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Characterisation of the leukaemia-associated ETO homologues

Doctoral Thesis

by

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Division of Hematology and Transfusion Medicine

Lund University, Sweden



LUND UNIVERSITY
Faculty of Medicine

This thesis will be defended on October 27, 2006 at 9.00
in Segerfalksalen, Wallenberg Neurocentrum, BMC, Sölvegatan 17, Lund

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To My Captain

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

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- II. Lindberg SR*, Olsson A*, Persson A-M, Olsson I. The leukemia-associated ETO homologues are differently expressed during hematopoietic differentiation.
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**These authors contributed equally to this work*
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Submitted for publication
- IV. Lindberg SR, Persson A-M, Olsson I. Upregulation and downregulation of the leukemia-associated ETO family of corepressors.
Manuscript

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Selected abbreviations

ALL	Acute Lymphoid Leukaemia
AML	Acute Myeloid Leukaemia
AML1	Acute Myeloid Leukaemia 1
Bcl-6	B-Cell Lymphoma 6
CBF	Core Binding Factor
cDNA	complementary DNA
C/EBP	CCAAT-Enhancer Binding Protein
ETO	Eight Twenty One
G-CSF	Granulocyte Colony Stimulating Factor
Gfi-1	Growth Factor Independence 1
GM-CSF	Granulocyte- Macrophage Colony Stimulating Factor
HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylase
HEB	Human E-box Binding factor
HEL	Human Erythroid Leukaemia
HHR	Hydrophobic Heptad Repeat
M-CSF	Macrophage Colony Stimulating Factor
MTG8	Myeloid Translocation Gene 8
MTG16	Myeloid Translocation Gene 16
MTGR1	Myeloid Translocation Gene Related protein 1
N-CoR	Nuclear Co-Repressor
NHR	Nervy Homology Region
PLZF	Promyelocytic Leukaemia Zinc Finger protein
RHD	Runt Homology Domain
SCL	Stem Cell Leukemia
SMRT	Silencing Mediator for Retinoid and Thyroid hormone receptors
TCR	T-Cell Receptor

Introduction

Haematopoiesis is a strictly controlled process, leading to the formation of cells necessary for a functional immune defence, oxygen transport and hemostasis. The high-rate production of blood cells and the firm control of the different processes make the system vulnerable to changes that affect the fine-tuned balance between proliferation, differentiation and cell death. In leukaemia, the production of mature blood cells is disrupted at some level, leading to an accumulation of non-mature blood cells at the expense of mature, functional cells. Frequently, leukaemia is associated with a chromosomal translocation involving a haematopoietic key transcription factor. The translocation results in the expression of an aberrant fusion protein with different properties and functions than those of the normal transcription factor. t(8;21) is the most frequent translocation found in patients with acute myeloid leukaemia. It results in the expression of the chimaeric protein AML1-ETO. AML1 functions as an essential transcription factor in the haematopoietic system. The normal function of ETO, on the other hand, is largely unknown. The ETO component of AML1-ETO possesses the fusion protein with features that are crucial for its actions. The focus of this work is on the function of ETO and its two homologues, MTGR1 and MTG16, with the aim to elucidate more about their functions in ordinary life as well as in leukaemogenesis.

Background

Haematopoiesis

Formation of blood cells

The cells of the blood take part in a variety of specialised functions including oxygen transport, blood clotting, phagocytosis, antibody production, and

destruction of invading microorganisms or particles. All cells of the haematopoietic system originate from a pool of primitive stem cells in the bone marrow. Even though the stem cells represent about 0,1 % of the bone marrow population, they are able to constitute all blood cells produced during a person's life span. Only during one day, about 300 billion blood cells are produced. The continuous formation of mature cells is dependent on the stem cell's multipotency, its capacity to self-renew and its ability to undergo asymmetric cell divisions. Thus, during cell division, it can either give rise to an identical daughter cell, or to an early progenitor cell, committed to differentiation. The early progenitor cells are multipotent and have the capacity to differentiate into several of the different lineages of the haematopoietic system. With time, their commitment becomes irreversibly restricted to one lineage. When maturation is complete, the blood cells leave the bone marrow and migrate into the blood and tissues to exert their functions. There are two main branches in the haematopoietic tree. The haematopoietic stem cell can either give rise to a common lymphoid progenitor (CLP) that is the origin of the lymphoid lineages, or it can turn into a common myeloid progenitor that subsequently differentiates into either megacaryocyte/erythrocyte progenitors (MEPs) or granulocyte/macrophage progenitors (GMPs). A schematic overview of the haematopoietic system is given in Fig 1 (1, 2).

Regulation of haematopoiesis

Given the vast amount of blood cells that are constantly needed for a proper body function, their production needs to be strictly regulated. Haematopoiesis is controlled by a combination of intrinsic transcription factors and external growth factors (cytokines). Transcription factors activate or inhibit genes involved in various differentiation programs. The effect of a transcription factor is both dependent on the differentiation stage of the cell and on other transcription factors simultaneously expressed. Cytokines are necessary for the survival, proliferation

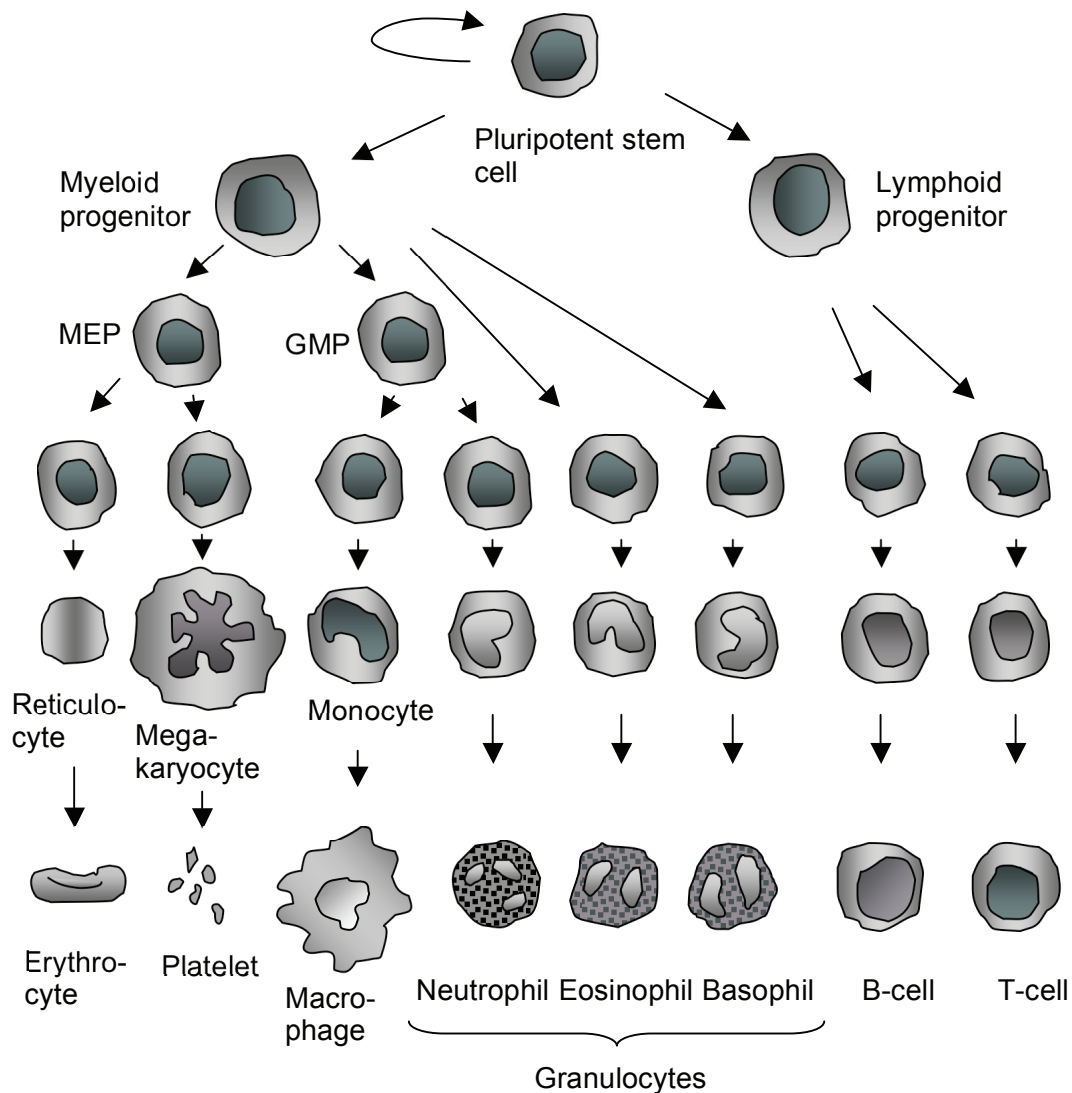


Fig 1. Haematopoiesis.

and differentiation of blood cells. Most of them are pleiotropic, i.e. they affect many different cell types. Only a few cytokines are lineage specific. Also, the combination of cytokines is critical for their effect. Thus, depending on the situation, the same cytokine can be either stimulatory or inhibitory. It is also known that many cytokines have overlapping functions and can substitute for each other (1, 2).

Granulopoiesis

Granulocytes characteristically contain lysosomes and secretory vesicles (granules). The granulocytic population consists of neutrophils (96%), eosinophils (3%) and basophils (1%), all involved in the defence against invading particles. The neutrophil has a critical role in the innate immune defence. Neutrophils are instantly recruited to the site of an infection where they function in the destruction of pathogens. Microorganisms are phagocytised by the neutrophils and killed by oxygen-dependent as well as oxygen-independent mechanisms. When the neutrophil has finished its task, it undergoes apoptosis. The production of neutrophils must for obvious reasons be fast and strictly controlled, and there must always be a pool of mature neutrophils that can be recruited when required. Shortage of neutrophils leads to a reduced immune defence and can result in a life threatening condition. The granulopoiesis is subdivided into several stages (Fig. 2). The maturation process in the bone marrow takes about 10 days. Once in the blood stream and tissues, the granulocyte has a very short life span of about two days. The major cytokine involved in granulopoiesis is granulocyte-colony stimulating factor (G-CSF) but several cytokines, synthesised by different cell types, act in concert to selectively produce granulocytes. The concentrations of these factors are rapidly upregulated when the body faces an infection (1, 2).

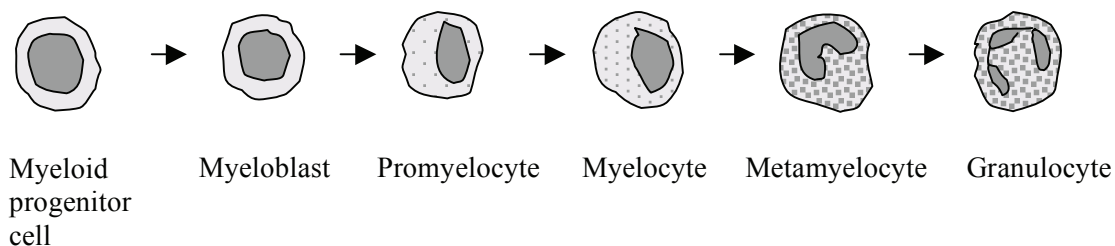


Fig 2. Granulopoiesis.

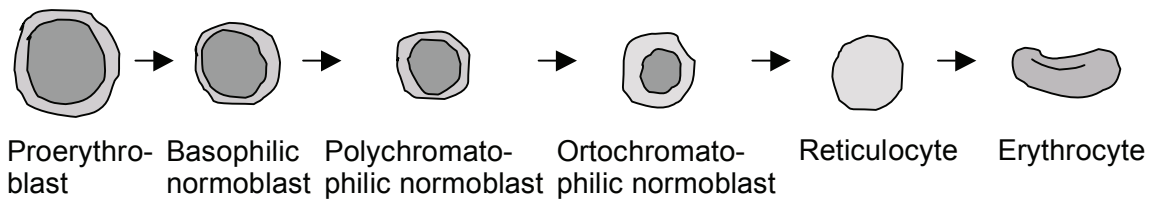


Fig 3. Erythropoiesis

Erythropoiesis

The erythrocyte is the most common cell type in the blood and it functions in the blood stream as a carrier of oxygen and carbon dioxide. The mature cell is filled with hemoglobin but lacks the usual cell organelles and is therefore unable to grow or divide. The different stages of erythropoiesis are presented in Fig 3. The first identifiable erythroid-restricted cell is the proerythroblast. During the maturation process the erythroid cell gets smaller, it starts to produce hemoglobin and the nucleus is reduced, to finally get excluded from the cell. At that stage the cell enters the blood stream as a reticulocyte. Once the cell loses its ribosomes it becomes a mature erythrocyte, with a survival of about 120 days. The major cytokine required for erythrocyte production is erythropoietin, which is synthesised in the kidney and is increased as a response to lack of oxygen or shortage of erythrocytes. The early erythroid stem cells are independent of erythropoietin but require IL-3, SCF and GM-CSF for proliferation and differentiation. However, the presence of erythropoietin is obligatory from the CFU-E stage and onwards (1, 2).

Transcriptional regulation

The phenotype, status and fate of a cell are mainly dependent on the combination of genes it expresses. The number of proteins involved in gene regulation reflects the complexity of the process; 5-10% of the estimated 30000 human gene products

are believed to act as transcriptional regulators. For transcriptional activation to occur, RNA polymerase must bind to DNA and form a transcription complex together with a number of conserved general transcription factors, where after the DNA helix is opened up and transcription can start. In the chromosomes, the DNA is bound to histone proteins and tightly packed into rigid nucleosomes. During transcription, these tight structures must be relieved so that the transcriptional machinery can access the DNA.

Transcriptional activators work in different ways to facilitate the work of RNA polymerase. They can interact directly with the transcription complex, either to assist in complex formation, or to facilitate the binding to DNA. They can also act by affecting the chromatin structure and thus make way for transcription. The major ways to induce chromatin changes are through covalent histone modifications (acetylations, methylations and phosphorylations) and nucleosome remodelling. Local histone acetylation is associated with transcription initiation. Thus, transcriptional activators frequently bind histone acetyl transferases (HATs) that catalyse histone acetylation. Transcriptional activators can also recruit chromatin remodelling complexes that act to make the chromatin accessible.

As opposed to transcriptional activators, the task of transcriptional repressors is to inhibit the binding or function of the RNA polymerase complex. This can be accomplished in several ways. For instance, a repressor can bind to the promoter to prevent the binding of activators. Or it can interact directly with the activator protein or the general transcription factors to prevent the actions of a transcriptional activator. It can also function in chromatin modifications, either by recruiting a repressive chromatin remodelling complex, or by removal of acetyl groups covalently bound to histones. Thus, many transcriptional repressors recruit histone deacetylases (HDACs) that renders the chromatin inaccessible for transcription.

Transcriptional regulators do not act as individual players but rather in cooperation with a number of other regulatory proteins. Thus they are parts of activation or repressor complexes where each protein has a specific function. The complex binds to a DNA consensus site to activate or repress transcription. The regulatory proteins of these complexes that do not bind directly to DNA are called coactivators or corepressors. A single regulator can participate in several different complexes and depending on context the same protein can act both as an activator or a repressor of transcription. This brings an enormous intricacy into the machinery of transcriptional regulation, where several outcomes are possible dependent of the final assembly of the transcription complex (2, 3).

Leukaemia

Leukaemia arises through a clonal expansion of non-mature blood cells combined with a partial or total differentiation block along one of the different lineages of haematopoiesis. The expansion of primitive cells leads to a shortage of mature cells of all lineages, resulting in hemorrhages, anaemia and susceptibility to infections. Depending on the haematopoietic lineage affected, leukaemias are termed either lymphoid or myeloid. Leukaemias can also be either acute or chronic based on the expansion rate. Acute leukaemias have a very fast and aggressive progression, whereas chronic leukaemias can evolve for a long time until they finally reach an acute phase (blast crisis). Chronic lymphoid leukaemia (CLL) is the most common form of leukaemia in the Western world and it is mainly found in elderly patients, which is also a feature of the more unusual disease chronic myeloid leukaemia (CML). Acute leukaemia on the other hand, is found in people of all ages with an increased incidence during the first years as well as after the age of 55. Acute lymphoid leukaemia (ALL) is the most common form of leukaemia in children, while acute myeloid leukaemia (AML) is the predominant form of acute leukaemia in adults (1, 4).

Acute myeloid leukaemia

AML is subdivided into 10 subgroups (M0-M3, M4, M4e, M5a&b, M6 and M7) according to the French-American-British classification. The classification is mainly based on the differentiation stage where the block occurs and on the degree of maturation observed. Each subgroup also shows a characteristic morphology. The etiology of acute myeloid leukaemia is mainly dependent on environmental factors and not on inheritance. However, some inherited diseases predispose individuals to AML, i. e. Down's syndrome, Fanconi anemia, neurofibromatosis, Shachman syndrome and Kostmann syndrome. AML development affecting a monozygous twin is associated with an increased risk for the other twin. However this is not inherited but dependent on prenatal twin-twin transfusion of a premalignant clone. Environmental factors with a strong association to AML development are exposure to ionisation radiation, benzene and chemotherapeutics. More vague associations proposed include ethanol and cigarettes. AML can also occur following therapy that includes the use of alchylating substances or topoisomerase II inhibitors (1, 4).

Chromosomal translocations

Chromosomal translocations are frequently associated with human cancer. The phenomenon is most prominent in haematological diseases. Epithelial tumours also display translocations but they are usually masked by a much more complicated karyotype. In leukaemia, chromosomal translocations are found in more than 50% of all cases. Characteristically, these translocations are balanced, representing a reciprocal exchange of gene segments without a net loss of DNA. Furthermore, the translocation is typically the only karyotypic change associated with the disease and in most cases a specific translocation leads to a specific sub-type of leukaemia (5-7).

The translocation results in one of two representative outcomes. The first is that the translocation occurs in the middle of two unrelated genes, which are fused

together to form a chimaeric protein with new properties. Commonly, one of these genes is a transcription factor with an important function in haematopoiesis (8). The translocation is thought to arise in an early progenitor cell and to block terminal differentiation. Additional mutations, still undefined, are believed to give the cell a survival advantage, manifested as an increased proliferation rate or a reduced cell death (apoptosis) (9). The other possible result of a translocation is that a gene is positioned close to a strong promoter sequence such as those for immunoglobulin or T-cell receptor genes. In this way proto-oncogenes can be activated and contribute to the progression of leukaemia.

The mechanisms behind translocations are basically unknown. Some inherited diseases affecting DNA repair, can predispose individuals to leukaemia. There seem to be some chromosomal hotspots that are prone to be involved in double stranded breaks of DNA. Proposed features of these sequences include purine and pyrimidine repeat regions, scaffold and matrix attachment regions (S/MARs) and DNA topo II cleavage sites. DNA sequences rich in purines and pyrimidines can form an unusual left-handed form of DNA, termed Z-DNA. Z-DNA is mainly situated in inter-nucleosomal regions and thus more susceptible for double-stranded breaks than histone-bound DNA. S/MARs are preferable cleavage sites during early apoptosis and it has been suggested that initiated but reversed apoptosis could lead to chromosomal translocations. The mechanism of topo II induced translocations are poorly understood but could simply represent a mis-repair of topo II induced double stranded DNA breaks (10).

t(8;21) and AML1-ETO

The most frequent translocation in patients with AML is the t(8;21). It is associated with the M2 subtype of the FAB system, constituting 40% of leukaemias found in this group. The M2 subtype is characterized by a partial differentiation block at the myeloblastic stage (11, 12). The t(8;21) rearrangement was first described in 1973 (13, 14) and in the early 90's the two gene parts

involved in the fusion were identified. It was shown that the previously identified haematopoietic transcription factor AML1 on chromosome 21 was fused to an unknown protein that was termed eight-twenty-one (ETO) or myeloid translocation gene 8 (MTG8), on chromosome 8. (15-18). The fusion results in the expression of the aberrant protein AML1-ETO that consists of the N-terminal part of AML1 fused to almost the entire ETO protein (Fig 4). There are three alternative breakpoint regions in AML1 and four in ETO that give rise to AML1-ETO, however they all result in the same fusion protein. The breakpoints of both AML1 and ETO are associated with topoisomerase II cleavage and DNase I hypersensitive sites (19). The reciprocal protein (ETO-AML1) is not expressed.

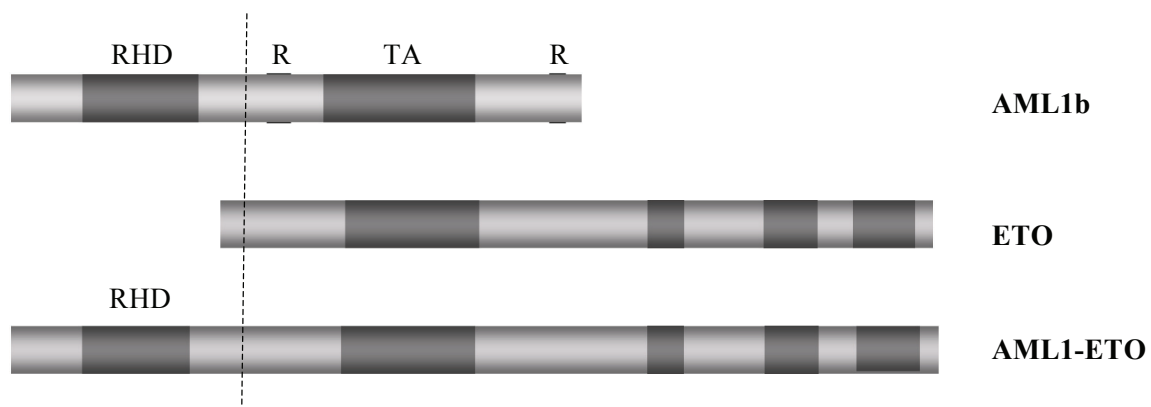


Fig 4. Fusion of AML1 to ETO in t(8;21). AML1 contains a DNA-binding Runt Homology Domain (RHD), a transactivation (TA) domain and two repression (R) domains. The translocation t(8;21), fuses the N-terminal part of AML1 to almost the entire ETO protein, resulting in the chimaeric protein AML1-ETO.

AML1

The Runx family

Acute myeloid leukaemia 1 (AML1, also termed RUNX1) belongs to the Runx gene family that also comprises AML2 (RUNX3) and AML3 (RUNX2) (20). The Runx proteins are key transcriptional regulators with distinct functions in development and with tissue-specific expression (21). The proteins are nuclear and

have two large functional domains, an N-terminal DNA-binding domain and a C-terminal transactivation domain. In addition, the C-terminal contains two repressor domains (Fig 4) The DNA-binding domain is called the runt homology domain (RHD) because of its homology to the drosophila protein Runt. Besides binding to DNA it also binds to the cofactor CBF β to form the core binding factor (CBF) complex (22, 23).

Gene structure of AML1

The gene for AML1 is situated on chromosome 21, band q22, and contains 12 exons spanning 260 kb. The gene can give rise to multiple isoforms through the use of two independent promoters and alternate splicing (24). The major isoforms that are formed are AML1b (453 aa) and AML1c (480 aa). AML1a (250 aa) is less abundant and represents a truncated form of AML1 that lacks the transactivation domain (25).

Transcriptional regulation by AML1

Transcriptional regulation exerted by AML1 is initiated through binding of the RHD to the DNA consensus sequence TGT/cGGT (23, 26). The binding is simplified by the cofactor CBF β (27, 28). AML1 then binds to other nuclear factors, in particular other lineage-specific transcription factors with DNA binding sites adjacent to that of AML1, forming a transcription complex. AML1 functions as a transcriptional organiser that brings the different transcription factors together, whereafter they work in synergy to regulate transcription. Examples of haematopoietic transcription factors that cooperate with AML1 are C/EBP α , PU.1, c-myb, ETS and GATA1 (29-34). Based on cell context, AML1 can act either as an activator or a repressor of gene expression. Transactivation of a gene is mediated through binding of the cofactors p300 and CREB-binding protein (CBP) (35). These factors have an intrinsic histone acetyl transferase (HAT) activity, allowing the acetylation of the chromatin that makes it accessible for transcription.

Furthermore, AML1 can bind to the coactivators ALY, YAP and MOZ (36-38). AML1 can also function as a transcriptional repressor through the binding to several factors involved in repression, like EAR-2, SIN3A, TLE and SUV39H1 (39-42). A number of genes have been shown to be activated by AML1. On the other hand, no genes have so far been identified that are repressed by AML1.

AML1 in haematopoiesis

AML1 regulates the expression of several genes that are necessary during haematopoiesis. Examples are interleukin-3 (IL-3), myeloperoxidase (MPO), neutrophil elastase (NE), granulocyte- macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), colony stimulating factor 1 (CSF-1), granzyme B and T cell receptor (TCR) subunits α , β , γ and δ (30, 43-51). The importance of AML1 in haematopoiesis is manifested through attempts to create mouse knockouts. Homologous disruption of either AML1 or its cofactor CBF β results in embryonal lethality. These mice lack a definitive haematopoiesis and die *in utero* due to hemorrhages in the central nervous system (52, 53).

AML1 in leukaemia

AML1 is one of the most targeted genes in chromosomal translocations leading to leukaemia. So far, at least 18 translocations, leading to AML, ALL or myelodysplastic syndrome (MDS), have been described that involves AML1 (54). Besides the t(8;21), some of the best described translocations include t(12;21), resulting in the TEL-AML1 fusion protein found in paediatric ALL patients, and t(3;21), resulting in the aggressive AML1-MDS1/EVI1 fusion protein associated with therapy-related MDS/AML and CML in blast crisis (55-58).

Besides the involvement of AML1 in chromosomal translocations, inherited and acquired point mutations of AML1 can lead to leukaemia. Point mutations can introduce a premature stop codon, producing a truncated protein lacking the

transactivation domain, which acts dominantly over full-length AML1. Alternatively, point mutations can target the RHD, resulting in an AML1 protein with abrogated DNA binding that can compete for binding of cofactors. Finally, recent reports have suggested a link between overexpression of AML1 and leukaemia, since AML1 is often overexpressed in patients with pediatric ALL, due to gene amplification or polysomy of chromosome 21. The linking of haploinsufficiency or overexpression of AML1 to leukaemia is reviewed in (54).

The ETO homologues

After the identification of ETO as a fusion partner of AML1 in t(8;21), two homologues of ETO have been identified: myeloid translocation gene 16 (MTG16) and myeloid translocation gene related protein 1 (MTGR1). MTG16 was identified as the partner of AML1 in another translocation leading to leukaemia, the t(16;21). This is less common and associated with therapy-induced AML. MTGR1 was identified through its strong interaction with AML1-ETO and through homology searches in EST databases. (59-61). The protein family has counterparts in other species including mouse (mETO and ETO-2, the murine homologues of ETO and MTG16 respectively), *Drosophila* (nervy), chicken (cETO/cMTG8, cMTGR1 and cMTG16) and *Xenopus* (XMTG8, XMTGR1/XETOR and XMTG16) (62-67). The conservation of this protein family throughout evolution suggests an important function. The terminology of ETO homologues and the identified counterparts in other species are summarised in Table 1.

Gene and protein structure of the ETO homologues

The gene for ETO is found on chromosome 8q22, spans 87 kb and contains 13 exons. Alternative splicing of exon 1 results in two isoforms: ETOa, containing 577 amino acids, and ETOb, containing 604 amino acids. There are also two

splicing variants of exon 9, where one isoform would result in a truncated protein. Expression of these truncated forms of ETO have not been shown, however the presence of two alternate exons might have implications for the onset of leukaemia as will be discussed later. The MTG16 gene, located on chromosome 16q24, also has 13 exons and spans 73 kb. The isoforms expressed are MTG16a with 653 amino acids and MTG16b with 567 amino acids. The gene encoding MTGR1 is situated on chromosome 20q11, spans 68 kb and contains 14 exons. The two identified isoforms of the protein are MTGR1a, containing 575 amino acids, and MTGR1b, containing 604 amino acids. The similar sizes and organisations of the three genes imply that they originate from a common ancestor (59, 68, 69).

Human ETO homologue	Additional names	Homologues in other species
ETO	MTG8 CDR CBFA2T1	mETO (mouse) XMTG8 (<i>Xenopus</i>) cETO (chicken) Nervy (<i>Drosophila</i>)
MTG16	MTGR2 CBFA2T3	ETO-2 (mouse) XMTG16 (<i>Xenopus</i>) cMTG16 (chicken) Nervy (<i>Drosophila</i>)
MTGR1	EHT CBFA2T2	XETOR (<i>Xenopus</i>) cMTGR1 (chicken) Nervy (<i>Drosophila</i>)

Table 1. Terminology of the ETO homologues and identified counterparts in other species.

Sequence alignment of the ETO homologues and the drosophila homologue Nervy reveals four conserved domains that are termed Nervy Homology Region (NHR) 1-4 (60). NHR1 shows homology to several TATA-binding protein-associated factors (TAFs) including drosophila TAF110 and human TAF105 and TAF130. Thus, NHR1 is sometimes referred to as the TAF domain (70, 71). Recently the NMR structure of NHR1 was modelled and it was found that it resembles the structure of the PAH2 domain of the SIN3 corepressors (72). NHR2 is also called the HHR domain because it represents a hydrophobic heptad repeat, capable of forming a coiled coil that is frequently observed in oligomerisation domains (73). Accordingly, NHR2 is involved in protein-protein interactions between the ETO homologues (discussed below). NHR3 also forms an α -helix structure, with no recognised homology. NHR4 is also termed the zinc finger (ZF) domain, since it includes two zinc fingers. Zinc fingers are recurrently involved in DNA binding, however, ETO does not seem to bind directly to DNA. Instead the zinc fingers take parts in protein-protein interactions (see below). NHR4 are homologous to the apoptosis-induced protein RP-8 found in mouse, rat and nematodes and the

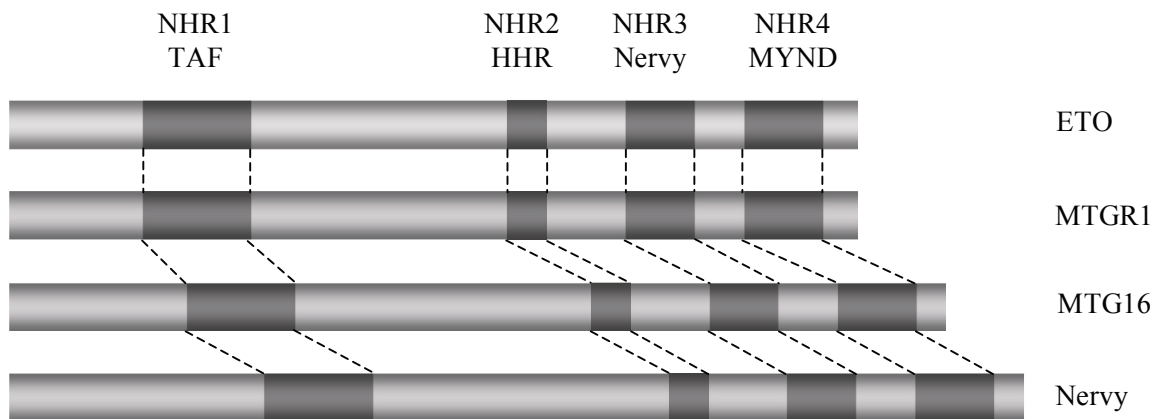


Fig 5. Schematic representation of the ETO homologues and Nervy. The Nervy Homology Regions (NHR) 1-4 are all conserved among the members.

Drosophila DEAF-1 protein. Therefore it is sometimes called the MYND domain (for myeloid nervy DEAF) (74, 75).

Expression of the ETO homologues

ETO is ubiquitously expressed in human tissues, albeit with a low expression in many of them. It is highly expressed in heart, brain and fetal brain (69). No ETO RNA is detected in peripheral blood (68). In haematopoietic cell lines, expression of ETO is restricted to the erythroleukaemic cell line HEL, myeloma cell lines and a few B-cell lines (76-78). In human bone marrow cells ETO is exclusively expressed in the erythroid lineage (78).

Both MTGR1 and MTG16 are ubiquitously expressed in human tissues. Except for the absence of MTG16 in kidney, both proteins are expressed in all tissues examined (59, 68). Also in most haematopoietic cell lines as well as bone marrow cells, both proteins are ubiquitously expressed, as opposed to the restricted expression of ETO in haematopoietic cells (68, 77-79).

Protein interactions of the ETO homologues

In 1998, three independent groups reported that ETO could bind to the corepressors N-CoR, SMRT and SIN3 and bind to histone deacetylases (HDACs) through the NHR4 domain (80-82). Recruitment of HDACs to a promoter closes the chromatin and makes it inaccessible for transcription. After these pioneering findings, it was established that several regions of ETO could bind to corepressors and different HDACs. In particular the NHR2 domain and the regions flanking it are important for binding of SIN3A (83, 84). ETO can bind to HDACs 1,2 and 3. The binding can either be direct or indirect through binding of SIN3 or N-CoR. The NHR2 important for corepressor interactions was also shown to be important for homo- or heterooligomerisation between the ETO homologues as well as AML1-ETO (60, 63, 85). This was first established through the binding between

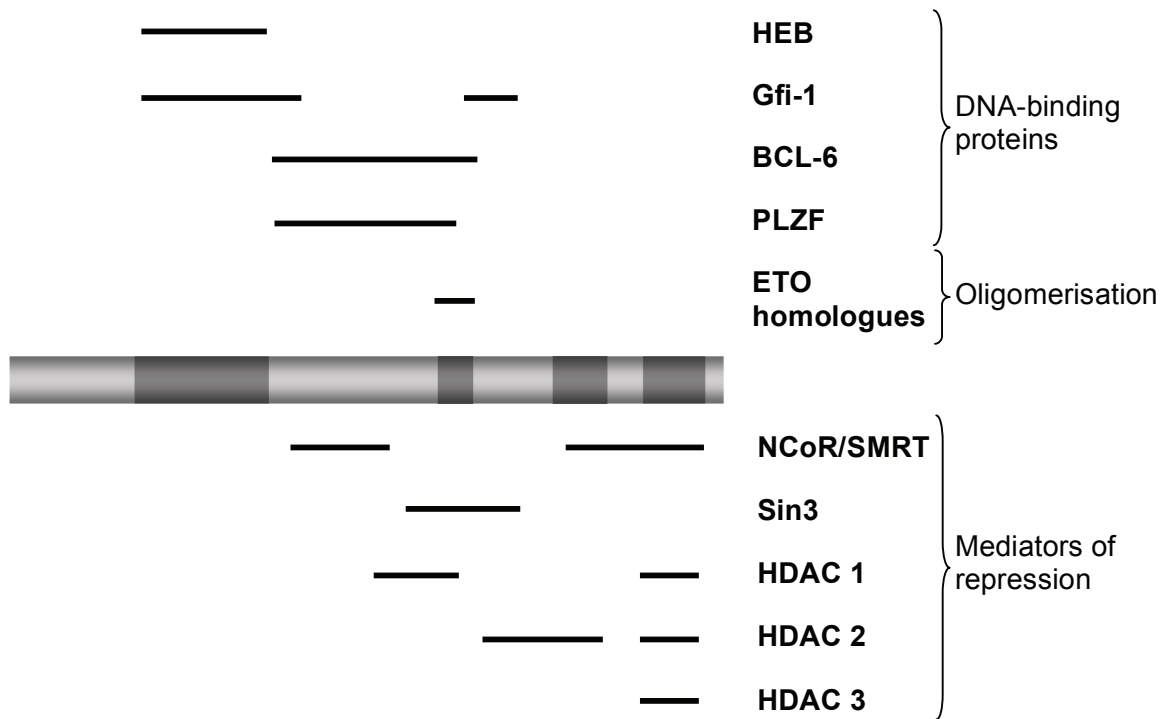


Fig 6. Regions of ETO involved in protein interactions.

AML1-ETO and MTGR1 (60). ETO has not been shown to bind directly to DNA. However it has been shown to bind to some DNA-binding proteins like Gfi-1, PLZF, Bcl-6, SCL1 and HEB (86-91). Another reported interaction partner of ETO is the neuronal protein Atrophin-1 (92). ETO has also been shown to interact with the regulatory subunit of type II cyclic AMP-dependent protein kinase (PKA RII α) (93). The different regions of ETO involved in protein- protein interactions are summarized in Fig 6.

Most studies on protein interactions are focused on ETO. The high homology between the ETO homologues implies that they all show a similar pattern of partner binding. However, the murine homologue of MTG16, ETO-2 does not bind to SIN3A, which either indicates that different homologues display different

binding patterns or that there is a species-difference in interaction partners. Amman *et al* showed that two amino acid residues in the NHR2 of ETO that is not conserved in ETO-2 could be mutated to disrupt association between SIN3A and ETO (83). These amino acids differ between ETO and human MTG16 as well, speaking in favour of a distinct corepressor binding pattern for each ETO homologue.

Function of the ETO homologues

The well-studied binding of corepressors to ETO has led to the general belief that the ETO homologues normally functions as parts of repressor complexes. Yet, the cellular responses of their actions are basically unknown. The differential expression and the maturation-associated regulation of the ETO homologues in bone marrow cells suggest a function for this protein family in haematopoiesis (78). The suggested DNA-binding interaction partners are in line with that theory, since Gfi-1, PLZF, Bcl-6 and SCL all have a role in haematopoiesis. Gfi-1 and its homologue Gfi-1B are involved in regulation of cell death, cell cycle progression and differentiation in haematopoietic cells (reviewed in (93)). Targeted disruption of Gfi-1B in mice showed that the protein was necessary for both erythroid and megakaryocytic differentiation (94). Gfi-1 has also been identified as a major regulator of haematopoietic stem cells (95, 96). Gfi-1 and ETO colocalise in the nuclear matrix and physically interact *in vivo* (87). PLZF is a transcriptional repressor that is expressed in CD34⁺ myeloid progenitor cells and is downregulated during differentiation. Furthermore, it suppresses growth, blocks differentiation and delays the cell cycle in myeloid cell lines, suggesting it needs to be downregulated for differentiation to proceed. Possible target genes are cyclin A and interleukin 3 (IL-3). ETO binds to PLZF and can augment transcriptional repression exerted by PLZF (88). Bcl-6 is normally expressed in B cells and mediates survival and proliferation. Bcl-6 is frequently overexpressed in human B cell myelomas. ETO and Bcl-6 are shown to interact in both normal and malignant

B-cells (86). SCL is a transcription factor that is required for erythroid and megakaryocytic differentiation. SCL only functions in complex with the ubiquitously expressed E proteins (E2A, HEB and E2-2) and it is generally expressed in multiprotein complexes. Studies of SCL-containing multiprotein complexes revealed that they contain ETO-2 and Gfi-1 (89, 91). The composition of the SCL/ETO-2 complexes differed between erythroid and megakaryocytic cells. ETO was also shown to bind directly to the E protein HEB (90).

Despite the suggested role for the ETO homologues in haematopoiesis, gene knock-outs in mice of either ETO or MTGR1 have affected the gastrointestinal system exclusively. Homozygous ETO deficient mice have a high mortality, but those who survive lack most of the intestine, which results in growth impairment (79). The MTGR1 knockouts have a reduced level of secretory cells in the small intestine (97). It is surprising that only the GI tract is affected in the mouse knock-outs, despite the ubiquitous expression of the ETO homologues. This could suggest that the different homologues can substitute for each other, but it can also represent a species difference between mouse and man.

ETO has also been proposed to have functions in the preadipocyte, where it physically interacts with and inhibits the function of the transcription factor C/EBP β . For adipogenesis to proceed, ETO must be downregulated so that C/EBP β can function in transcription activation (98). A role in neurogenesis for all the ETO homologues is suggested from studies in chicken and *Xenopus*, where the ETO homologues are expressed sequentially during neuronal development. Early expression of MTGR1 is followed by expression of ETO and MTG16. A dominant negative MTG protein inhibits neuronal differentiation in these systems (67). A function in the nervous system is also proposed by the binding of ETO to the neuronal protein Atrophin-1 (92). The ETO homologues might also have other functions than acting as corepressors. For instance it has been shown that

MTG16b can work as an A kinase anchoring protein (AKAP) in the cytoplasm in T-cells. AKAPs serves as scaffolds that helps to spatially and temporally colocalise the cell signalling molecule protein kinase A (PKA) and other signalling molecules in the cytoplasm (99).

Cellular localization of the ETO homologues

In line with their roles as transcriptional corepressors, the ETO homologues are mainly located to the nucleus (63, 100). Studies on ETO mutants have shown that ETO contains an unusual form of Nuclear Localisation Signal (NLS) that is situated between NHR1 and NHR2. The critical basic residues necessary for nuclear entry are conserved between the ETO homologues. It is also shown that the subnuclear distribution of ETO is dependent on NHR1 (101). Hoogeveen *et al* showed that MTG16a has a nucleolar localisation (77). In neural cells the expression of ETO can either be nuclear or cytoplasmic. This suggests that the intracellular localisation can be spatially and temporally regulated. Interactions with other proteins might dictate the direction of the ETO homologues in a cell (102).

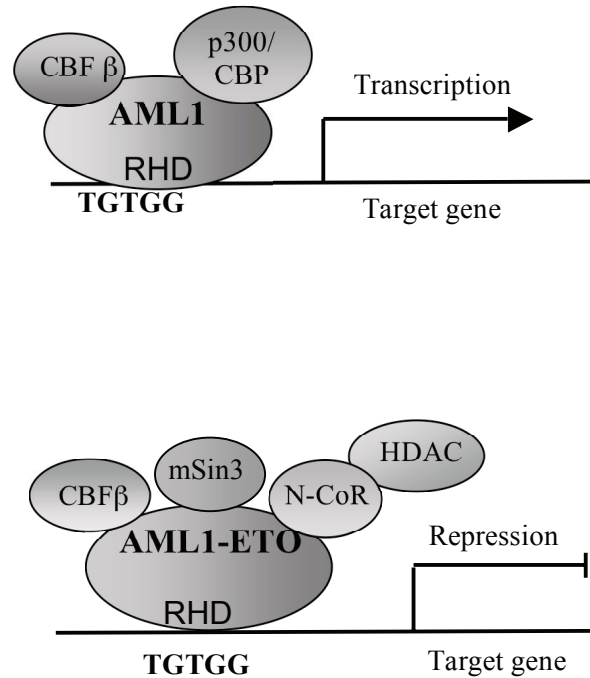
AML1-ETO and AML1-MTG16 in leukaemia

The translocations t(8;21) and t(16;21) are believed to arise in early CD34⁺ progenitor cells. For transformation into leukaemia there seems to be a requirement for additional genetic alterations. During the past decade there have been an extensive exploration of the function of AML1-ETO and its involvement in leukaemia. It has been shown that AML1-ETO can adverse the effects of AML1, but also equip the target cells with gains-of-function that are independent on AML1 function. The sequence of ETO seems to be critical for the function of the fusion protein, pointing at the need for a better understanding of the role of the ETO homologues.

Transcriptional regulation by AML1-ETO

When AML1 is fused to ETO through t(8;21), it retains the RHD domain and thus the ability to bind to DNA and to its cofactor CBF β . The transactivation domain on the other hand is lost and substituted by almost the entire ETO protein. The functional consequence of this substitution is that AML1 can no longer bind coactivators to activate transcription but instead binds to corepressors, through the ETO sequence, and represses transcription (Fig 7). Accordingly, AML1-ETO can repress AML1 target genes. Reporter gene studies have shown that AML1-ETO can adverse the activation of AML1 in the promoters of MDR-1, GM-CSF, IL-3, c-fos, TCR β enhancer, Ig α and p14^{ARF} (43, 103-108).

Fig 7. The basis of AML1-ETO mediated repression of AML1 genes. AML1 binds to the core sequence TGT/cGGT through the RHD domain. The recruitment of the coactivators p300 or CBP results in histone acetylation and transcriptional activation. In AML1-ETO, the RHD of AML1 is retained but the transactivation domain is substituted by the sequence of ETO. ETO can bind to the corepressors mSIN3 or NCoR and recruit HDACs. This results in the closing of chromatin and transcriptional repression.



AML1-ETO has also been shown to act as a positive regulator of transcription. AML1-ETO can activate transcription from the M-CSF promoter and enhance the AML1 mediated transcription dose-dependently. High expression of AML1-ETO

though, had an adverse effect on AML1 activity (109). AML1-ETO has also been shown to positively regulate the promoters of bcl-2 and G-CSFR (110, 111). Direct binding of AML1-ETO to an AML1 site in the promoter activates bcl-2. On the other hand, regulation of G-CSFR is indirect through AML1-ETO-mediated upregulation of C/EBP ϵ , which in turns activates transcription of G-CSFR.

AML1-ETO does not only act as a regulator of AML1 targets, but it can also bind to other transcription factors and regulate transcription that is independent of AML1 consensus sites. Through the RHD, AML1-ETO can bind and negatively regulate C/EBP α , PU.1 and MEF (33, 112-114). The ETO portion of the fusion protein can bind to PLZF or HEB (90, 115). Surprisingly, the effect of AML1-ETO on PLZF-mediated repression is diverse form that of ETO. While ETO enhances the repression exerted by PLZF, AML1-ETO acts as a repressor of PLZF activity, resulting in gene activation. The effect on the function of HEB on the other hand is the same as for that of ETO, that is transcriptional repression.

Cellular effects of AML1-ETO

The cellular respons to the presence of AML1-ETO have been studied in both haematopoetic cell lines and primary bone marrow cells, showing effects on differentiation as well as proliferation and cell survival. In cell lines, AML1-ETO can block erythroid and myeloid differentiation (39, 60, 105, 111, 113, 116-119). In line with this, suppression of AML1-ETO with siRNA induces differentiation of a t(8;21) positive cell line (120). Regarding proliferation, AML1-ETO promotes G-CSF dependent proliferation of 32D and L-G cells, (60, 117). On the other hand, inducible expression of AML1-ETO in U937 cells, showed that AML1-ETO has a negative impact on both cell cycle progression and survival (119, 121, 122). Some studies have been made that aimed at defining the gene expression profile of AML1-ETO expressing cells (123-125). One interesting finding was that genes that are downregulated by AML1-ETO are mainly involved in haematopoietic

differentiation (for instance the granulocytic transcription factors C/EBP α and C/EBP β and the erythroid/megakaryocytic transcription factor NFE2) whereas downregulated genes (for instance BCL11A and JAG1) have a role in the maintenance of a stem cell phenotype. Gene expression profiling also revealed that AML1-ETO can disrupt the expression of genes involved in DNA repair (123). Another observation was that AML1-ETO can induce several granule protein genes. This indicates that the fusion protein induces a partial differentiation, in line with the myeloblastic phenotype of t(8;21) leukaemic cells, but that it prevents terminal differentiation (124)

In primary human bone marrow cells, the presence of AML1-ETO initially blocks proliferation and colony formation. However this is followed by an increase in self-renewal (126, 127). Nevertheless, long-term cultures of AML1-ETO expressing CD34⁺ cells retain the ability to differentiate and they are not able to promote leukaemia when transferred to NOD/SCID mice (128). AML1-ETO expressed in progenitor cells has also been shown to block erythroid and myeloid but not monocytic differentiation. AML1-ETO transiently inhibits the growth of early progenitor cells but promotes the expansion of erythroid or myeloid progenitors. (127, 129, 130).

To summarise the effects of AML1-ETO in cellular systems, the role of the fusion protein in leukaemia development is contradictory. On the one hand, AML1-ETO blocks the differentiation of haematopoietic cells, which is favourable for leukaemia progression. On the other hand, at odds with its role as an oncogenic protein, AML1-ETO can inhibit proliferation and induce apoptosis. This suggests that t(8;21) positive cells must overcome the antiproliferative and apoptosis-promoting effects of AML1-ETO, to be fully transformed.

Cellular localisation of AML1-ETO

AML1 contains a nuclear matrix targeting signal (NMTS) that is not retained in AML1-ETO. Instead, the fusion protein is directed to an alternate nuclear matrix site by the NMTS of ETO. This relocation of AML1 in AML1-ETO in the nucleus might lead to the deregulation of AML1 target genes (131). Hoogeveen *et al* have shown that AML1-MTG16 lacks the nucleolar targeting signal found in MTG16a. AML1-MTG16 is therefore present in the nucleoplasm, where it binds to and redirects MTG16a from the nucleoplasm, thus disturbing its function (77).

Murine model systems of AML1-ETO leukaemia

Many attempts have been made to create a mouse model of AML1-ETO induced leukaemia. The first was a heterozygous “knock-in” of AML1-ETO that resulted in embryonal mortality. The mice displayed essentially the same phenotype as AML1 knock-out mice, suggesting that AML1-ETO exerts a dominant negative effect over the normal AML1 allele product (132, 133). After that, several models were created to circumvent the embryonal lethality. These include: 1) Inducible AML1-ETO expression, activated by tetracycline or Cre-mediated recombination (134, 135), 2) Directed expression of AML1-ETO into myeloid cells or haematopoietic stem cells through the promoters of MRP8 and Sca-1 respectively (136, 137) or 3) Bone marrow reconstitution with cells transduced with AML1-ETO (138, 139). Noticeably, in neither of these models, expression of AML1-ETO is sufficient for leukaemia development. Some AML1-ETO models display a blood phenotype similar to that of t(8;21) patients, whereas others have a near to normal haematopoiesis. Interestingly, in the inducible systems, treatment with mutational agents results in leukaemia. These models suggest that the spatial expression of the fusion protein is critical for its effects, but most importantly it points to the importance of additional genetic alterations for the onset of leukaemia. Potential genetic alterations that cooperate with AML1-ETO in the induction of leukaemia have been described and include the activated receptor

tyrosine kinases TEL-PDGF β R or FLT3, involved in intracellular signalling, and Wilms' tumor gene 1 (WT1), a protein frequently overexpressed in human leukaemias (140-142). Co-expression of either of these proteins with AML1-ETO can induce leukaemia in mice, supporting the proposed model of leukaemia as a result of the combinatorial effects of at least two genetic alterations. Another finding, strengthening this model, is that t(8;21) can be detected in healthy individuals and persist in AML-patients in long-term remission (143, 144).

The role of the Nery Homology Regions for the function of AML1-ETO

The fact that different regions of AML1-ETO can bind to different corepressors raises the question as to how the individual domains contribute to the function of the fusion protein. The corepressors SIN3, NCoR and SMRT are involved in the formation of numerous corepressor complexes whose compositions differ in a complicated manner (127). The interaction of AML1-ETO with all these corepressors implies that the fusion protein can recruit different complexes. Which interactions are then essential for the function of AML1-ETO? Are the actions of the fusion protein dependent on a single complex or do different complexes synergise to make a functional protein? The question is further complexed by the fact that the NHR2 does not only bind to SIN3A, but also contains the oligomerisation motif that is claimed to be critical for the function of AML1-ETO. Oligomerisation is a phenomenon proposed to have an important role for fusion protein function in leukaemia. AML1-ETO as well as the leukaemic fusion protein PML-RAR α are both found in high molecular weight complexes and the ability to oligomerise seems to be critical for the function of both fusion proteins. Thus, elimination of their oligomerisation domains impairs their ability to bind to N-CoR, repress transcription and block differentiation (145).

A number of studies with deletion mutants of the NHR2 and/or NHR4 of AML1-ETO have been performed in cell lines (summarized by Hug *et al.*(127)). The

results from these assays are contradictory. Simultaneous deletion of NHR2 and NHR4 always leads to a non-functional AML1-ETO. However, in some assays, AML1-ETO activity is NHR4-dependent, while in other assays, NHR4 is dispensable. Similarly, in some cases NHR2 accounts for all AML1-ETO activity, while in others, deletion of NHR2 is without functional significance. The diverse outcomes could reflect the different compositions of repressor complexes found in different cell lines. When AML1-ETO was expressed in enriched primary bone marrow cells, representing a more authentic environment for the fusion protein, it was found that individual deletions of NHR2 or NHR4 only had small effects on AML1-ETO function, whereas simultaneous deletion of both domains restored the function (127).

A surprising finding was that a truncated form of AML1-ETO, lacking the NHR4 domain, alone could induce leukaemia in mice, as opposed to wild-type AML1-ETO. The truncation eliminates the growth arrest exerted by the full-length protein (142). One theory that could explain the effect of the truncation is that an oncoprotein needs to be expressed at an optimal level to induce leukaemia. It was found in other leukaemia model system, that high levels of an oncoprotein might be toxic and that a reduced expression was needed for the penetrance of disease (146, 147). Thus, a truncated AML1-ETO could resemble the reduced fusion protein activity required for leukaemia progression. Recently, an extended study following the first interesting finding, showed that a high fraction of t(8;21) AML patients express a truncated form of AML1-ETO, due to alternate splicing of exon 9 in ETO. As mentioned earlier, the usage of exon 9a in ETO would introduce a premature stop codon after exon 8 in ETO resulting in a truncated protein (69). Expression of the truncated AML1-ETO (AML1-ETO9a) in mice resulted in a phenotype similar to that of t(8;21) AML. Most importantly, patients expressing AML1-ETO9a also express full-length AML1-ETO simultaneously, and coexpression of AML1-ETO9a with full-length AML1-ETO in mice resulted in an

early onset of leukaemia, showing that these proteins, working in concert, are highly potent inducers of AML (148).

A recent study on the NHR2 of AML1-ETO could give some further clues into the role of corepressor binding versus oligomerisation for the function of AML1-ETO (149). Fine tuning mutations of NHR2 that either disrupted oligomerisation or SIN3A binding were introduced in AML1-ETO. It was then found that AML1-ETO activity is abolished by mutations that disrupt oligomerisation, but not by mutations in the mSIN3A binding site, suggesting that oligomerisation is the major determinant of AML1-ETO function.

The SIN3 family of proteins

The SIN3 proteins are a family of conserved transcriptional regulators, involved in a multitude of cellular functions. In humans, there are two homologues, SIN3A and SIN3B. The proteins have several conserved domains involved in protein-protein interactions; 4 paired amphipathic α -helices (PAH 1-4), a HDAC interacting domain (HID) and a highly conserved region (HCR) (150, 151). SIN 3 acts as a component of the SIN3/HDAC repressor complex (152). The core of this complex contains 7 conserved proteins namely SIN3, HDAC1, HDAC2, retinoblastoma associated protein (RbAp) 46, RbAp 48, SIN associated protein (SAP) 30 and SAP 18. SIN 3 serves as a scaffold onto which the other proteins bind. The HDACs provide enzymatic activity to the complex and the RbAps and SAPs serves as stabilisers. This core complex is located to the PAH3 and HID domains of SIN3. The other conserved domains of SIN3 can then participate in a multitude of protein-protein interactions that targets the protein to DNA. SIN3 cannot bind directly to DNA but binds to different DNA binding proteins involved in transcriptional repression. It can also bind indirectly to DNA through other corepressors like NCoR or SMRT. There is an increasing list of proteins that

interact with SIN3 (summarised in (153)). Examples include the MAD family of proteins, Pfl, AML1, ETO, p53, Bcl-6 and many more. Despite its major role as a transcriptional corepressor, there is also evidence that SIN3 can act as an activator of transcription. Furthermore, besides the binding of HDACs, SIN3 can also recruit other enzymatic activities to the core complex, including nucleosome remodelling enzymes, histone methyl transferases and N-acetylglucosamine transferases. Hence, SIN3 serves as a flexible scaffold for binding of DNA-binding proteins and histone modifying enzymes and can function as a global regulator of gene expression (153).

The present investigation

Aims

The general aim of this work was to increase the knowledge about the ETO homologues in order to understand how they function in their natural environment as well as in an aberrantly expressed fusion protein.

Specific aims:

- I. To characterise the interactions between the different ETO homologues (Paper I).
- II. To study expression of the ETO homologues in haematopoietic cells of different lineages and maturation stages (Paper II).
- III. To investigate if the ETO homologues interact with the corepressor SIN3B (Paper III).
- IV. To search for functional consequences of increased or decreased ETO homologue expression (Paper IV).

Experimental considerations

Detection of ETO homologue proteins

To detect a protein it is crucial to have a functional antibody. In the start-up of this work we produced an antibody directed against the N-terminal third of ETO (Paper II). This antibody functions in Western blot and immunoprecipitation and easily detects endogenous proteins. However, because of the high sequence homology between the ETO homologues, this antibody does not discriminate between the different proteins. The similar sizes of the ETO homologues, the presence of two isoforms of each protein and the possibility of posttranslational modifications, further complicates the analysis of Western blot data. Therefore, to be able to study one protein at a time it was necessary to produce specific

antibodies for each protein. This was accomplished by choosing specific peptide sequences for each protein and use them for immunisation of rabbits (Paper I). These peptide antibodies are highly specific, but their drawback is that they are not as sensitive as the first antibody produced. Thus, since the endogenous expression of the ETO homologues is relatively low, we either needed to overexpress the proteins in order to detect them, or to use a large amount of cells for detection of endogenous protein with the help of IP-Western.

Detection of ETO homologue transcripts

We used two methods to examine the RNA levels of the ETO homologues, reverse transcriptase PCR (RT-PCR) (Papers II and III) and real-time PCR (Papers II, III and IV) using the TaqMan system. An advantage of these methods is that they are very sensitive as compared to for instance Northern Blot. In both methods you reverse transcribe extracted RNA to achieve complementary DNA (cDNA). The cDNA is subsequently used as a template in a PCR reaction. In RT-PCR you analyse the reaction end product on an agarose gel. Since PCR reactions reach saturation after a certain repeat cycle it can be difficult to compare the relative expression between samples, unless you optimise the number of cycles run. However, RT-PCR can give qualitative answers as to how a gene is expressed or not. If you want to compare the relative amount of RNA expressed between different samples, real-time PCR is a preferable method of choice. In the TaqMan system you make use of two gene specific primers and a gene specific probe (TaqMan probe) containing a reporter and a quencher. During PCR, the DNA polymerase specifically cleaves TaqMan probes that are bound to DNA. The reporter is then separated from the quencher, resulting in light emission. By measuring the light during the exponential phase of the PCR reaction it is possible to follow the amplification in real time. The higher expression of a gene, the earlier the amplification will be detected, thus making a relative comparison between samples possible. To compensate for differences in efficiency of cDNA

production and pipetting errors, an internal control is used and expression of the gene of interest is normalised to the expression of the internal control. The internal control should ultimately be a gene that is stably expressed over time and during different experimental conditions. The sensitivity of real-time PCR proved to be useful for expression analysis in cases when there were drawbacks in the detection of proteins, for instance when the sample material was small. However it must be taken into account that the RNA levels might not truly reflect the levels of expressed protein, due to posttranslational modifications and differences in RNA and protein stability.

Cell lines vs. bone marrow cells

Cell lines are immortalised cells of different origins. The cell lines used in this work are mainly derived from connective tissue cells or from clones of human leukaemia. Cell lines provide a resource to obtain *in vivo* data in a relevant environment. Due to their indefinite divisions, it is possible to rapidly acquire a large amount of working material. Furthermore, cell lines are often easy to transfect to receive a high expression of an exogenous protein. However it must be taken into account that during the process of immortalisation, these cells have gone through several genetic changes that distinguish them from their natural counterparts. These discrepancies might have an influence on the results. Therefore it is optimal to confirm findings from cell line experiments in the more authentic environment of human bone marrow cells. In paper II, we collected data in cell lines but also extracted the experiments to human bone marrow cells. The disadvantage of using bone marrow cells is that the working material is limited and it is usually difficult to exert gene transfer to these cells.

Differentiation of cell lines and bone marrow cells

Human leukaemic cell lines can be induced to undergo maturation through the use of different inducing agents like hemin, all trans retinoic acid (ATRA), Vitamin

D₃, phorbol myristate acetate (PMA), TGFβ and combinations thereof. These assays were used in papers II and IV. It is also possible to direct the maturation of primary bone marrow cells into different lineages of haematopoiesis, through the addition of specific combinations of cytokines. This was exploited in paper II. In these ways it is possible to mimic the differentiation process, even though the complexity of maturation never can be authentically imitated. The differentiation can be confirmed using functional assays like NBT reduction (Paper II), by studying morphological changes (Paper II) or by examining the upregulation of lineage specific surface markers by FACS (Paper IV).

Interaction studies

There are several ways to examine protein- protein interactions. In this work we mainly used IP-Western as a mean of determining interactions (Papers I and III). In this method you immunoprecipitate cellular extracts with an antibody directed against protein A. The precipitate is then run in a Western blot using an antibody against protein B. If protein B interacts with protein A it will be co-precipitated with protein A in the IP and a band will appear on the blot. The interaction can be confirmed in a reciprocal experiment. It is important to perform control experiments that out-rule the possibility of unspecific binding to the sepharose used in the IP or unspecific binding of the proteins to the antibodies used. We either studied interactions of proteins that were overexpressed in COS-7 cells, or endogenously expressed proteins in human leukaemic cell lines. The advantages of the COS system is that it is easy to obtain high levels of protein expression making the detection possible through the use of our specific antibodies. Furthermore, endogenous expression of the proteins studied was low, providing a clean and easily interpreted system. On the other hand, the forced expression of proteins represents an artificial system that can result in false positives. Therefore it is preferable to examine interactions of endogenous proteins. However, this could be problematic if the expression of the proteins of interest is low.

Interactions between low abundance proteins might be disregarded due to detection problems. Interactions confirmed through IP-Western do not necessarily represent interactions that take place in the cell, since the proteins might bind to each other in the test tube, subsequent to cell lysis. One way to examine if the interactions take place in the cells is through the mammalian two-hybrid system used in Paper III. Here the two proteins are fused to either a DNA binding domain that recognises a specific promoter sequence or to a transcriptional activation domain. If the two proteins interact the activation domain is brought to the promoter and a reporter gene is activated, in this case luciferase. Another way to indirectly confirm an interaction is through co-localisation studies (Paper III). Cells are fixated and permeabilised and the proteins of interest are immunostained with antibodies labelled with two different fluorochromes that are recorded at different wavelengths. By overlaying the individual pictures recorded at different wavelengths, colocalisation between the two proteins can be shown. A colocalisation does not prove that there is a physical interaction between the proteins, but it can act to support the proposed existence of an identified interaction.

Results and general discussion

Do all the ETO homologues interact with each other?

In Paper I we examined the proposed interactions between the ETO homologue members. It had been previously shown by Kitabayashi *et al* that AML1-ETO could interact with MTGR1 through the NHR2 domain (60). Furthermore Davis *et al* showed in vitro interactions of the NHR2 domain of ETO homologues (63). Thus, we aimed at making a thorough investigation and systematically examine every possible protein interaction combination of the ETO homologues. Overexpression studies followed by IP-Western experiments led us to the conclusion that all the ETO homologues as well as AML1-ETO could interact

with each other. NHR2 was confirmed to be responsible for the complex formation. As to what extent these interactions occur in a normal environment is not clear, since we could not confirm interactions of endogenously expressed proteins. This might be due to detection problems, given the weak sensitivity of the peptide antibodies used in the studies and the low abundance of the ETO homologues. Furthermore, if only a small fraction of the ETO homologues in a cell is present in a heterocomplex, it might be hard to detect.

If the ETO homologues indeed interact with each other in real life, there are several possible implications of these interactions:

- 1) Different combinations of ETO homologues might result in different cellular responses, for instance a certain combination of ETO homologues might be responsible for the regulation of a specific target gene. If all three homologues are expressed in a cell, the interaction pattern could be rather complex, and the presence of AML1-ETO in a cell would further complicate the picture. There are several examples where different patterns of homo- or heterodimerisation between transcriptional regulators lead to different cellular responses, including the Mad-Max-Myc system and the RXR receptors (154, 155).
- 2) AML1-ETO might sequester the ETO homologues and thus inhibit their normal actions. Indeed, AML1-ETO decreases the interaction between ETO-2 and N-CoR, suggestedly as a result of ETO-2 sequestration by AML1-ETO (118). Furthermore, AML1-MTG16 can sequester MTG16 leading to its translocation from the nucleolus to the nucleoplasm (77).
- 3) The ETO homologues might affect the function of AML1-ETO. Examples from transcriptional assays show that MTGR1 can reinforce the transcriptional repression exerted by AML1-ETO (60) whereas in another system ETO was shown to have the opposite effect and relieve AML1-ETO mediated repression (103).

How are the ETO homologues expressed in haematopoietic cells and are they involved in haematopoietic differentiation?

In paper II, we focused on two major topics. The first was the expression pattern of the ETO homologues in different populations of haematopoietic cells and the second was how they are expressed during haematopoietic differentiation. We found that MTGR1 and MTG16 were ubiquitously expressed in leukaemic cell lines and human bone marrow cells of different lineages. The expression of ETO on the other hand was most strikingly restricted to erythroid cells. Induced differentiation of leukaemic cell lines showed that the protein levels of the ETO homologues were downregulated during the differentiation process, supposedly in a lineage specific manner, since ATRA-induced granulocytic differentiation but not Vitamin D₃-induced monocyte differentiation resulted in their downregulation. The observed downregulation was a result of decreased protein stability combined with reduced RNA levels. When human haematopoietic progenitor cells were differentiated into the granulocytic or erythroid lineages and analysed by real-time PCR, there was a prominent transient upregulation of ETO during erythroid differentiation. MTG16 was downregulated during both erythroid and granulocytic differentiation. The levels of MTGR1 transcripts were unaffected during both erythroid and granulocytic differentiation.

Importantly, our findings suggest the following:

- 1) The restricted expression and the distinct upregulation of ETO during erythroid differentiation suggest a role for this protein in erythropoiesis. In their description of the ETO knockout mouse, Calabi *et al* proposed that ETO is not expressed in haematopoietic cells and that disruption of the ETO gene does not affect haematopoiesis (156). We show that ETO is indeed expressed in the erythroid compartment and our findings suggest that there is a species difference between mouse and man concerning the expression of ETO. AML1-ETO has been shown to inhibit erythroid

- differentiation (129). One explanation could be that it interferes with the actions of ETO. AML1-ETO might either bind directly to ETO and redirect it in the nucleus, or it could compete for cofactors.
- 2) The downregulation of MTG16 during both erythroid and granulocytic differentiation propose a function for MTG16 in haematopoietic progenitor cells. Possibly, downregulation of MTG16 is a prerequisite for differentiation. One theory is that MTG16 is part of a repressor complex that helps to keep the cells at the progenitor stage, by repressing genes involved in differentiation. When this complex is dissolved, the cells can differentiate.
 - 3) The differential expression of the ETO homologues in the haematopoietic system suggests that they all have a distinct function. The high homology between the ETO homologues could imply that they have overlapping functions. However their specific expression patterns points to unique functions for each homologue. This does not necessarily rule out the possibility that they can substitute for each other when necessary.

Do the ETO homologues interact with the corepressor SIN3B?

In paper III, we examined if the ETO homologues could interact with the human corepressor SIN3B. It had previously been shown that ETO and AML1-ETO can bind to SIN3A. However, the murine homologue of MTG16, ETO-2, does not bind to SIN3A (82, 83). Due to the homology between SIN3A and SIN3B, a similar interaction pattern could be expected for interactions between SIN3B and the ETO homologues. Cotransfection of SIN3B with either of the ETO homologues, followed by IP-Western, showed that SIN3B can bind to ETO, MTG16 and MTGR1. Conversely, SIN3B does not bind to AML1-ETO. Studies with deletion mutants suggested that NHR1, NHR2 and NHR3 of ETO all take part in the binding to SIN3B. The interactions between SIN3B and the ETO homologues were also confirmed in a mammalian two-hybrid system.

Furthermore, colocalisation studies showed that SIN3b colocalised with ETO, MTG16 and MTGR1 but not with AML1-ETO in the nucleolus.

The following findings deserve to be commented on:

- 1) Human SIN3B can bind to ETO, MTG16 and MTGR1. It is not shown if human MTG16 can bind to SIN3A, but its murine homologue ETO-2, does not bind to SIN3A, suggesting a discrimination between ETO and MTG16 in SIN3 binding (83). Here we show that both ETO and MTG16 can bind to human SIN3B. It must be remembered though that all studies in Paper III are made on overexpressed proteins. As discussed above, care needs to be taken to draw conclusions about interactions in the authentic environment. It still remains an open question if endogenous SIN3B and the ETO homologues do interact in normal cells.
- 2) SIN3B as opposed to SIN3A does not bind to AML1-ETO. ETO binds to SIN3A through a region that is concentrated around the NHR2 domain (84). Our studies on deletion mutants suggest that a much larger region, that includes NHR1-3 of ETO, takes part in the interaction with SIN3B. This could explain why AML1-ETO fails to bind to SIN3B. Either the very N-terminal region of ETO, which is not included in AML1-ETO, has a role in SIN3B binding, or the linking of AML1 to ETO inhibits the recruitment of SIN3B through steric hindrance.
- 3) SIN3B colocalise with the ETO homologues in the nucleolus. According to previous findings, MTG16 but not ETO or MTGR1 are localised to the nucleolus (77). Our findings are at odds with this since we see that both ETO and MTGR1 colocalise with the nucleolar marker B23. The discrepancy between these results could be due to the dynamic structure of the nucleolus. The nucleolus assembles and disassembles throughout the cell cycle and the constituents of the nucleolus are constantly changing (157).

- 4) The binding of the ETO homologues to SIN3B adds further complexity into the corepressor binding pattern of ETO. Besides binding to SIN3A and SIN3B, ETO can also bind to the corepressors NCoR and SMRT to recruit HDACs. Furthermore, ETO can directly bind to HDAC 1-3 (80-84). Accordingly, ETO can repress transcription through a multitude of protein interactions in a flexible manner depending on the temporal and spatial expression of the different factors in the repressor complex.

What are the consequences of increased or reduced expression of the ETO homologues?

In paper IV we aimed at defining phenotypes that would result from upregulation or downregulation of the ETO homologues. Even though we faced some methodological problems, our findings could still provide some information about the ETO homologues. Through the siRNA approach we were able to stably knock down the RNA expression of both ETO and MTG16 in an erythroid cell line. Interestingly, the downregulation of MTG16 RNA was not reflected at the protein level. ETO on the other hand was downregulated at the protein level as well and we studied the effects of ETO knock down on proliferation, differentiation and apoptosis. However, we could not determine a phenotype that resulted from the low expression of ETO. Attempts to conditionally overexpress ETO and mutants of ETO resulted in the establishment of inducible clones of the respective proteins. Unfortunately these clones could not be further characterised, because they were not possible to expand due to slow proliferation and poor survival.

Our findings suggest the following:

- 1) Downregulation of ETO does not have an obvious impact on proliferation, differentiation or apoptosis. However, our experimental approaches naturally do not cover all aspects of these complex processes and alternate experiments might have unravelled phenotypes that could not be

- distinguished in our experiments. As for differentiation, it is possible that the role, if any, of ETO in erythropoiesis is outplayed in the maturation stage that the HEL cells represents. The expression of ETO in these cells is already high and it might correspond to the peak expression of ETO that we found during induced differentiation of bone marrow cells (Paper II).
- 2) The discrepancy between RNA levels and protein levels of MTG16 in the siRNA experiments, could imply an important function for MTG16. The stable levels of protein could be the result of a regulatory mechanism that aims at keeping the protein levels of MTG16 stable in the cell. This regulation could take place at different levels of transcription and translation. The persistent levels of MTG16 protein throughout the downregulation of RNA could suggest that protein stability increases as a response to a decreased amount of transcripts. Moreover, some siMTG16 clones that were not further characterised, displayed a significant upregulation of MTG16 transcripts. Thus it is possible that the regulation could take place at the transcript level as well, either through increased transcription or through increased RNA stability. The finding also points at the danger of making assumptions about protein expression based on RNA expression, even though at most times they probably go hand in hand.
 - 3) The strong impact of ETO, even by the very low levels derived through leakage of the Tet-system, on survival and propagation of the inducible clones, could imply a role for this protein in proliferation and apoptosis. The response could be cell type specific since we could not confirm a role for ETO in these processes in the siRNA experiments. Indeed, at odds with its role as a leukaemic protein, AML1-ETO has been shown to both increase the expression of apoptosis associated genes and to induce growth arrest (119, 121, 122). Thus, our findings suggest that these characteristics are dependent on the ETO sequences of the fusion protein.

Conclusions and future perspectives

The major findings of this work were the following:

- 1) The ETO homologues as well as AML1-ETO can interact with each other. This might have implications for the function of AML1-ETO in leukaemogenesis.
- 2) The differential expression of the ETO homologues in different populations of haematopoietic cells suggests a specific function for each of these proteins.
- 3) The downregulation of MTG16 during erythroid as well as granulocytic differentiation proposes a role for this protein in the early phases of haematopoiesis.
- 4) The restricted expression of ETO to erythroid cells supports a role for this protein in erythropoiesis.
- 5) The ETO homologues, but not AML1-ETO can interact with the corepressor SIN3B.
- 6) siRNA experiments show a discrepancy between RNA levels and protein levels of MTG16. This could be indicative of a mechanism that keeps MTG16 protein levels constant in the cell.
- 7) Results from conditional overexpression experiments imply a role for ETO in proliferation and apoptosis.

Future studies could include the search for interactions between endogenous proteins of the ETO homologues as well as SIN3B. It would also be of interest to elucidate the proposed role of ETO in erythroid cells and the role of MTG16 in early haematopoiesis. Furthermore, one could explore the mechanism behind the stable levels of MTG16 protein found in Paper IV.

Summary

Acute myeloid leukaemia (AML) is commonly associated with balanced chromosomal translocations that fuse two unrelated genes. This results in the expression of an aberrant fusion protein. t(8;21) is one of the most common translocations found in patients with AML. It results in the expression of the chimaeric protein AML1-ETO. AML1 is a transcription factor of crucial importance during haematopoiesis. The function of the fusion partner eight-twenty-one (ETO) is much less understood. The aim of this thesis was to characterise ETO and its two homologues, myeloid translocation gene 16 (MTG16) and myeloid translocation gene related protein 1 (MTGR1), to elucidate their role in normal and dysregulated haematopoiesis. We studied the interaction patterns of the ETO homologues as well as their expression pattern in haematopoietic cells. We also examined the consequences of upregulation or downregulation of the proteins. We found that all the ETO homologues as well as AML1-ETO can interact with each other. We also found that the ETO homologues, but not AML1-ETO can bind to the corepressor SIN3B. The proposed interactions of the ETO homologues might have implications for the onset of leukaemia, since it opens up for an AML1-ETO mediated disturbance of ETO homologue function as well as a regulation of AML1-ETO function by the ETO homologues. Examination of the expression patterns of ETO homologues in haematopoietic cells showed that the expression of ETO was restricted to erythroid cells, suggesting a role for ETO in erythropoiesis. MTG16 and MTGR1 are ubiquitously expressed in haematopoietic cells. The expression of MTG16 decreases during erythroid and granulocytic differentiation, suggesting a role for MTG16 in early haematopoiesis. The differential expression of the ETO homologues in haematopoietic cells implies a specific function for each protein in haematopoiesis. Attempts to knock-down MTG16 showed a discrepancy between RNA levels and protein levels, which could propose a mechanism to keep the expression of MTG16 constant. Finally, overexpression experiments indicate a role for ETO in proliferation and apoptosis.

Populärvetenskaplig sammanfattning

Cellerna i blodet har en rad skilda funktioner, såsom att transportera syre, levera blodet vid behov eller att på olika sätt delta i vårt immunförsvar. Alla blodets celler härstammar från en s k stamcell som har förmåga att genom en rad förändringar mogna ut till vilken typ av cell som helst i blodsystelet. Leukemi är en sjukdom som drabbar blodet och som uppstår p g a att en omogen cell börjar att föröka sig kraftigt och på så sätt tar överhanden. Detta leder till en brist på mogna celler och följaktligen till blödningar, blodbrist och ökad infektionskänslighet.

Man har funnit att leukemi ofta uppstår p g a en s k translokation: genom ett fel som uppstår när cellerna delar sig, kan två kromosomdelar byta plats med varandra. Ofta leder en sådan translokation till att två gener som egentligen inte har med varandra att göra, länkas samman. Följden blir att ett onormalt protein uttrycks, där ena delen härstammar från ett protein och den andra delen från ett helt annat protein. Ett av dessa onormala proteiner heter AML1-ETO och det uttrycks relativt ofta i cancercellerna hos patienter med leukemi. Man vet att AML1, som utgör den ena delen av det onormala proteinet, under normala förhållanden har en viktig funktion för utmognaden av cellerna i blodet. Däremot vet man inte så mycket om vad ETO, den andra delen, har för normal funktion. Jag har i mitt arbete studerat den proteinfamilj som ETO tillhör och som består av sammanlagt tre proteiner: ETO, MTG16 och MTGR1. Min uppgift har varit att göra olika experiment som kan leda till att vi bättre förstår hur de här proteinerna fungerar i cellerna. En ökad förståelse av deras funktion kan också leda till en ökad förståelse för vilken roll AML1-ETO spelar vid uppkomsten av leukemi.

En av de studier vi gjorde var att undersöka om de olika proteinerna i ETO-proteinfamiljen kan binda till varandra. Genom att undersöka parvisa kombinationer, dels av de tre proteinerna inbördes och dels av dessa och AML 1-ETO, kunde vi visa att de alla kan binda till varandra. Dock användes ett system där man uttrycker väldigt stora mängder av proteinerna jämfört med de mängder

som uttrycks i normala celler. Vi har alltså visat att proteinerna i ETO-familjen kan binda till varandra, men inte att de verkligen gör det i sin naturliga miljö. Om de faktiskt gör det, finns möjligheten att de samverkar i cellerna. Dessutom kan AML1-ETO, genom att binda till ETO-proteinerna, på olika sätt störa deras normala funktion och det kan bidra till uppkomsten av leukemi.

I en annan studie undersöktes uttrycket av ETO-proteinerna i olika typer av blodceller. Vi fann att ETO uttrycks endast i erytropoesen, dvs den utmognadslinjen som bildar de syretransporterande röda blodkropparna. Det kan tyda på en roll för ETO vid utmognaden av röda blodkroppar. Vi fann att MTG16 och MTGR1 uttrycks även i andra typer av blodceller. MTG16, minskar i mängd i takt med att cellerna mognar ut. Det skulle kunna innebära att MTG16 spelar en roll i de tidiga omogna blodcellerna, men att det måste minska i mängd för att utmognad ska ske.

Man tror att ETO-proteinerna har en funktion vid avstängningen av vissa gener. Det tror man därför att de har visats binda till andra proteiner som man vet stänger av gener. I den tredje studien vi gjorde kunde vi visa att ETO-proteinerna binder till ytterligare en genhämmare. Den bindningen har inte visats tidigare. Även här är det oklart om denna bindning sker i normala celler. Om så är fallet, öppnar det möjligheten för en ytterligare komplexitet i kompositionen av genhämmande proteinkomplex.

I den sista studien försökte vi att öka eller minska uttrycket av ETO-proteinerna för att på så sätt få information om deras påverkan på cellerna. När vi försökte öka uttrycket av ETO visade det sig att cellerna växte långsammare eller att de dog. Det kan tyda på att ETO spelar en roll vid celldelning och celldöd. Man vet att även AML1-ETO paradoxalt nog kan påverka celler till att dela sig långsammare och att dö snabbare. Det är motsägelsefullt med tanke på att cancerceller delar sig

snabbt och har en bättre överlevnad än normala celler. Våra studier tyder på att ETO är ansvarigt för de här egenskaperna hos AML1-ETO.

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