observed using anti-Myc or anti-HA respectively in cells that were singly transfected.

Applying immunofluorescence analysis and confocal microscopy we observed partial co-localization between Evi1 and SUV39H1 in small-punctuated speckle like structures in Cos cells transiently transfected with both constructs (Figure 1B). These findings confirm the immunoprecipitation experiments showing that the two proteins may be present in a complex *in vivo*.

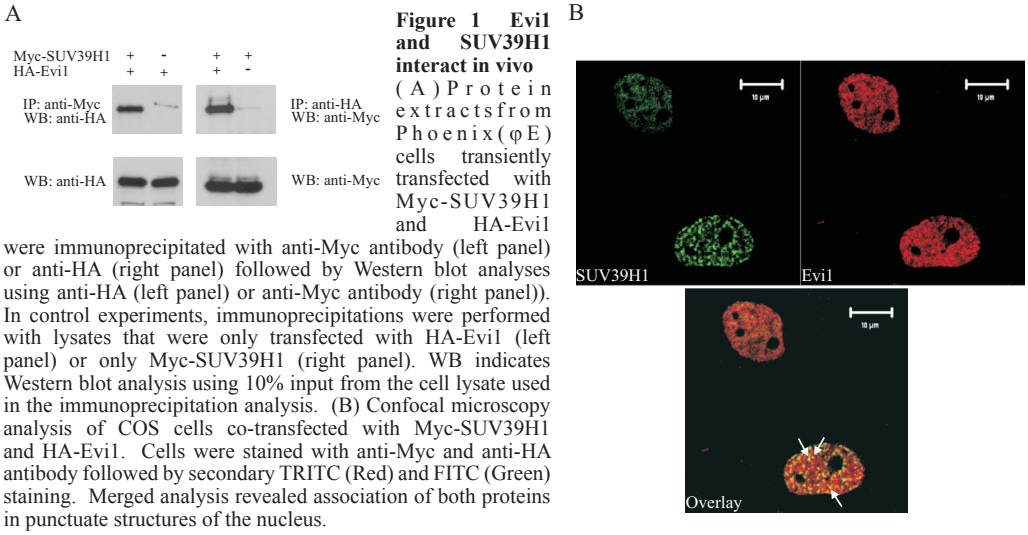

Figure 1 Evi1 and SUV39H1 interact in vivo (A) Protein extracts from Phoenix (φE) cells transiently transfected with Myc-SUV39H1 and HA-Evi1 were immunoprecipitated with anti-Myc antibody (left panel) or anti-HA (right panel) followed by Western blot analyses using anti-HA (left panel) or anti-Myc antibody (right panel)). In control experiments, immunoprecipitations were performed with lysates that were only transfected with HA-Evi1 (left panel) or only Myc-SUV39H1 (right panel). WB indicates Western blot analysis using 10% input from the cell lysate used in the immunoprecipitation analysis. (B) Confocal microscopy analysis of COS cells co-transfected with Myc-SUV39H1 and HA-Evi1. Cells were stained with anti-Myc and anti-HA antibody followed by secondary TRITC (Red) and FITC (Green) staining. Merged analysis revealed association of both proteins in punctuate structures of the nucleus.

The SET domain of SUV39H1 and the N-terminal zinc finger domain of Evi1 are required for interaction.

To identify which domain of SUV39H1 is responsible for the interaction with Evi1 we carried out co-immunoprecipitation experiments with full length HA-Evi1 and various Myc-SUV39H1 mutants (Figure 2A). *SUV39H1* possesses several functional domains that have all been shown to play an important role in the function of the protein. We used for this analysis Myc-*SUV39H1* constructs (1) lacking the chromatin organization modifier domain (Myc-*SUV39H* -Δchromo) shown to be important for chromatin targeting, (2) containing only the small chromo domain (Myc-*SUV39H1*-chromo), and (3) mutant lacking the SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain and post-SET domain *(*Myc-*SUV39H1*-ΔSET) required for methylation of H3-K9. The mutants were co-transfected with full length FLAG-*Evi1* into φE cells and lysates were immunoprecipitated using anti-Myc antibody. The results showed that absence of the SET domain abolishes the interaction (Figure 2B). In contrast, expression of constructs possessing the SET domain, Myc-SUV39H1-Δchromo or Myc-SUV39H1, preserves the interaction, as a specific product was obtained after staining with anti-FLAG antibody.

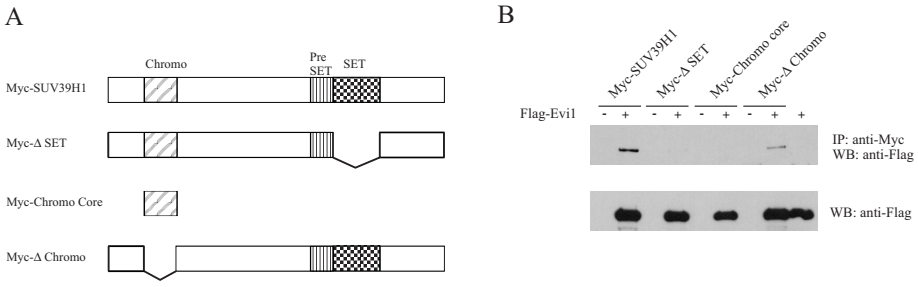

Figure 2 The SET domain of SUV39H1 is required for interaction with Evi1
(A) SUV39H1 domains: Chromo (CHRomatin Organization MOdifier), SET (Su(var), Enhancer of zeste, Trithorax) and Pre-SET. SUV39H1 and its mutants used for the analysis. ΔSET: mutant lacking the SET domain; ΔChromo: mutant with deletion of the chromo domain; Chromo Core: Chromo domain only. (B) Full length Myc-SUV39H1 and corresponding mutants were transfected with full length of FLAG-Evi1. Following anti-Myc immunoprecipitation on cell lysates, Western blot analysis was performed using anti-FLAG antibody to detect FLAG-Evi1. Input of 10% from total proteins was used for Western Blot (WB) analysis and is depicted in lower panel.

To identify the region of Evi1 required for interaction with SUV39H1 we constructed a panel of FLAG-*Evi1* mutants (Figure 3A). We used deletion mutants within zinc finger domains, FLAG-*Evi1*Δ1-7, FLAG-*Evi1*Δ4-7, FLAG-*Evi1*Δ1-10, and a mutant that only possesses the zinc fingers 8 to 10 plus the remaining C-terminal part of the protein, FLAG-Evi1-8-10+AD. Full length Myc-SUV39H1 showed interaction only with FLAG-Evi1 mutants that contained an intact N-

terminal zinc finger domain or that only missed zinc finger 4-7. This indicates that the region required for interaction is located within the first zinc finger domain (Figure 3B).

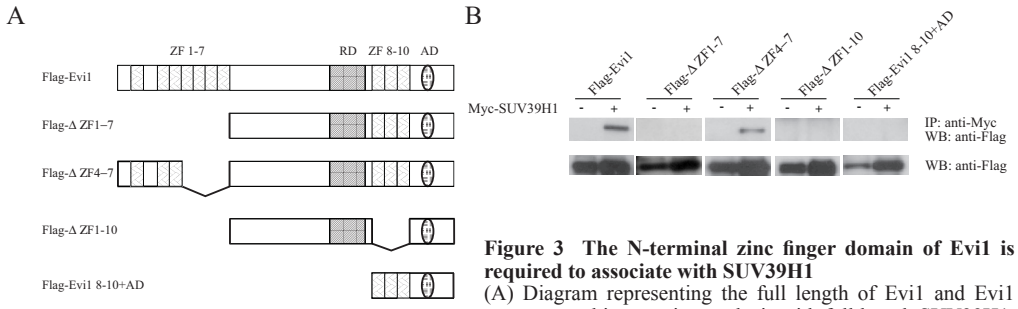

Figure 3 The N-terminal zinc finger domain of Evi1 is required to associate with SUV39H1
(A) Diagram representing the full length of Evi1 and Evi1 mutants used in mapping analysis with full length SUV39H1. Evi1 domains: ZF 1-7 (N-terminal domain containing 7 zinc fingers), RD (repression domain), ZF 8-10 (C-terminal domain containing 3 zinc fingers), AD (acidic domain). (B) Myc tagged full length SUV39H1 was transiently transfected with full length FLAG-Evi1 or with one of its mutants into φE cells. Immunoprecipitation was performed using anti-Myc antibody. Proteins were detected by Western blot analysis using anti-FLAG antibody. Input of 10% from total proteins was used for Western Blot (WB) analysis and is depicted in lower panel.

Evi1 binds histone H3 N-terminus in the presence of SUV39H1

H3-K9 is a specific target for the SUV39H1 enzyme. To investigate if SUV39H1 and Evi1 can form a complex at the H3 tail we performed GST-pull down experiments using a GST-H3 histone tail fusion protein. φE cells transfected with HA-Evi1 alone or in combination with Myc-SUV39H1 were lysed and incubated with an equal concentration of GST-H3 (Figure 4). The complex bound to H3 was analyzed using either anti-Myc or anti-HA antibodies. Staining with anti-HA antibody revealed the presence of Evi1 in the samples that also contained SUV39H1, whereas no HA-Evi1 signal was observed in the control samples with HA-Evi1 and GST-H3 alone. Using purified GST only we did not observe any binding of SUV39H1 or Evi1 (Figure 4).

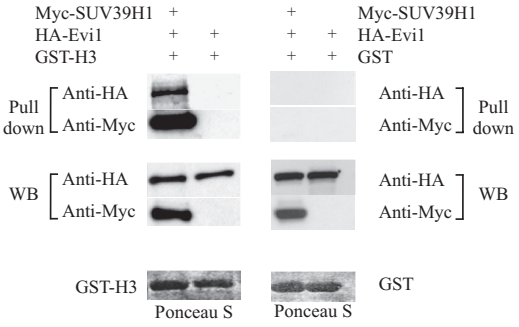

Figure 4 Evi1 forms a complex with SUV39H1 on histone H3 amino termini
Pull down assays using GST recombinant protein fused to histone H3 N-terminal tail (amino acids 1-57) (GST-H3) (left panel) or GST only (right panel) on total cell extracts from φE cells transiently transfected with HA-Evi1 plus Myc-SUV39H1 or HA-Evi1 only. Presence of HA-Evi1 was determined by Western blot analysis using anti-HA antibody, and Myc-SUV39H1 was detected by anti-Myc antibody (top panel). The expression level of proteins in each sample is shown by Western blot (WB) (middle panel). Presence of purified GST-H3 or GST was determined by Ponceau S staining (bottom panel).

The Evi1/SUV39H1 complex displays methyltransferase activity

SUV39H1 possesses histone methyltransferases activity specific for H3-K9 [24]. To investigate whether the Evi1-SUV39H1 complex possesses methyltransferases activity, we transfected φE cells with FLAG-Evi1, Myc-SUV39H1 or with both constructs. Evi1, SUV39H1 or the Evi1-SUV39H1 complex were immunoprecipitated and tested for in vitro H3 methylation in a histone methyltransferase assay (HMT). Immunoprecipitation of SUV39H1 clearly showed methylation of histone 3 as expected (Figure 5, lane 1), while immunoprecipitated Evi1 did not show any activity (Figure 5, lane 2). Notably, Evi1 immunoprecipitated by anti-FLAG from cells transfected with both constructs showed weak but significant signal indicating that Evi1-SUV39H1 complex displays methyltransferases activity (Figure 5, lane 3).

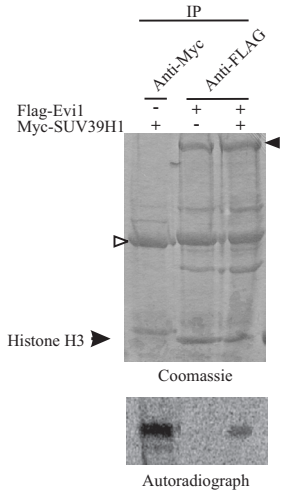

Figure 5 Evi1 shows methylation activity of histone H3 when in a complex with SUV39H1
Immunoprecipitates obtained from FLAG-Evi1/Myc-SUV39H1 or FLAG-Evi1 alone using anti-FLAG antibody were analyzed in HMTase assays with 1μg purified H3 peptide in presence of S-Adenosyl-L-[methyl-^{14}C] methionine as a methyl donor. Myc-SUV39H1 was purified with anti-Myc antibody and used as a positive control (left lane). Closed arrows indicate purified FLAG-Evi1; open arrows indicate Myc-SUV39H1 and purified histone H3 is depicted by an arrow on Coomassie Brilliant Blue R-250 staining (upper panel). The presence of Histone H3 is demonstrated in each lane. Histone H3 methylation is demonstrated by autoradiography in the lower panel.

Evi1 does not interfere with SUV39H1 activity

Our in vitro binding analysis suggests that the interaction between Evi1 and SUV39H1 requires a specific region within the SET domain of SUV39H1. As this domain is also required for the enzymatic activity of SUV39H1, we asked the question if interaction between Evi1 and SUV39H1 might influence histone methyltransferase activity. φE cells were co-transfected with Myc-SUV39H1 and FLAG-Evi1 and the proteins were subsequently immunoprecipitated with anti-Myc and anti-FLAG antibodies. The methyltransferase activities of the complexes were analyzed in a HMT assay (Figure 6). The results showed that even in the presence of high concentrations of FLAG-Evi1 the activity of Myc-SUV39H1 was unchanged (Figure 6, lane 2) as compared to a sample without FLAG-Evi1 (Figure 6, lane 3). Single purification of each of the proteins and subsequently combining the two samples in a HMT assay did not change

the outcome of the SUV39H1 methylation activity (data not shown). Western blot analysis revealed equal amounts of SUV39H1 in samples with or without Evi1 (Figure 6, lower panel). The results indicate that HA-Evi1 binding to Myc-SUV39H1 does not interfere with histone methyltransferase activity.

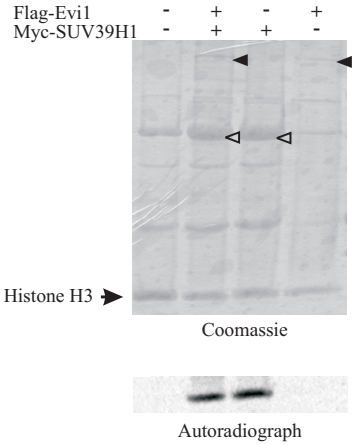

Figure 6 Evi1 does not interfere with SUV39H1 histone methyltransferase activity
FLAG-Evi1 and Myc-SUV39H1 were transfected alone or together into φE cells. Purified complexes using corresponding antibodies were analyzed in HMTase assay. Arrows indicate presence of purified proteins or histone H3. Specific H3 methylation was demonstrated by autoradiography (lower panel).

SUV39H1 binding enhances transcriptional repression of Evi1

To determine if interaction between SUV39H1 and Evi1 may influence Evi1 transcriptional repression properties we performed transcription reporter assays. A GAL4-DBD-*Evi1* construct in combination with Myc-*SUV39H1* plasmid and luciferase reporter gene (GAL4-AD-Luc) were transiently transfected into φE cells. Expression of low concentration of Evi1 did not significantly repress luciferase expression (Figure 7A). φE cells transfected with GAL4-DBD-Evi1 plus increasing concentrations of Myc-SUV39H1 showed significant repression of luciferase activity (Figure 7A). On the contrary, in an experiment in which the SET domain of SUV39H1 was deleted we did not observe significant changes in repression of the luciferase activity (Figure 7B). These results showed that SUV39H1 may cooperate with Evi1 and enhance its transcriptional repression activity in a dose dependent manner and requires the SET domain of SUV39H1 for this functional interaction.

Evi1 interacts with selected SET domain containing proteins

SET domains are found in many proteins and consist of a 130 amino-acid motif first identified in members of the polycomb group (PcG) and Trithorax group (TrG) of genes [26]. To date several mammalian SET domain proteins have been identified, and have been shown to directly

or indirectly associate with chromatin [27,28]. To address the possibility that Evi1 interacts with other SET containing proteins we performed immunoprecipitation assays with two other known methyltransferase proteins, i.e., SET9 and G9a.

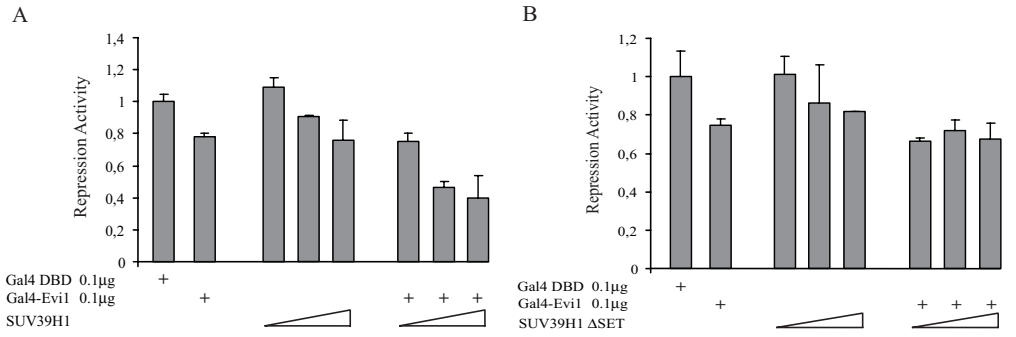

Figure 7 SUV39H1 enhances transcriptional repression activity of Evi1
(A) φE cells were cotransfected with a luciferase reporter plasmid containing four GAL4 binding sites upstream of the thymidine kinase promoter (4XGAL4-TK-Luc) with Evi1 fused to GAL4 DNA binding domain (GAL4-Evi1) and different concentration of SUV39H1 expression plasmid. The concentration of GAL4-Evi1 was kept constant at 0.1µg of DNA. The amount of SUV39H1 DNA for each plasmid increase as follow: 0.1µg, 0.2µg, and 0.3µg. 4XGAL4-TK-Luc expressions were normalized to 1.0. Transfection efficiencies were normalized using LacZ assay. All luciferase assays were performed in two independent duplicated experiments. Error bars indicate variations of all experiments.
(B) Luciferase experiment using SUV39H1ΔSET instead of SUV39H1. The experimental settings were identical as described for SUV39H1.

HA-*Evi1* was co-transfected either with FLAG-SET9, FLAG-G9a, or Myc-SUV39H1 into φE cells. Complexes were precipitated with anti-FLAG antibodies and analyzed by Western blotting using anti-HA antibodies to determine the interaction between HA-EVI1 with FLAG-SET9, FLAG-G9a or Myc-SUV39H1. The experiments in Figure 8A demonstrate that FLAG-G9a (lane 2) as well as SUV39H1 (lane 3) form a complex with Evi1, whereas FLAG-SET9 and HA-Evi1 (lane 1) did not show any interaction. No HA-EVI1 was identified in the immunoprecipitation experiments in the absence of anti-FLAG/anti-Myc antibody (beads only; lane 4). Vice versa, when anti-HA was used for the immunoprecipitation experiments, only G9a and SUV39H1 could be detected by anti-FLAG Western blot analysis (data not shown).

To specifically demonstrate that the enzymatic activity of G9a could be precipitated in a complex with EVI1 we performed an HMT assay on H3-histones using HA-Evi1/FLAG-G9a transfected φE cells. H3 peptide methylation was demonstrated using purified FLAG-G9a methyltransferase (Figure 8B, lane 3), whereas this activity was not detectable when the same lysate was exposed to beads without anti-FLAG antibody in the immunoprecipitation experiment (Figure 8B, lane 5). Using anti-HA antibody we immunoprecipitated HA-Evi1 with G9a and observed weak but significant methylation of purified H3 in an HMT assay (Figure 8B, lane 1). Autoradiography

revealed no signal in the precipitate obtained in the absence of anti-HA or anti-FLAG (Figure 8B, lanes 4 and 5) or when no G9a (lane 2) or EVI1 (lane 4) was introduced into φE cells. We conclude that Evi1 might physically interact with G9a and causes methylation of histone-H3.

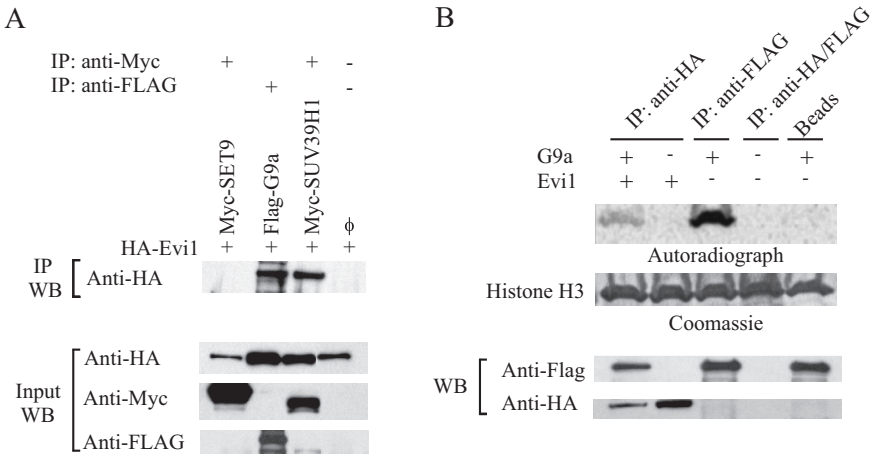

Figure 8 Evi1 binds to methyltransferase protein G9a but not to SET9
(A) φE cells were transiently transfected with Myc-SET9, FLAG-G9a, Myc-SUV39H1, HA-Evi1 or control (φ). Pre-cleared cell lysates were immunoprecipitated with anti-Myc or anti-FLAG antibodies to immunoprecipitate the histone methyltransferases. The immunoprecipitates were analyzed with anti-HA antibody for the presence of Evi1. Cell lysates were analyzed by Western blotting for the presence of input proteins (lower three panels). (B) HMTase assay with FLAG-G9a and HA-Evi1. Lysates from φE cells transiently transfected with FLAG-G9a and HA-Evi1 were immunoprecipitated with anti-HA antibody and analysed for specific histone H3 activity (top panel). FLAG-G9a was purified with anti-FLAG antibody and used as a positive control. Presence of G9a and Evi1 proteins in samples was detected using Western blot analysis.

Discussion

Evi1 encodes for a nuclear zinc finger containing protein, which is required for early development [29]. Inappropriate activation of the gene in hematopoietic precursor cells disturbs normal cellular proliferation and differentiation pathways as observed in patients with MDS or AML harboring translocation or inversion of chromosome 3 involving band q26 [2]. Although the mechanism of transformation by this nuclear zinc finger protein is still unknown, it has been shown that Evi1 may act as a transcriptional repressor [9-11,30]. Evi1 protein has been shown to physically and functionally interact with different classes of nuclear proteins involved in transcriptional control [9,10,12,31]. Here we demonstrate association between Evi1 and SUV39H1, a member of the histone methyltransferase family of genes, and show that this interaction enhances Evi1 mediated transcriptional repression in a dose dependent manner. Moreover we also show that

Evi1 may interact with another repressor belonging to the family of histone methyltransferase, i.e. G9a. The ability of Evi1 protein to directly bind DNA and its capability to interact with these strong repressors of transcription further substantiates the idea that deregulation of gene expression consequently leading to abnormal homeostasis may be one of the effects of Evi1 in transformation.

Methylation of histones within promoter regions is thought to be the main cause of transcriptional silencing of target genes by SUV39H1, resulting in changes in chromatin structure [32,33]. In accordance to the "histone code", modification of the histone H3 terminus influences the chromatin state, resulting in either silenced heterochromatin due to specific lysine methylation or active euchromatin due to acetylation. Recently, it has been shown, that SUV39H1 is capable of interacting with transcription factors involved in development. Interaction between SUV39H1 and AML1 interferes with DNA binding of AML1, whereas TAL1/SCL association with SUV39H1 facilitates specific histone methylation within the DNA target regions of TAL1/SCL [34,35]. Retinoblastoma protein (Rb) interacts with SUV39H1 resulting in repression of cyclin E and E2F promoter activity [36,37]. Interaction with Smad family members by histone methyltransferases enhances silencing of the muscle creatine kinase (MCK) promoter [38]. Thus SUV39H1 appears to be a transcriptional repressor [39] that methylates histone residues, but requires specific DNA binding proteins to be recruited to particular promoter regions. This could provide a model in which Evi1 targets methyltransferases to specific promoter regions, leading to transcriptional silencing. The hypothesis that Evi1 may recruit histone methyltransferases towards target genes is in line with a recently reported finding, showing that the Evi1 family member BLIMP-1 interacts with G9a and this complex is recruited to the BLIMP target gene *IFBN1* [25] Promoter binding by this complex resulted in silencing of *IFBN1* through histone H3-K9 methylation.

Evi1 recognizes and binds to specific nucleotide sequences via fingers 4-7 of zinc finger domain 1 or fingers 8-10 of zinc finger domain 2, whereas the first three zinc fingers have been demonstrated not to interact with DNA. Therefore, we may have identified a specific function of zinc fingers 1-3 of the N-terminal zinc finger domain of Evi1, which are highly conserved between mouse and human. It is also possible that Evi1 does not directly target DNA itself, but mainly acts as an adaptor that may attract multiple repressor complexes. Recently, it has been demonstrated that zinc finger containing protein friend of Gata1 (Fog1) modulates Gata1 activity via interaction with multiprotein complexes [40,41]. In any case, it appears that Evi1 is able to interact with repressor complexes and bring them to particular sets of genes either through

DNA binding of Evi1 itself or via other DNA-binding transcription regulators. It suggests that Evi1 may play a key role in the different steps in the process of gene silencing, i.e. it may affect histone deacetylation, histone methylation and possibly even DNA methylation. Unveiling all these possibilities requires the application state-of-the-art proteomic approaches such as mass spectrometric analysis of purified Evi1 containing protein complexes. Such studies combined with gene array analysis in patient samples and well-defined in vitro and in vivo animal models are indispensable to unravel the mechanisms of transformation by Evi1.

Domain mapping studies revealed that the SET domain of SUV39H1 is involved Evi1 interaction. The SET domain and surrounding pre-SET domains are required for specific enzymatic activity towards histone H3 lysine 9 methylation [24]. The luciferase reporter assays revealed that enhancement of transcriptional repression by Evi1 required the SET domain as well. These data are in agreement with reports showing that SUV39H1 transcriptional repression is impaired in mutants lacking the SET domain [38]. Thus the absence of repression enhancement by *SUV39H1*-ΔSET can be explained by the lack of Evi1 interaction and by a defect in its function. The interaction, between Evi1 and SUV39H1 through the SET domain, appeared not to influence the histone methyl transferase acitivity. In addition to our findings showing that Evi1 interacts specifically with SUV39H1 we also showed an interaction between another methytransferase G9a. G9a methylates histone H3 at lysine 9 and lysine 27 (H3-K9 and H3-K27) [42] also leading to transcriptional repression. On the contrary, we did not observe interaction with SET9 methyltransferases, which methylates histone H3 at lysine 4 (H3-K4) [22]. In contrast to SUV39H1 and G9a, SET9 is involved in transcriptional activation rather than repression [22]. The reason for Evi1 binding selectively to SUV39H1 and G9a remains unclear, but it again emphasizes that transcriptional repression may be the major function of Evi1. The fact that Evi1 is frequently involved in myelodysplastic disorders and AML may indicate a function in the repression of genes required for normal myeloid proliferation and differentiation.

Materials and Methods

Expression constructs

Evi1 cDNA was fused in frame with FLAG (FLAG-*Evi1*) or HA (HA-*Evi1*) nucleotide sequences and inserted into the pCMV mammalian expression vector (Clontech, Palo Alto, CA) under the early cytomegalovirus promoter. All deletion mutants were generated from the original cDNA of FLAG-*Evi1* using available restriction sites (available upon request).

Full length *SUV39H1* and its mutants; *SUV39H1*-ΔSET, *SUV39H1*-Δchromo, and *SUV39H1*-chromo core, all cloned into a mammalian expression vector under the CMV promoter were kind gifts from Dr. A.H. Peters (IMP, Vienna, Austria). All constructs were Myc tagged at the N-terminus.

pCMV-FLAG-*G9a* expression vector was a kind gift from Dr. I Talianidis (Institute of Molecular Biology & Biotechnology, Crete, Greece) and has been described previously [43].

The cDNA of *SET9* was amplified from HL-60 human cell line using *Pfu* polymerase. The amplified fragment was cloned into a pCMV-FLAG expression vector to generate pCMV-FLAG-*SET9* construct. The insert was sequenced and the expression was confirmed using Western blot analysis.

Immunoprecipitation and Western Blot analysis

$2x10^6$ Phoenix (φE) cells were seeded in $100mm^2$ dishes (Becton Dickinson, Franklin Lakes, NJ) and cultured in Dubelco's modified Eagle medium (DMEM, Life Technologies, Paisley, UK) supplemented with 10% FCS and transfected with 20μg DNA using calcium phosphate co-precipitation [44]. Cells were harvested, washed with cold PBS and lysed in Carin lysis buffer (20mM Tris pH 8.0, 137mM NaCl, 10mM EDTA, 100mM NaF, 1% NP-40, 10% Glycerol) containing complete protease inhibitor mix and Pefablock (Roche, Zwijndrecht, NL). Supernatants were incubated at 4^0C with an appropriate antibody (1mg) for 2hr, followed by 2hr incubation with sepharose G-protein beads (Sigma-Aldrich, St.Louis, MO). Bound proteins were collected, washed extensively with cold lysis buffer and resuspended in 1X SDS Laemmli buffer [45]. Complexes were denatured, separated on 8-10% SDS-PAGE gel and transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked in 4% milk (Bio-Rad, Hercules,CA) and incubated with the appropriate antibodies. Detection was performed using a secondary horseradish peroxidase-conjugated antibody (Dako Diagnostic BV, Denmark) and visualize by enhanced chemiluminescence according to the manufacturers protocol (ECL, Boston, MA). The following antibodies were used in this study: anti-HA (Santa Cruz Biotechnology, Santa Cruz, CA), anti-FLAG, and anti-Myc (Sigma-Aldrich, Zwijndrecht, NL).

Immunofluorescence microscopy

COS-7 cells were grown on cover slips and transfected with equal concentrations (4μg) of Myc-SUV39H1 and HA-Evi1 using FuGENE transfection reagent according to the manufacturer's

(Roche, Zwijndrecht, NL) specification. 48 hours post transfection cells were washed with cold PBS and processed as described previously [46]. Cells were stained with anti-HA and anti-Myc primary antibodies. Detection of primary antibodies was performed using tetramethylrhodamine isothiocyanate (TRITC) and fluorescein isothiocyanate (FITC) conjugated secondary antibodies (Dako Diagnostics BV, Denmark). Cells were analyzed using Zeiss confocal laser scanning microscopy (LSM510).

Protein purification and in vitro histone methyltransferase assay (HTMase)

φE cells were grown in DMEM supplemented with 10% FCS and transfected with corresponding plasmid by calcium phosphate co-precipitation. Cells were grown for 48 hours, collected, washed once with cold PBS and lysed as described above. Purified sepharose G-protein beads with bound proteins were washed extensively in Carin lysis buffer, and used directly for the methyl transferase assay. HTMase was performed as described previously [42]. Briefly, purified sepharose bound proteins were incubated in HMTase buffer (50 mM Tris, pH 8.5, 20 mM KCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 250 mM sucrose) containing 250nCi S-Adenosyl-L - [methyl-^{14}C] methionine (MP Biomedicals, Amsterdam, NL) as a methyl donor and 1μg purified native histone H3 as substrate (Roche Molecular Biochemicals). Reactions were incubated at 30^{0}C for 1 hour under constant rotation. Tubes were centrifugated and the reaction was terminated by addition of 3X SDS-Laemmeli sample buffer [45]. Samples were heated at 95^{0}C for 2 minutes and separated on 12.5% SDS-polyacrylamide gel. Proteins were visualized by staining SDS-polyacrylamide gel with Comassie Brilliant Blue R-250 (N.V Life Technologies S.A. Merelbeke, NL).

Histone H3 pull down assay

Mouse histone H3 cDNA cloned into pGEX-4T-3 (Amersham Pharmacia Biotech) bacterial expression vector was a kind gift of Dr. Y. Shinkai (Department of Cell Biology, Kyoto University, Japan). The isolation and purification of the fusion was performed as described previously [42]. In short, transfected DH12 or JM110 *Escherichia coli* were grown to OD_{600} 0.6 and induced with 1mM IPTG for 3 hours at 37^{0}C. Cells were collected by centrifugation at 4^{0}C and lysed in TEND buffer (1X TBS, 0.5% NP-40, 1mM EDTA, 1mM DTT) containing protease inhibitor mix and Pefablock (Roche, Zwijndrecht, NL). Clear lysates were incubated with Glutathione beads (Amersham Pharmacia Biotech) for 2 hours at 4^{0}C. Recombinant proteins were washed extensively and the concentration was verified using Coomassie Brilliant Blue R-

250 staining. Equal concentrations of the GST-H3 recombinant protein were incubated with φE purified cell extract transfected previously with Myc-*SUV39H1* with or without HA-*Evi1*. The complexes were incubated overnight at 4^0C, washed with cold Carin lysis buffer, and separated by 8% SDS-polyacrylamide gel. Detection with antibodies was performed as described above.

Luciferase reporter assays

The luciferase assay was performed as described previously [47]. In short, φE cells at $1x10^5$ cells per well were transfected with 300ng 4xGAL-TK-Luc, 100ng pcDNA3-DBD-*Evi1*, 300ng pRSVLacZ and various concentration of pCMV-*SUV39H1*. Total amount of plasmid was normalized using empty pCMV expression vector. 48 hours post transfection cells were lysed in 100µl lysis buffer (25mM Tris phosphate pH 7.8, 15% glycerol, 1% Triton X-100, 1mM DTT, 8mM $MgCl_2$). 25µl of cell lysate was transferred to 96-well flat bottom plates (Costar, Corning Inc, Corning, NY) and 25µl of a 16mg/ml luciferase substrate-containing buffer (Steady-Glo luciferase assay system, Promega, Madison, WI) was added to each well. Light emission intensity was measured using a TopCount luminometer (Packard, Meriden, CT). In parallel, 25µl of cell lysate was incubated with 75µl β-galactosidase buffer containing 0.56mg/ml o-Nitrophenyl β-D-galactopyranoside (ONPG, Sigma). Absorption was measured at 450nm in a microplate reader (BioRad 450, Veenendaal, NL). All assays were carried out in triplicate.

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Chapter 6

General Discussion

General Discussion

The application of retroviral insertion mutagenesis in mouse models to identify potential proto-oncogenes, has led to identification of a substantial number of genes that may be involved in human pathogenesis. One of the genes that was identified as a common integration site in murine leukemia was the ecotropic viral integration 1 (*Evi1*) gene[1]. This finding suggested a possible role for the aberrantly expressed gene in myeloproliferative disorders such as myelodysplastic syndrome (MDS) and human acute myeloid leukemia (AML). Analysis of chromosome 3, the locus where the gene resides, in AML patients revealed a group of cases that were harboring translocations or inversions involving chromosome 3, e.g. t(3;3)(q21;26) or inv(3)(q21;q26)[2]. Inappropriate expression of *EVI1* as a result of these chromosomal aberrations has been shown to be directly or indirectly involved in dysregulation of cellular homeostasis and induction of tumorigenesis. To elucidate the role of *Evi1* in cellular transformation we generated and analyzed a mouse model for Evi1. Furthermore, we extended the analysis of Evi1 in the context of complex formation with novel binding partners of Evi1.

Evi1 in vivo model

AML is a cancer due to uncontrolled cell proliferation of hematopoietic cells that can result from dysregulation of the balance between cellular proliferation, differentiation and apoptosis. The traditional paradigm of cancer is that it is the result of a multi-step process involving an accumulation of mutations in multiple genes that contribute in the control of cell proliferation (gain-of-function or overexpression) or growth inhibition (mutational inactivation).

Louz *et al,* using the murine stem cell antigen 1 promoter *Sca-1* (Ly-6E.1), for the first time presented experiments that suggested the consequences of aberrant expression of Evi1 *in vivo*. Overexpression of Evi1 in multipotent hematopoietic stem cells (HSC) negatively affected the number of erythroid colony forming units (CFU-E). Additional genetic alteration delivered by retroviral induction increased the incidence of tumor development as proposed by the multi-hit theory of cancer development[3].

Another approach to deliver *Evi1* into progenitors cells, using retroviral transduction of lineage negative bone marrow cells followed by transplantation, was undertaken. The animals showed abnormalities 10 months post-transplantation, characterized by a phenotype similar to that observed in patients with MDS i.e., pancytopenia, hypercellular bone marrow and

dyserythropoiesis[4].

In **Chapter 2** and **Chapter 3** of this thesis we describe the generation of a novel mouse model for Evi1, targeting exclusively the hematopoietic system. In addition, the expression of Evi1 gene is conditionally expressed using specific transcriptional control DNA sequences enclosed by LoxP sites. Using this model we analyzed the expression and effect of Evi1 during fetal erythropoiesis. Using specific lines in which Cre was under the β-globin regulatory elements the expression pattern of the Cre gene was predominantly active in the erythroid lineage (Figure 1). Analysis of E12.5 fetal liver from double positive littermates revealed interference of Evi1 with normal erythroid development. Morphologically the fetal livers showed a distinguished paleness and significant decrease in size, that correlated with a decrease of late, differentiated cells and lower cell counts. The expression of TER119/CD71 cell markers validates our findings that the differentiation program within the erythroid lineage was impaired. In agreement with the block in differentiation, fetal livers obtained from double positive embryos showed reduced numbers of colony forming units-erythroid (CFU-E).

Our results support the notion that inappropriate expression of Evi1 interferes with cellular homeostasis and contributes to neoplastic development. However, the mechanism behind cellular transformation remains elusive. Observations in the transplantation model described by Buonamici *et al,* suggested interference of Evi1 with factors that are indispensable for normal erythroid development. For instance, expression of the erythropoietin receptor (*EpoR*) was affected[4]. Although a direct link between Evi1 and down regulation of *EpoR* expression *in vivo* has not been shown, it suggested more an indirect effect in combination with other specific erythroid factors. In contrast, our findings demonstrate that both genes are unaltered in the presence of Evi1. The observed effects could be partly due to the methods that were used to deliver *Evi1*. In both models, using the bone marrow or an established cell line the *Evi1* gene was introduced using the retroviral transduction method. The random proviral insertion of the enhancers within the genome could affect the overall outcome of these experiments.

We showed that the expression of transcription factors *Pu.1* and *c-Myb* was altered in presence of Evi1 in double heterozygous fetal erythroid cells. This could provide some explanation for the block in differentiation. The *Pu.1* gene plays a key role in granulocytic development[5,6]. Alteration in *Pu.1* expression due to proviral insertion as shown in Friend virus induced murine erythroleukemia (MEL) leads to cellular transformation[7]. Several groups have demonstrated that Gata1 and Pu.1 mutually antagonize the transactivation activity of each other[8-10]. This cross-antagonizing effect is achieved as a result of direct physical interaction between Gata1 and

Pu.1[11]. Pu.1 binds to the DNA binding domain of Gata1, thereby blocking Gata1 ability to bind DNA. Gata1 can bind Pu.1 and displaces the c-Jun protein, an important coactivator of Pu.1 for proper transactivation of myeloid target genes. As the fine-tuning balance between those two factors is necessary for maturation of erythroid cells *in vivo*, fluctuation of levels due to ectopic expression of Evi1 may perturb the equilibrium. Our findings that *c-myb* expression levels had also changed are not unexpected. The hierarchical transcription factor model proposed for MEL cells in which Pu.1 is able to block differentiation of proerythroblasts is in part achieved by inducing the c-*myb* gene, which in turn is able to deregulate the expression of c-*myc* gene, and possibly other genes. Interestingly, downregulation of c-*myb* is necessary for the terminal differentiation of erythroid cells, as ectopically expressed c-*myb* block differentiation of MEL cells, even though *PU.1* is downregulated[12].

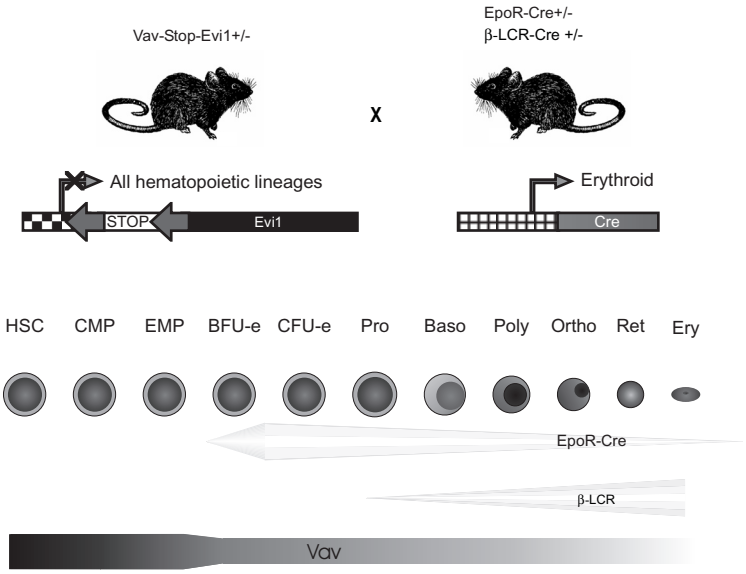

Figure 1 Lineage specific expression of Evi1 in novel mouse model
The expression of Evi1 is under the control of Vav hematopoietic promoter (chess box). The Evi1 gene is silent, due to transcriptional stop box flanked with Cre sites (arrows) inserted between the gene and the promoter. Cross-breeding between Evi1 mouse with specific Cre recombinase mouse (PEV-Cre) removes the transcriptional box and allows for Evi1 expression in a lineage specific manner. The expression pattern of the Vav promoter as well as the β-LCR promoter within the erythroid lineage is depicted. Stages during erythroid differentiation are as follow: HSC, hematopoietic stem cell; CMP, common myeloid progenitor; EMP, erythroid myeloid progenitor; BFU-e, burst forming units-erythroid; CFU-e, colony forming units-erythroid, Pro, proerythroblasts; Baso, basophilic erythroblast; Poly, polychromatic erythroblast; Ortho, orthochromatic erythroblast; Ret, reticulocyte; and Ery, erythrocyte.

The next important step to understand the impediment of Evi1 in erythropoiesis will be to elucidate how Evi1 interferes with other essential factors. In addition, experiments with

transgenic animal models bearing expression of the Cre gene at different stages during erythroid development, such as EpoR-Cre, may help to understand additional genetic changes resulting in the outgrowth of malignant clones.

Evi1 possesses DNA binding motifs, which have been shown in vitro to bind artificial AGATA sequences and repress transcription of synthetic promoters[13,14]. While it remains to be established whether this mechanism is also operational *in vivo*, other mechanism could be the cross-antagonistic activity of Evi1. This phenomenon is observed during normal cell development where genes are shutdown to allow lineage commitment[15,16]. On the other hand, in leukemia development inappropriate gene expression due to chromosomal translocation, viral insertion, or epigenetic changes can cause adverse effects in an uncontrolled manner by interference with normal transcription factors. In such a hypothetical scenario, Evi1 can directly cross-react and antagonize a specific, until now unidentified, gene thereby producing an arrest in cell maturation.

Although analysis of fetal erythropoiesis in the *Evi1* transgenic mouse model is important to understand the role and function of Evi1 in cellular transformation, the ultimate goal is to comprehend its role in myeloid lineage in view of its role in AML. For this purpose, our animal model can be crossed with specific Cre model(s) to induce the expression within the granulocytic lineage. Finally, as Evi1 belongs to a transcription factor family, that possess affinity for specific recognition sites within the genome, it seems logical to perform a large scale screen using genomic approaches to bring closer the molecular changes within the cell in the presence of Evi1.

In the sea of interactions

Transcription factors function is not solely restricted to interactions with DNA. In addition they form protein-protein interactions with other proteins, which can transform their functions in different ways. The interaction between Evi1 and several different proteins has been described in **Chapter 1**. The question for each and all of those interactions is to sort out which ones are functionally significant and play a role in Evi1 transformation, and what their functional relationship is.

It has been shown for many different transcription factors that physical interaction with other proteins or protein complexes modulates their activity. The changes in activity can be achieved through different levels of direct protein modifications, such as phosphorylation, methylation,

acetylation, or ubiquitination[17,18]. It can also be accomplished by interaction with multiprotein complexes, such as Sin3/HDAC, nucleosome remodeling and histone deacetylation (Mi-2/NuRD), N-CoR/SMRT, Sin-associated proteins (Sin3-SAP), and SWI/SNF[19-21]. Since in general multiprotein complexes do not have intrinsic DNA-binding activity, they can, directly or via an adaptor protein, recruit transcription factor, which can then target the complex to specific gene promoters. The activity of a transcription factor bound complex can be modulated to carry out a positive or negative effect on transcription. This depends on the relation between different complexes to each other, as the composition might change during cell development in both the spatial and temporal context. Furthermore, the regulation can be achieved by additional, as yet unidentified, factors, which may participate and contribute further functions. Finally, the activity associated with different complex points toward specialization.

The modulation characteristics of large complexes are achieved via functionally different members of a given complex. Most complexes combine and exhibit multiple enzymatic activities such as histone acetylation, histone methylation, and nucleosome modulation. The most common subunits of several complexes are histone deacetylases (HDAC1 and HDAC2). HDACs are found in complexes such as Sin3-SAP, NuRD, CoREST-HDAC, and N-Cor/SMRT[22-26]. In addition some complexes contain more common subunits, such as retinoblastoma protein (Rb) associated proteins RbAp-46 and RbAp-48 found in Sin3-SAP and NuRD complex[27]. This indicates that the core HDAC complex recruits additional proteins that impart distinct functional roles for each complex. Furthermore, the close relationship between DNA methylation and gene silencing via methylated CpG dinucleotides can be achieved via direct interaction between methyl-CpG-binding protein MeCP2 and HDACs. This transient physical interaction occurs without MeCP2 being a subunit of the N-CoR/SMRT complex[28].

In **Chapter 4** we have identified a novel interacting partner of Evi1, the methyl binding domain 3b protein, a member of the nucleosome remodeling complex (NuRD)[29,30]. The analysis was extended to show that the complex between those two proteins does exist in vivo, and therefore Evi1 is found to interact directly with the NuRD complex.

The characterization of the NuRD complex and its components have been described in details previously[29]. The complex exerts two major functions, chromatin remodeling via the Mi2 subunit, and histone deacetylase activity through HDAC-1 and HDAC-2. Those two functions seem to be of importance in altering chromatin structure and control of gene transcription.

In general, eukaryotic repressors can exhibit this function in many different ways: 1) By competitive DNA binding, when the repressor occupies the binding site, and thus does not allow

binding of an activator, 2) By interaction between repressor and activator at the binding sites, masking the activation surface of the activator, 3) By direct interference of the repressor with the general transcription factors and thereby inhibit transcription, 4) By recruitment of repressive chromatin remodeling complexes and 5) By recruitment of histone deacetylase complexes, which might change the pattern of acetylation at the histone level.

As Evi1 contains several interesting motifs, it is tempting to propose several different option how Evi1 can exhibit its dominant repressive function. Many transcription factors contain repressor motifs that are generally highly charged and mediate direct interactions with basal transcription factors. They also interact with activators or co-activators or with co-repressors, which physically modulate binding to the ultimate target. In **Chapter 5** we investigated the interaction between Evi-1 and a histone methyltransferases enzyme SUV39H1.

Covalent post-translational modifications of core histone amino (N) terminal tails (H2A, H2B, H3, and H4) at specific amino acid residues is achieved by diverse enzymes. The array of different modifications includes acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and ADP-ribosylation[31,32]. This combinatorial pattern of modifications, "histone code", is a main determinant of chromatin structure and gene activity. In general, acetylation of histones correlates with transcriptional activity, whereas histone methylation was shown to be involved in both activation and repression. These modifications are reversible, and the level of specific modifications is associated with transcriptional activity.

SUV39H1 belongs to a family of enzymes that possess specific methyltransferase activity on histone H3 lysine 9 (H3-K9)[33]. This is achieved through a conserved domain, SET, found amongst many different proteins. The specificity of methylation is highly selective for each enzyme, as different modifications of histone tails dictate different states of chromatin. We have found a direct interaction between Evi1 and SUV39H1 and shown found that Evi1 can selectively interact with other methyltransferases, namely G9a, but not with SET9. Interestingly, SUV39H1 and G9a were shown to modify the active chromatin into an inactive state via histone methylation[34,35]. On the contrary, SET9 modifications modified chromatin into an active state[36]. This indicates that Evi1 may predominantly co-operate with methyltransferases that are involved in transcriptional repression rather than activation. Both proteins (SUV39H1 and G9a) require specific DNA binding proteins to be recruited to particular promoter regions. As Evi1 can interact with specific DNA sequences it may target methyltransferases to specific promoter regions, leading to transcriptional silencing (Figure 2).

The question that arises is who recruits whom? Is the multiprotein complex recruited to a specific

DNA motif by a DNA-binding transcription factor, or does the transcription factor bind to the DNA upon already established access by the remodeling complex? Experiments with different transcription factors point to a possibility where both scenarios may be true. Some factors are able to bind compact chromatin DNA, whereas others can not. Thus, the model proposes is that transcription factors that can bind the recognition sites of chromatin DNA recruit a remodeling complexes alleviates chromatin structure, and thus allow other factors to bind DNA.

In our studies we have found that Evi-1 interferes with factors that are involved in chromatin remodeling (Mbd3b) and histone modification by methylation (SUV39H1 and G9a). Direct interaction with those factors showed an inhibitory effect at the transcriptional level. However, EVI-1 has also been shown to interact with group of molecules, C-binding protein (CBP) and p300/CBP-associated factor (P/CAF), that are involved in transcriptional activation rather then repression[37].

So, how can one trans-acting protein be both an activator and a repressor? To answer this question the best example that can be referred to is the tumor suppressor p53 protein. As mentioned earlier proteins may undergo different post-translational modifications. Each of the specific protein modifications may play a role and dictates the state either to be an activator or repressor. The C-terminal part of p53 contains regulatory domain that undergoes multiple modifications at different residues. It has been shown that acetylation of several lysine residues (K372, K373, K381, K382, and K320) can be achieved by histone acetyltransferase (HAT) enzyme, which increases the DNA-binding activity, and transactivation of p53[38,39]. However, DNA bound p53 is in an unmodified form and after DNA damage several lysine residues are acetylated, which in turn recruits HDAC complexes that release the HAT enzymes from the promoter site and induce rapid promoter repression. Such highly selective changes regulate the p53 status from being an activator towards a repressor during specific phases of the cell cycle[40].

Future directions

Although our knowledge about the role of aberrantly expressed transcription factors in AML improved largely over the years, we still lack the insight into the pathways that could be disrupted in AML by those transcription factors. Animal model studies are crucial to comprehend the pathogenesis of human diseases. Analysis of our animal model generated to specifically expressed Evi1 in a control manner in all hematopoietic lineages may be a useful tool to dissect and reveal the full significance of Evi1 interference with lineage specific differentiation.

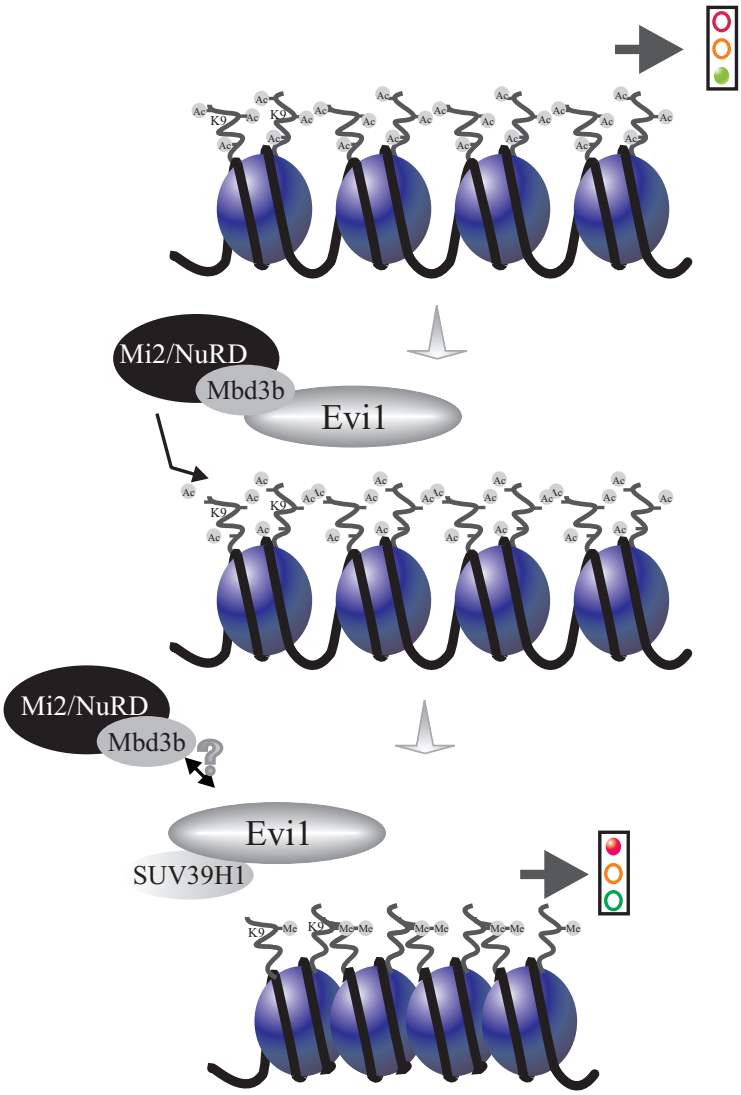

Figure 2 Proposed model for Evi1 transcriptional repression
Schematic representation of nucleosomes and histone tails. The DNA strands are wrapped around the nucleosome and extrude the histone N-terminus tails. The tails undergo specific modifications at different amino acids. The acetylated status of histone tails dictates transcriptional activation as the nucleosomes are in a relaxed form. Interaction between Evi1 and Mbd3b (Chapter 3) brings in the histone deacetylation complex (NuRD), which is able to deacetylase, the histones. As Evi1 also interacts with SUV39H1 (Chapter 4) this interaction can exhibit specific methylation at histone H3 lysine 9 (H3-K9). Methylation of H3-K9 is found to be associated with histone condensation and thus transcriptional repression.

Our working hypothesis that Evi1 interferes with erythroid lineage differentiation was recapitulated by showing a direct link between Evi1 expression and erythroid lineage impediment during the development of fetal erythropoiesis. Although the interference of Evi1 with fetal erythropoiesis is an important observation, it still does not fully explain in details the genetic abnormalities behind the transformation. Therefore, to further study the changes at the molecular level on a genome wide scene, analysis of selective population of fetal hematopoietic cells could be analyzed by means of genomic approaches like microarray analysis.

In addition, of great interest is to study inappropriate expression of Evi1 in myeloid lineage. Analysis of blast cells from AML patients with aberrant expression of Evi1 clearly shows a lineage- and stage-specific block of differentiation, supporting the idea that the stem cells compartment is also involved. To elucidate fully on the involvement of Evi1 in myeloid leukemia development, it might be worthwhile to intercross the Evi1 transgenic mice with various transgenic mice lines that specifically express Cre enzyme at a stage specific time point during myelopoiesis. Furthermore, this will also help to understand if Evi1 is solely responsible for AML development or additional genetic abnormalities are needed to recapitulate human AML. In situation when Evi1 is not able to induce AML on its own, cooperating mutations can be introduced by crossing Evi1 mice with transgenic mice expressing other genes frequently found to be involved in AML development and progression.

Equally interested are more detailed studies of Evi1 protein-protein interactions and contribution to the block of differentiation found in AML. In particular, Evi1 interactions with factors implicated in epigenetic gene control and/or with multi subunit complex involved in chromatin remodeling can provide further knowledge of aberrantly disrupted transcription factors in critical cellular processes such as cell growth, proliferation, differentiation, and apoptosis.

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Summary & Samenvatting

Summary

Ecotropic viral integration site 1 (Evi1) is a zinc finger containing nuclear protein, which is involved in transcriptional control. Although Evi1 expression during normal embryonic development is essential for survival, the level of expression appears less necessary in the later stages of development.

Inappropriate "re-expression" of Evi1 in patient's hematopoietic precursor cells due to chromosomal abnormalities has been found to be a contributing factor in the development and progression of malignancies such as myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). Patients with high levels of Evi1 transcript have a poor survival rate and current therapy is not suitable to treat the disease.

Chapter 1 gives an overview of current knowledge in development of two hematopoietic lineages, i.e. erythroid and myeloid. The role and function of the most important transcription factors in these specific lineages will be described. Furthermore, the Evi1 gene is introduced in the context of 1) leukemia development, 2) interaction with known proteins and 3) formation of fusion proteins in human disease. Finally, regulation of transcription by histone modification is briefly described.

The involvement of Evi1 in cellular transformation has been shown *in vitro* using several different models. However, its role *in vivo* was not addressed as extensively. To elucidate on the effects of Evi1 *in vivo* we generated and analyzed a novel Evi1 transgenic mouse model as described in **Chapter 2**. Using conditionally expressed Evi1 in a restricted manner, i.e. within the erythroid lineage we have found a lethality rate of almost 40% in double heterozygous embryos. Analysis of E12.5 fetal livers from double transgenic animals revealed profound anemia, characterized by abnormal maturation of erythroid cells, with the presence of large dysplastic cells. The differentiation capacity of cells was also affected as significant reduction in the numbers of colony forming units-erythroid (CFU-E) was obtained upon culture of fetal liver progenitors. Moreover, several different erythroid markers genes were affected: We observed an increase in expression of Pu.1 and c-Myb, but a decrease in beta-major and Alas2. No changes were observed in Gata1 and EpoR mRNA expression levels. The results indicate and support the notion that erythroid specific overexpression of Evi1 interferes with erythroid development in the mouse. In **Chapter 3** we described analysis of Evi1 expression in early erythroid progenitors using Cre mouse under the erythropoietin receptor promoter. The analysis revealed severe

phenotype characterized by profound cell death, decrease number of progenitor cells, inability of erythroid precursors to terminally differentiate, and dysplasia.

Chapter 4 of this thesis describes identification and analysis of a novel Evi1 binding partner found in yeast two-hybrid screen. Methyl binding domain 3b (Mbd3b) was found to interact with Evi1, a component of histone deacetylase complex (NuRD). The association between several other transcriptional factors and NuRD indicate its role in transcriptional repression. The interaction requires zinc finger domain of Evi1 and the MBD domain plus a short stretch of amino acids close to the MBD domain of Mbd3b. Biochemical analysis revealed that both proteins interact in vivo, and are found in subcellular speckles in the nucleus. Although the nature of the nuclear speckles has been not identified up to date, it may represent specific loci where Evi1 interacts with DNA. Interaction of Evi1 with Mbd3b may recruit the NuRD complex to those specific areas. In luciferase reporter assay the direct interaction between those two proteins was able to repress transcriptional activity. The binding between Evi1 and Mbd3b showed that it might be involved in epigenetic chromatin modification and leukemic transformation.
Evi1 belongs to the PRDM family of genes. The features of the PRDM3 (MDS-Evi1) gene are highly analogous to the PRDM2 (RIZ) and PRDM1 (Blimp-1) genes, in which each express a truncated protein missing the PR domain. It has been demonstrated that another member of this family of proteins PRDM1 interacts with the histone methyltransferases G9a. In **Chapter 5** we analyze whether Evi1 may also interact with histone methyltransferases. The findings indicate that Evi1 indeed physically interacts with methyltransfereases, however the interactions are selective as it interacts with SUV39H1 and G9a but not with Set9. The binding domain of SUV39h1 has been mapped to involve SET domain, a common motif found in several different methyltransferases. As the binding does not interfere with SUV39H1 enzymatic activity, we showed that upon interaction with SUV39H1, Evi1 is able to exhibits methylation activity. Further analysis showed that the complex of SUV39H1/Evi1 can be found bound to histone tails. Since specific methylation of histone tails is involved in transcriptional repression, we could demonstrate that presence of SUV39H1 enhances Evi1 transcriptional repression in a dose dependent manner. These results establish Evi1 as a transcriptional regulator that is able to form higher order complexes with histone methyltransferases and may cause transcriptional repression of putative target genes during tumorgenesis.
The general discussion described in **Chapter 6** summarizes our findings and provides possible explanation for the role of Evi1in leukemic development.

Samenvatting

EVI1 (Afkorting voor: Ecotropic Viral Integration site 1) is een nucleair eiwit met specifieke domeinen (zogenaamde zinkvingers) die kunnen binden aan DNA. EVI1 is oorspronkelijk geïdentificeerd als een eiwit dat betrokken is bij de ontwikkeling van leukemie in de muis. Daarnaast is abnormale expressie van EVI1 in de hematopoietische voorlopercellen, als gevolg van bijvoorbeeld chromosomale translocaties, (is) aangetoond in patiënten met myelodysplastisch syndroom (MDS) en acute myeloïde leukemie (AML). Deze bevindingen suggereren dat EVI1 mogelijk een rol speelt bij de ontwikkeling en progressie van leukemie. Patiënten met verhoogde expressie van EVI1 reageren over het algemeen relatief slecht op de huidige therapieën.
Alhoewel de exacte rol van EVI1 nog onduidelijk is lijkt het eiwit betrokken te zijn bij transcriptionele genregulatie. Ondanks dat EVI1 expressie noodzakelijk is voor embryonale ontwikkeling, blijkt het niveau van EVI1 minder essentieel later in de ontwikkeling.

Hoofdstuk 1 geeft een overzicht van de normale bloedcelvorming, in het bijzonder de erythroïde en myeloïde bloedcelontwikkeling. De rol en functie van de belangrijkste transcriptiefactoren in deze twee specifieke bloedcel lijnen worden bediscussieerd. In dit hoofdstuk wordt EVI1 besproken met betrekking tot 1) leukemieontwikkeling, 2) interactie met andere eiwitten en 3) de EVI1-fusie eiwitten die worden gevonden in humane leukemieën. Tot besluit, wordt de regulatie van gentranscriptie door histon modificatie beschreven.
Cellulaire transformatie als gevolg van verhoogde expressie van EVI1 is aangetoond in *in vitro* modelsystemen. Echter, het effect van abnormaal verhoogde expressie van EVI1 in *in vivo* systemen was nog niet bestudeerd. Om deze *in vivo* effecten te onderzoeken hebben we een nieuw EVI1 transgeen muizenmodel gegenereerd en geanalyseerd, hetgeen staat beschreven in **Hoofdstuk 2**. Door EVI1, in dit conditionele muizenmodel, specifiek tot expressie te laten komen in de erythroïde bloedcellen, tonen we aan dat EVI lethaal is voor bijna 40% van de muizenembryo's. De foetale levers van muizen met verhoogde EVI1 expressie vertoonden een anemie, d.w.z. een duidelijk verlaagde hoeveelheid aan rode bloedcellen. De rode bloedcellen die aanwezig waren vertoonden een abnormale erythroïde ontwikkeling, gekarakteriseerd door karakteristieke grote displastische cellen. De capaciteit van de rode cellen om te differentiëren is ook gestoord aangezien er een duidelijke reductie in kolonie vormende cellen werd aangetoond wanneer de cellen uit de foetale levers *in vitro* werden gekweekt. Verhoogde EVI1 expressie resulteerde ook in abnormale expressie van verschillende erythoïd- specifieke genen. De genen

die coderen voor de transcriptiefactoren Pu.1 en c-Myb kwamen verhoogd tot expressie, terwijl betaglobine (major) en AlaS verlaagd waren. Geen veranderingen in expressie werden aangetoond voor de erythroïd-specifieke genen die coderen voor Gata1 en erythropoietine receptor. We tonen aan dat verhoogde EVI1 expressie in een *in vivo* muizenmodel de normale ontwikkeling van erythroïde cellen in het embryonale stadium verstoord. In **Hoofdstuk 3** onderzochten we de effecten van EVI1 in vroege erythroïde voorloper cellen door de in hoofdstuk 3 beschreven transgene EVI1 muizen te kruisen met muizen waarbij het Cre-gen onder controle staat van de Epo-receptor promoter. Dubbel transgene embryo's vertonen een ernstig fenotype, namelijk veel celdood van foetale lever cellen, een sterke afname van het aantal erythroïde voorloper cellen, onvermogen van voorlopercellen om te differentiëren en dysplasie.

Hoofdstuk 4 beschrijft de identificatie en analyse, met het zogenaamde two-hybrid systeem, van een nieuw eiwit dat aan EVI1 bindt. Dit eiwit, Mbd3b behoort tot een familie van eiwitten dat specifiek bindt aan gemethyleerd DNA. Het is onderdeel van een groter complex (NuRD) dat betrokken is bij de modificatie van histonen en kan daarbij de expressie van genen verlagen door middel van deacetylering. EVI1 wordt over het algemeen gezien als een transcriptionele repressor. Het feit dat EVI1 kan binden aan het NuRD complex suggereert dat EVI1 de expressie van specifieke genen mogelijk verlaagd als gevolg van de NuRD functie. EVI1 en Mbd3b binden door middel van specifieke domeinen in EVI1, de zogaande zinkvingers, en het methyl bindingsdomein van Mbd3b. Met biochemische analyses werd aangetoond dat de binding tussen EVI1 en Mbd3b optreedt *in vivo* en dat beide eiwitten zich bevinden in specifieke nucleaire structuren. Ondanks dat de exacte functie van deze structuren onbekend is, zouden het mogelijk de plaatsen aangeven waar EVI1 aan het DNA bindt. Het NuRD complex zou als gevolg van de interactie van EVI1 met Mbd3d naar deze specifieke plaatsen worden getransporteerd. Middels specifieke *in vitro* transcriptie analyses tonen we aan dat de directe interactie tussen EVI1 en Mbd3b inderdaad transcriptionele repressie tot gevolg heeft. Deze resultaten zouden kunnen suggereren dat EVI1 zijn leukemische transformatie ontleent aan de binding met Mbd3b dat, als onderdeel van het NuRD complex, resulteert in de modificatie van het chromatine.
EVI1 behoort tot de familie van zogenaamde PRDM (PR domein-bevattende) eiwitten. De eigenschappen van het *PRDM3* (MDS-EVI1) gen zijn analoog aan de genen die coderen voor PRDM2 (RIZ) en PRDM1 (BLIMP-1), waarbij naast het volledige gen product ook een verkort eiwit tot expressie kan komen, zonder het PR domein. In het verleden is aangetoond dat PRDM1 bindt aan de histon methyltransferase G9a. In **Hoofdstuk 5** hebben we onderzocht of EVI1 ook

bindt aan histon methyltransferases. We hebben aangetoond dat EVI1 inderdaad een selectieve interactie aangaat met twee specifieke methyltransferases, SUV39H1 en G9a. EVI1 bindt echter niet aan een ander methyltransferase Set9. Het zogenaamde SET domein, een domein dat frequent wordt gevonden bij methyltransferases, van SUV39h1, is noodzakelijk voor de binding aan EVI1. Deze binding interfereert niet met de normale enzymatische functie van SUV39h1 en we laten zien dat EVI1 door middel van de interactie met SUV39h1 methyltransferase activiteit krijgt. We hebben tevens laten zien dat het EVI1/SUV39h1 complex aan histonen bindt. Specifieke methylering van histonen resulteert in transcriptionele repressie. We hebben aangetoond dat de transcriptionele repressie van EVI1 versterkt naar mate er meer SUV39h1 aanwezig is. Onze resultaten impliceren dat EVI1 onderdeel is van complexen van eiwitten met methyltransferase activiteit. Deze activiteit is mogelijk betrokken bij de remming van de transcriptie van EVI1-gereguleerde genen en EVI1-gemedieerde tumorgenese.

De algemene discussie beschreven in **Hoofdstuk 6** geeft een samenvatting van de bevindingen en belicht de rol van EVI1 in leukemie ontwikkeling naar aanleiding van de bevindingen beschreven in dit proefschrift.

List of abbreviations

293T	Human primary embryonal kidney cells
32D	Growth factor-dependent mouse bone marrow cells
7-AAD	7-amino actinomycin D
AML	Acute myeloid leukemia
BFU-e	Burst forming unit erythroid
cDNA	Complementary deoxyribonucleic acid
CD 71	Transferrin receptor
CFU-e	Colony forming unit erythroid
c-kit (CD117)	Stem cell factor receptor
COS-7	African green monkey kidney cell line
DNA	Deoxyribonucleic acid
E	Embryonic day
EGFP	Enhanced green fluorescence protein
Epo	Erythropoietin
Evi	Ecotropic integration site
FACS	Fluorescence-activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GST	Glutathione S-transferase
HDAC	Histone deacetylase
HSC	Hematopoietic stem cell
HTMase	Histone methyltransferases assay
IL	Interluekin
IP	Immuno precipitation
kb	Kilo base
kD	Kilo Dalton
LCR	Locus control region
MDS	Myelodysplastic syndrome
MEL	Mouse erythroleukemia
ONPG	o-nitrophenyl-β-d-galactopyranoside
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
φE	Retrovirus producer cells based on the 293T cell line
SCF	Stem cell factor
TAP	Tandem Affinity Purification
TER119	Erythroid cell marker
TRITC	Tetramethylrhodamine isothiocyanate
WB	Western blotting
Y2H	Yeast-two hybrid

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