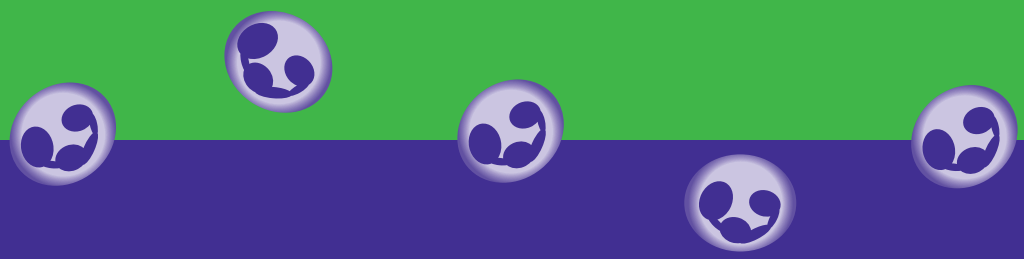
Molecular Mechanisms in Differentiation-Induction in Acute Promyelocytic Leukemia



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Molecular Mechanisms in Differentiation-Induction in Acute Promyelocytic Leukemia

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Introduction

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1. Hematopoiesis

Hematopoiesis is the process that generates blood cells of all lineages. It is a continuously and closely regulated process that proceeds from the bone marrow. Every type of blood cell has its origin in common pluripotent stem cells. These hematopoietic stem cells (HSCs), have the ability to self-renew and, upon stimulation by different hematopoietic growth factors and cytokines, these HSCs will become progenitor cells committed to a particular lineage. Further progression of a progenitor cell towards a specific mature and functional blood cell takes place via several differentiation stages, which can be distinguished by morphological characteristics and cell-surface markers (Figure 1). During maturation, immature blood cells lose their proliferative capacity and gain their functional characteristics, whereupon they leave the bone marrow and enter the peripheral blood. Once matured, most blood cells have a limited life span and will soon leave the blood circulation system via the apoptotic pathway. During normal hematopoiesis HSC homeostasis reflects the balance between self-renewal and proliferation, differentiation and apoptosis. Each day, an adult produces hundreds of billions new blood cells, which is largely controlled by feedback mechanisms. When the demand for blood cells of a particular lineage increases or when the levels of certain cells fall in blood, the HSCs will be stimulated to generate sufficient numbers of mature blood cells. A complex network of growth factors and their receptors, signal transduction pathways and transcription factors that regulate the expression of lineage specific genes is involved in the regulation of blood cell differentiation. HSCs can undergo either self-renewal or differentiation into one of the two multipotent progenitor cells: a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP). These cells then give rise to more differentiated progenitors and ultimately, they will mature towards unilineage committed progenitors for natural killer cells, B-cells, T-cells, dendritic cells, monocytes, granulocytes, erythrocytes and megakaryocytes.[1-3]

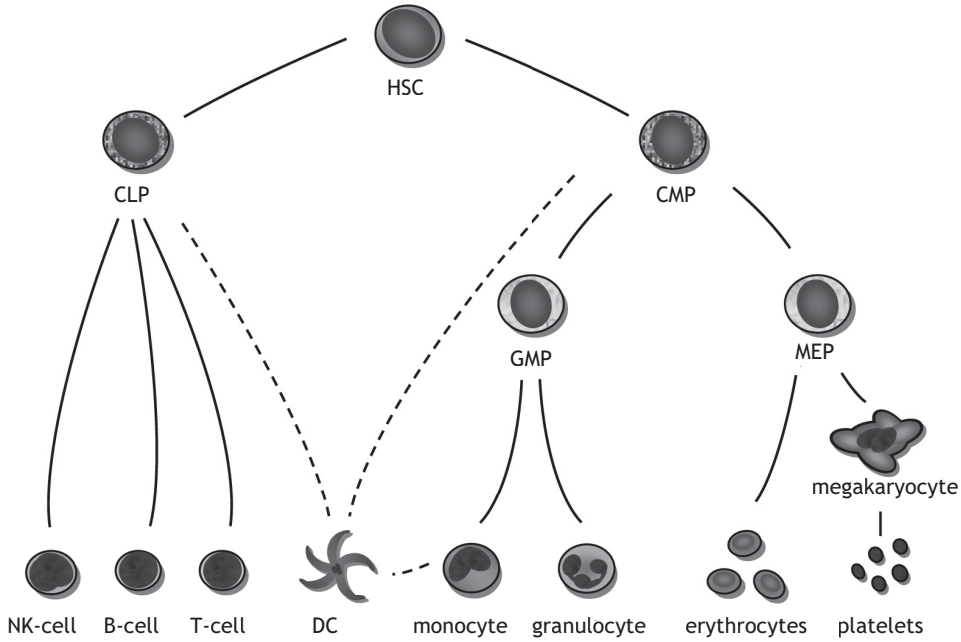

Figure 1. Schematic representation of hematopoiesis. All mature blood cells originate from a pluripotent hematopoietic stem cell (HSC), which can differentiate into either a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). These multipotent progenitor cells give rise to more committed progenitors, including the granulocyte-monocyte progenitor (GMP) and megakaryocyte-erythroid progenitor (MEP). The lymphoid progenitor cells ultimately generate the natural killer (NK) cells, B- and T-cells and lymphoid dendritic cells (DC), and the myeloid progenitor cells differentiate into myeloid DCs, monocytes, granulocytes, erythrocytes and megakaryocytes/platelets.

2. Leukemia

Disturbance of the normal process of hematopoiesis leads to the development of blood disorders and diseases. Leukemia affects the immature stem cell population that resides in the bone marrow. It is characterised by abnormal proliferation of immature bone marrow cells that fail to differentiate and remain in the bone marrow. Eventually the bone marrow starts to release the malignant, immature cells into the

blood. The displacement of normal bone marrow cells by malignant cells, results in a shortage of normal blood cells and a concomitant range of symptoms, such as anemia, malaise, abnormal bleeding, excessive bruising, infection and fever.

There are several types of leukemia, which can be grouped according to how quickly the disease develops and becomes worse. When the disease develops quickly, it is classified as acute, whereas a more slowly progressing disease is classified as chronic. Early during chronic leukemia, the presence of abnormal blood cells, which have the ability to partially differentiate, may not cause any symptoms. Slowly, patients with chronic leukemia will get symptoms as the number of malignant cells rise. In the case of acute leukemia, a complete block in differentiation of malignant cells causes a rapid developing disease. According to the type of white blood cell that is affected, leukemia can be classified as myelogenous or lymphocytic. The four most common types of leukemia are: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL). AML is the most frequent form of leukemia that accounts for about 25% of all leukemias in adults in the Western world.[4]

Leukemogenesis is a process in which immature blood cells evade the tight coordination of proliferation, differentiation and apoptosis. The aberrant progenitor cells not only lose their differentiation potential but also gain uncontrolled proliferation ability and in addition apoptosis is reduced in these abnormal cells (Figure 2). The disturbed balance between proliferation, differentiation and apotosis is caused by a variety of mutations in pathways that are required for normal hematopoiesis. Specific growth factors and their receptors, signal transduction molecules, regulators of the cell cycle and apoptosis machinery and transcription factors that regulate lineage-specific genes, these may all be mutated in leukemia. Transformation of a normal progenitor cell towards an abnormal leukemic blast is a multistep process involving the accumulation of genetic mutations over time. Not a single, but several mutations are required to obtain a full-blown malignancy.[5] Studies on the molecular events that occur during normal and malignant hematopoiesis increase our knowledge of the complex network of protein interactions and signaling pathways, providing multiple targets for therapeutic intervention. At the same time, molecular markers may be used to predict prognosis.

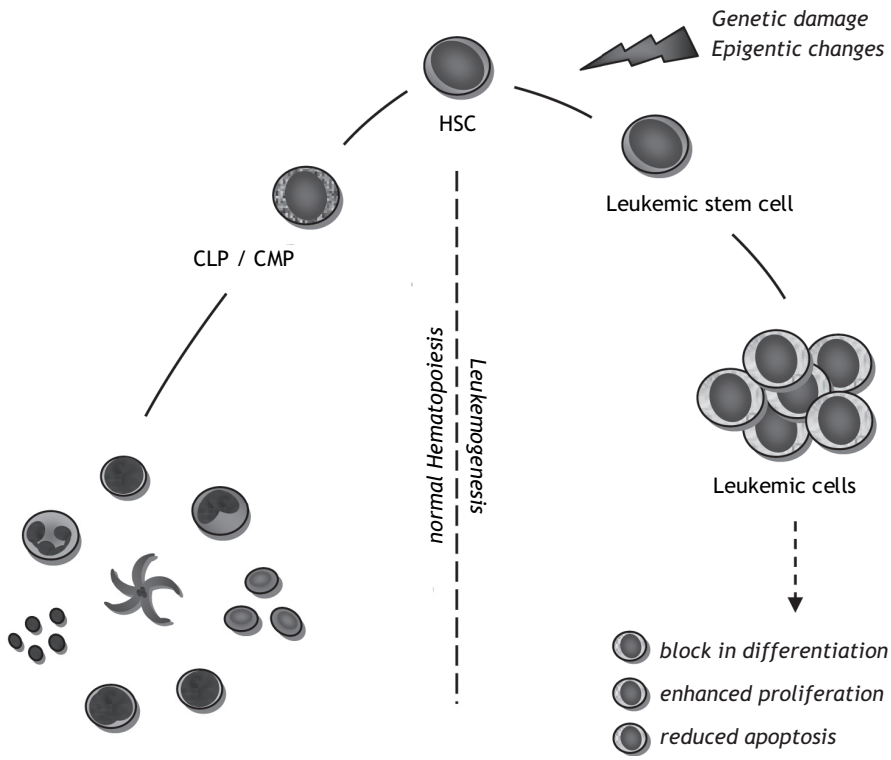

Figure 2. Schematic representation of leukemogenesis. Due to an accumulation of multiple genetic alterations together with epigenetic changes, normal cells may undergo leukemic transformation. A common characteristic of leukemic cells is their loss of maturation ability, their uncontrolled proliferation and increased survival. Hematopoietic stem cell (HSC); common myeloid progenitor (CMP); common lymphoid progenitor (CLP).

3. Acute promyelocytic leukemia

Acute promyelocytic leukemia (APL) accounts for 5-10% of all AML cases, and is classified as AML-M3 according to the French-American-British (FAB) classification system that is predominantly based on morphological criteria.[6-12] Recently, the World Health Organization (WHO) published a new classification system that includes many of the criteria of the FAB classification but also incorporates genetic, biologic and

clinical features to allow more appropriate categorization and therapy.[13] In APL the leukemic blasts are blocked at the promyelocytic stage of development and they fail to differentiate towards mature granulocytes. Mutations that affect the retinoic acid receptor α gene *(RARA)* are characteristic for APL and in 98% of the APL cases, a chromosomal translocation occurs involving chromosome 15 and 17, fusing the *RARA* and the promyelocytic leukemia *(PML)* genes.[14-17] The resulting chimeric fusion protein, PML-RARα, has oncogenic potential and may interfere with the normal function of PML and RARα ([18] and references therein). Besides this unique genetic lesion, mutations in the FLT3 gene have been detected in approximately 45% of the APL cases.[19;20]

RARα is a nuclear hormone receptor that acts as a ligand-dependent transcription factor. In order to regulate gene expression, RARα dimerizes with the retinoic X receptor (RXR).[21] RARα/RXR heterodimers bind to specific DNA sequences or retinoic acid response elements (RAREs),[22;23] which are present in retinoic acid responsive genes. Retinoic acid, such as all-*trans* retinoic acid (ATRA), and their receptors are important in the development and homeostasis of a wide variety of vertebrate tissues through their regulatory roles in cell proliferation, differentiation and apoptosis.[24] In addition, ATRA exerts profound effects on the growth and maturation of hematopoietic cells. For example, ATRA is capable of suppressing the formation of erythroid and macrophage colonies, while inducing an increase in the number of neutrophil colonies from primary human progenitors.[25-28] In the absence of ATRA, RARα/RXR heterodimers inhibit transcription through the recruitment of corepressor complexes containing histone deacetylase (HDAC) activity, resulting in chromatin compaction and a transcriptional inactive state. Upon ligand binding, the corepressor complexes are replaced by coactivator complexes with histone acetylase (HAT) activity, creating an accessible chromatin configuration that facilitates transcriptional activation[29] (Figure 3A).

PML is a ubiquitously expressed protein, which is a component of nuclear substructures termed nuclear bodies (NBs). Over 50 protein partners, with a wide variety of functions, have been described for PML, including transcription factors, chromatin remodelling factors and DNA replication factors.[30] PML has multiple tumour suppressor functions and is involved in growth control, replicative senescence and apoptosis.[31] In APL cells the NBs are disrupted and PML is dispersed into numerous small microspeckles. Treatment of APL cells with ATRA, as discussed below, induces re-localization of PML into normal-appearing NBs.[32;33]

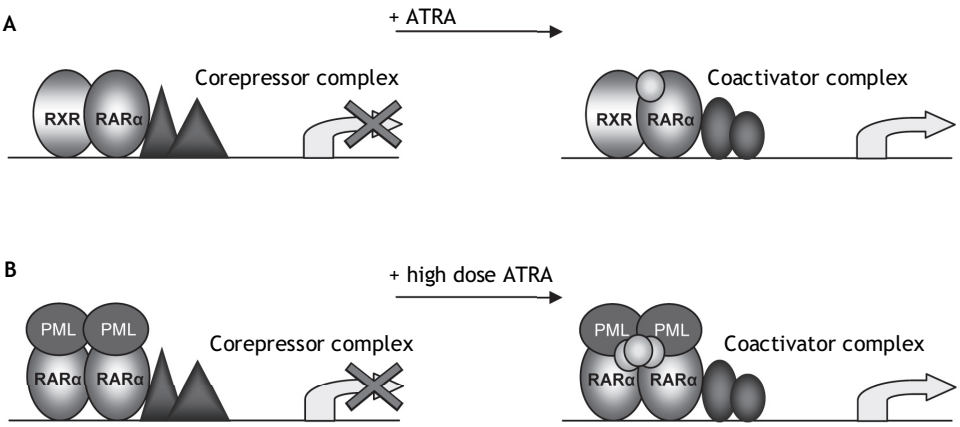

Figure 3. Transcriptional activation by RARα/RXR and PML-RARα. (**A**) In the absence of ATRA, wild type RARα/RXR heterodimers repress transcription through the recruitment of corepressor complexes. Upon binding of ATRA, conformational changes in the receptor occur resulting in the dissociation of the corepressor complexes and binding of coactivator complexes allowing transcriptional activation. (**B**) Comparable to wild-type receptors, PML-RARα fusion proteins are able to transcriptionally repress their target genes by the recruitment of corepressor complexes. In contrast to wild-type receptors, physiological doses of ATRA do not induce the release of corepressor proteins. Only in the presence of high doses of ATRA the corepressor complexes are replaced by coactivator complexes resulting in transcriptional activation.

PML-RARα fusion proteins have DNA binding capability by forming homodimers and ATRA is able to bind to PML-RARα [34;35]. Compared to RARα, PML-RARα shows enhanced binding with corepressor proteins. Physiological concentrations of ATRA (10^{-9} M) are not able to release the corepressor complex and PML-RARα target genes remain silenced. However, at pharmacological doses of ATRA (10^{-6} M), the corepressors are replaced by coactivators allowing transcriptional activation of PML-RARα target genes[36-42] (Figure 3B). Several important RARα target genes have been identified that are deregulated by PML-RARα in APL cells, including $p21^{waf1/Cip1}$ [43-45] and the transcription factors CAAT/enhancer-binding-protein-β and -ε (C/EBPβ and C/EBPε).[46;47] $p21^{waf1/Cip1}$ is a cyclin-dependent kinase inhibitor that is involved in the regulation of the activity of several cyclin-dependent kinases. The C/EBP transcription factors are involved in the regulation of granulocytic differentiation.

Unlike other types of AML, APL is uniquely sensitive to treatment with ATRA. APL patients benefit from the addition of pharmacological doses of ATRA to standard chemotherapy, leading to cure rates from 40% with chemotherapy alone to up to 90% when combined with ATRA. ATRA causes natural cell death of the malignant cells by inducing granulocytic differentiation.[48-57]

4. Transcriptional regulation of hematopoiesis

Hematopoiesis is controlled by specific combinations of transcription factors, which define lineage commitment and stage differentiation. Improved technologies to identify and isolate highly purified populations of HSCs and lineage restricted progenitor cells allow us to map specific transcription factor expression patterns that correlate with specific stages of differentiation. Further understanding of the function of these transcription factors during hematopoietic differentiation is determined by two general approaches: overexpression and inhibition of specific transcription factors. Some transcription factors are expressed in HSCs, such as Runt-related transcription factor 1 (RUNX1), whereas others have a more lineage-specific expression pattern. GATA1, PU.1, the transcription factors CCAAT/enhancer-binding-protein-α and -ε (C/EBPα and C/EBPε) and the transcriptional repressor Gfi1 are lineage-specific transcription factors that are implicated in myeloid development. The results of both knockout and overexpression studies have shown that GATA1 participates in the differentiation of common myeloid progenitors to megakaryocytic/erythroid progenitors, PU.1 is involved in the development of monocytes/macrophages and C/EBPα, C/EBPε and Gfi1 have a role in granulopoiesis.[58;59] Transcription factors may specify cell lineage and promote differentiation actively by auto-stimulation of their own expression. In addition, they may inhibit the expression of transcription factors that are involved in other differentiation routes, as has been described for PU.1 and GATA1.[60] Thus, both transcriptional activation and transcriptional repression are important for lineage decisions.

In general, transcription factors are involved in many protein-protein interactions and different combinations of interactions may reflect different functions of the same protein. One example is the RARα protein, which can function as a transcriptional repressor in the absence of ligand by binding to corepressor proteins, but when ligand-

bound turns into a transcriptional activator through interaction with coactivator proteins. In addition, changes in composition of a complex may direct the complex to distinct enhancers or may inhibit DNA binding. The presence of a mutated gene within these complex networks may change the expression of many genes contributing to malignant transformation. In addition to these genetic changes that have been demonstrated to be implicated in tumorigenic processes, there are many other genes contributing to tumorigenesis that do not show any mutation. Deregulated expression of genes is not only determined by genetic alterations in transcription factors or their interacting proteins, also epigenetic changes play an important role in the level of gene expression. Several epigenetic modifications exist that change the gene expression capacity without DNA sequence alterations. DNA methylation, which occurs at cytosine residues in CpG dinucleotides, is the major epigenetic modification of mammalian genomes that is required for processes such as genomic imprinting and X chromosome inactivation and is indispensable for normal embryonic development. Methylation of DNA, mediated by specific DNA methyltransferases and methyl-CpG-binding domain proteins, is associated with an inactive chromatin state and transcriptional repression.[61] Altered methylation patterns have been associated with cancer cells. CpG islands located in promoter regions of many tumour suppressor genes show hypermethylation and, as a consequence, these genes are transcriptionally inactive in cancer cells. In addition, loss of methylation at sites that are normally methylated may be associated with genomic instability and increased chromosomal rearrangements or with the potential activation of oncogenes ([62-65] and references therein).

Recently, the discovery of microRNA (miRNA) molecules has led to new insights into the regulation of gene expression. MiRNAs are small, non-coding, single stranded RNAs that are able to bind specific mRNA sequences and due to inhibition of translation or mRNA degradation miRNAs induce repression of protein expression [66] (Figure 4). MiRNAs have been linked to several important biological processes such as early stage development, cell growth, cell differentiation and apoptosis and strong evidence exists for a role of miRNAs during hematopoiesis. In addition, impaired miRNA expression has been implicated in tumorigenesis.[67-69]

Box 1. Epigentics, cancer and therapeutic interventions

The epigenetic control of gene expression involves the formation of chromatin that is either transcriptionally inactive or transcriptionally active. The two most studied modifications of chromatin include DNA methylation, which is associated with transcriptionally inactive chromatin, and histone acetylation, which is associated with transcriptionally active chromatin. Nowadays, strong evidence exists for a correlation between DNA hypermethylation, hypoacetylation of histones, tightly packed chromatin, and transcriptional repression. On the other hand, unmethylated DNA and hyperacetylation of the histones are associated with an open chromatin structure and transcriptional activation. The process of DNA methylation and histone acetylation involves several proteins. DNA methyltransferases (DNMTs) and methyl-CpG-binding proteins (MBDs) are involved in the process of DNA methylation, mediating silencing of gene expression. Histones are acetylated by histone acetyltransferases (HATs), which create an accessible chromatin configuration that facilitates transcriptional activity. Histones are deacetylated by histone deacetylases (HDACs), which facilitate chromatin compaction and transcriptional inactivation. In cancer, aberrant epigenetic silencing of tumour suppressor genes frequently occurs by DNA hypermethylation and/or by decreased histone acetylation. The reversible characteristic of epigenetic modifications is being explored to revert the tumour phenotype using hypomethylating agents in combination with agents that inhibit the deacetylation. For instance, the combination of either 5-azacytidine or 5-aza-2'-deoxycytidine, which are potent hypomethylating agents when used in a low dose, with an HDAC inhibitor has significant activity in myelodysplastic syndrome and acute myelogenous leukemia[167-169] (Figure box 1).

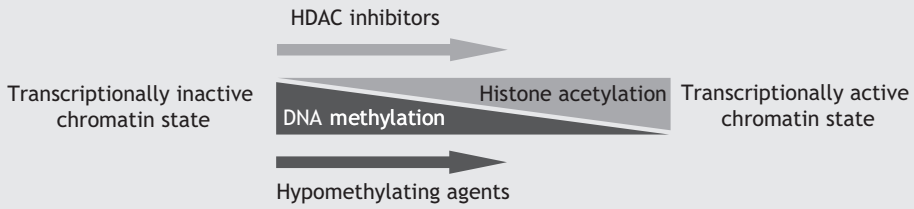

Figure box 1. Schematic representation of two important ways to epigenetically modify the DNA. DNA methylation is associated with a transcriptionally inactive chromatin state, while histone acetylation is associated with a transcriptionally active chromatin state. With HDAC inhibitors acetylation of histones can be increased resulting in transcriptional activation. With hypomethylating agents methylation of DNA can be decreased also resulting in transcriptional activation.

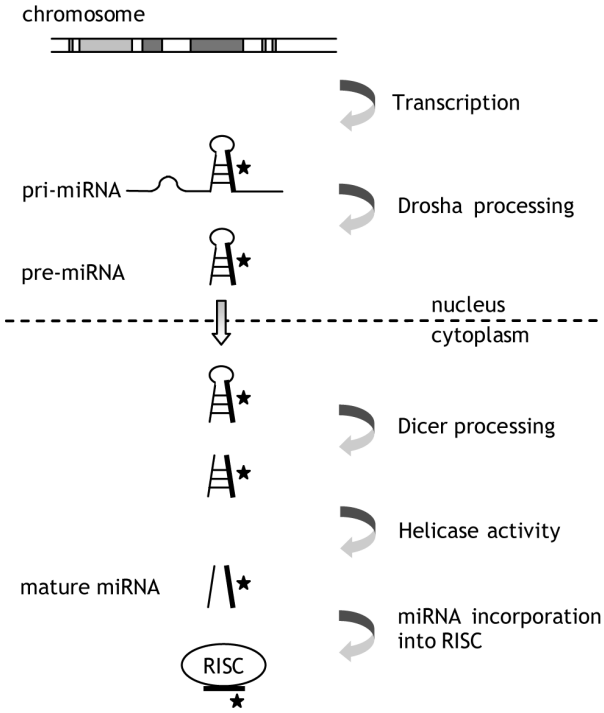

Figure 4. Schematic overview of miRNA processing. The majority of miRNA genes are transcribed by RNA polymerase II. First, a primary miRNA transcript (pri-miRNA) is transcribed from the genome and this pri-miRNA forms stem-loop structures of about 70-100 nucleotides in length. The precursor miRNA (pre-miRNA) is subsequently processed from the pri-miRNA by the enzyme Drosha, which cleaves both strands of the stem-loop. The pre-miRNA is then transported from the nucleus to the cytoplasm by the export receptor Exportin-5. In the cytoplasm, an enzyme called Dicer further processes the pre-miRNA. Dicer cuts the loop from the pre-miRNA to generate the mature miRNA (indicated by an asterisk) as part of a short RNA duplex and subsequently, this double stranded RNA is unwound by helicase activity. The mature miRNA is incorporated into a complex that is similar to the RNA-induced silencing complex (RISC) that participates in RNA interference. After incorporation of the miRNA strand into the RISC complex, the other strand of the duplex is degraded. When the mature miRNA is incorporated into the RISC complex it is ready to perform its function.

The identification of tissue- and cell-specific gene and miRNA expression profiles and the comparison of these expression profiles between normal and malignant cells is a step towards the unravelling of the involvement of certain genes and miRNAs

in specific biological processes. Advances in the understanding of the molecular mechanisms of normal and malignant biological processes will offer the possibility of identifying new and more rational therapeutic strategies. Rather than a common therapy for all leukemic patients, ongoing research focuses on drugs that target unique alterations in protein networks, or deregulated cellular pathways that are specific for certain subgroups of patients, to obtain more disease pathogenesis-based and individualized therapy with a better over-all survival.

5. Basic helix-loop-helix transcription factors

One major class (>240 members) of regulatory factors that have been implicated in cell growth, cell differentiation, and cell fate decisions in many different tissues, including myogenesis,[70] neurogenesis[71] and hematopoiesis,[72] is the helix-loop-helix (HLH) family of proteins.[73] Developmental processes may be positively or negatively controlled by basic helix-loop-helix (bHLH) transcription factors and their inhibitors. As a common theme, tissue-specific bHLH factors form heterodimers with members of the more widely expressed E-protein bHLH family members E12/47, HEB and E2-2. For example, E-protein bHLH family members are heterodimerization partners for the tissue-specific bHLH factors such as the MyoD family of muscle regulatory bHLH proteins, the NeuroD family involved in nervous system development and the hematopoietic bHLH factor SCL/TAL1. In addition, E-proteins also have the capacity to homodimerize.[74] Dimerization of these positively acting bHLH factors is essential for binding to DNA sequences that contain the (G/A)CANNTG(G/A) E-box consensus sequence present in the promoter region of responsive genes[75] (Figure 5A).

HLH factors play important roles in hematopoiesis. For example, the bHLH transcription factor SCL/TAL1 is essential for primitive hematopoiesis and is therefore a critical factor in the development of HSCs towards all hematopoietic cell lineages.[72;76] Disruption of the *SCL/tal1* gene in mice leads to a complete absence of blood and early embryonic lethality. The *E2A* gene is required for lymphocyte development, as revealed by *E2A*$^{-/-}$ mice, which show both severe defects in early B-cell development[77;78] as well as abnormalities during the early stages of thymocyte differentiation. In addition, a high incidence of T-cell tumours was observed in these mice.[79-81] Several mutations affecting bHLH proteins have been implicated in

lymphoid leukemia. Three bHLH family members (*SCL/TAL1*, *LYL1* and *E2A*) are located at the sites of recurrent chromosomal translocations breakpoints in leukemia.[82-84]

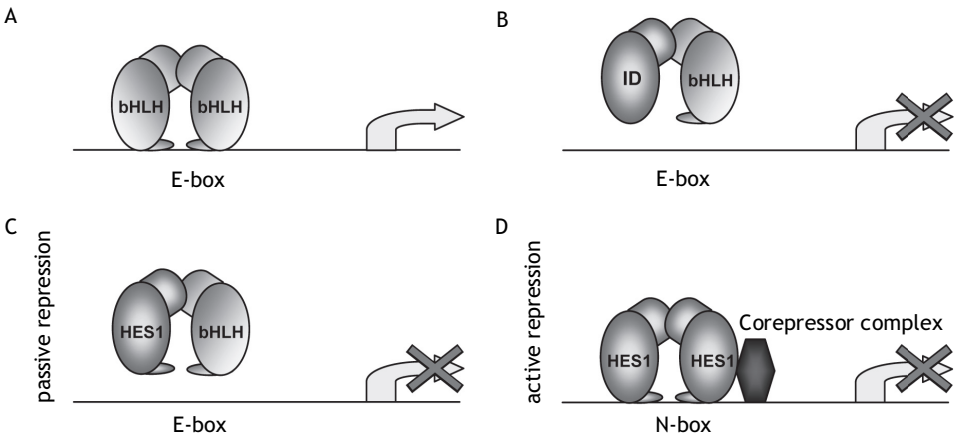

Figure 5. Transcriptional regulation by HLH proteins. (**A**) bHLH transcription factors contain a HLH motif, through which hetero- and homodimeric interactions are mediated. Furthermore, the bHLH proteins contain a conserved basic region that interacts with DNA. By forming dimers bHLH transcription factors can bind to specific DNA sequences, known as E-boxes, resulting in transcriptional activation of their target genes. (**B**) ID proteins constitute a separate class of HLH proteins lacking the DNA-binding domain and acting as dominant-negative regulators of bHLH transcription factors by trapping them in heterodimeric complexes that are unable to bind DNA. (**C**) Comparable to ID proteins, HES1 proteins function as dominant-negative regulators of bHLH transcription factors, but in contrast to ID proteins, HES1 proteins do contain a DNA-binding domain. This mechanism of transcriptional repression by HES1 is called passive repression. (**D**) By forming homodimers HES1 can bind to specific DNA sequences, known as N-boxes. Upon DNA binding corepressor complexes are recruited to mediate active transcriptional repression.

6. ID proteins

The inhibitor of DNA binding (ID) proteins constitute a HLH subfamily and act as dominant-negative regulators of bHLH transcription factors.[85] While capable of forming dimers with other HLH proteins, the four mammalian ID members (ID1-4)[85-90] lack a basic DNA-binding domain, thereby trapping bHLH factors in heterodimeric

complexes which are unable to bind DNA. Therefore, ID proteins prevent the bHLH protein complex from activating or inactivating downstream events. ID proteins contain a highly conserved HLH domain and are capable of binding to various common partner proteins[91] (Figure 5B). In line with this, they were shown to exert similar or redundant biological functions in various experimental models. However, the differential expression patterns of different ID proteins in various tissues indicate different biological functions for each of the ID proteins under normal physiological conditions.[90;92-95] Their intracellular expression level and their cytoplasmic or nuclear localization determine the specificity and functional activity of the individual ID proteins.[96-98] In addition, posttranslational modifications may alter the activity of the ID proteins.[99;100] Although E-proteins have been established as bona fide ID-interacting proteins *in vivo*, a range of other partners have been identified, including transcription factors that do not contain a HLH domain, such as retinoblastoma (Rb) proteins,[101;102] Ets-family proteins,[103] Pax,[104] MIDA1,[105-107] SREBP-1c[108] and PC2.[109] The relevance of these various interactions in mediating biological functions of ID proteins in a truly physiological context *in vivo* has yet to be established.

Traditionally, ID proteins have been considered to function as negative regulators of cell differentiation. Nowadays this family of regulatory proteins has been ascribed much wider biological roles. Recent work has revealed that ID proteins act as negative as well as positive regulators dependent on the specific cell lineages and developmental stages analysed. They have been implicated in developmental biology, cell cycle regulation, tumorigenesis and angiogenesis.[110;111]

Besides the ID proteins, Hairy-Enhancer of Split (HES) proteins (HES1-7)[112-119] also act as transcriptional repressors of bHLH-regulated genes. Just like ID proteins, HES1 acts by sequestration of positive bHLH transcription factors into non-functional heterodimeric complexes[113;116] (Figure 5C). In addition to this passive repression mechanism, HES1 proteins function as active repressors by binding to DNA. HES1 proteins contain a DNA binding domain that preferentially recognizes the consensus sequence CACNAG (or N-box).[113] HES1 homodimers bind to the N-box sequence and the corepressor TLE/Groucho is subsequently recruited to mediate repression by interaction with HDACs (Figure 5D). A third repression mechanism is mediated by the orange domain/helix3-helix4 in combination with the DNA-binding domain.[120]

Box 2. ID2 as a functional target of the retinoblastoma protein

The retinoblastoma (Rb) protein is involved in the molecular machinery controlling the cell cycle by acting as a negative regulator of cell cycle progression. The G1/S transition is a key step for cell cycle progression and the Rb protein is able to repress this transition by inhibiting gene transcription that is required for S-phase entry. An important target of Rb proteins is the E2F family of transcription factors, which plays a pivotal role during entry into the S-phase. Rb family members are posttranslationally regulated and their phosphorylation leads to functional inactivation and the release of E2F factors. The *Rb* gene has an important role in suppressing tumorigenesis and is functionally inactivated in many human tumour types.[170] Another Rb-interacting protein is Id2. By binding to Rb, Id2 is able to abolish its growth-suppressing activity. In addition, it has been suggested that Id2 is a downstream target of Rb, since rescue of embryonic lethality of $Rb^{-/-}$ mice by loss of Id2 was observed.[101;102;150;152] By keeping control of Id2, Rb might prevent Id2-mediated inhibition of transcription factors that control normal development. The relationship between the Rb-E2F interaction and the Rb-Id2 interaction is poorly understood and remains to be further clarified. Rb might simultaneously bind E2F and Id2, but alternatively E2F and Id2 might compete for binding to Rb (Figure box 2).

$Rb^{-/-}$ mice show profound abnormalities of fetal liver macrophages and loss of Id2 rescues this defect. A molecular cross-talk between Rb, Id2 and PU.1 in the transcriptional activation of a gene that controls macrophage development (CSF-1R) has been proposed as a model for the rescue of the Rb-deficient phenotype by loss of Id2. It has been suggested that by restraining Id2-mediated inhibition of the transcription factor PU.1, Rb promotes differentiation of macrophages.[152]

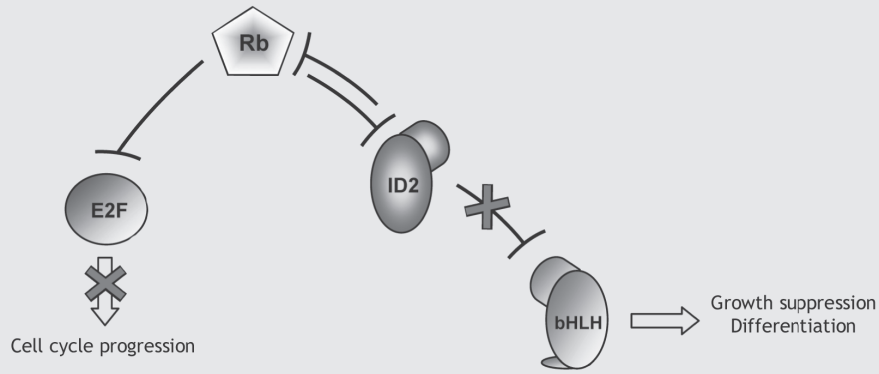

Figure box 2. Targets of the retinoblastoma protein. By inhibiting E2F family members, Rb negatively regulates cell cycle progression, and by preventing Id2-mediated inhibition of transcription f actors, such as bHLH transcription factors, Rb stimulates growth suppression and differentiation. Furthermore, by binding to Rb, Id2 abolishes its growth-suppressing activity.

6.1 ID proteins and lineage commitment

Knockout studies revealed a role for ID proteins during hematopoiesis (Table 1). Recently, it has been found that HSCs of *Id1*$^{-/-}$ mice showed a loss of self-renewal capacity and an increased tendency to myeloid differentiation.[121] *Id3*$^{-/-}$ mice displayed defects in B-cell proliferation and a block in thymocyte maturation[122;123] and *Id2*$^{-/-}$ mice have the most severe phenotype of all single Id knockouts showing retarded growth and high neonatal morbidity. The surviving *Id2*$^{-/-}$ mice demonstrated an essential role for Id2 in the generation of peripheral lymph nodes and Peyer's patches. These mice display disturbed differentiation of natural killer cells, they lack Langerhans cells and splenic CD8α^+ dendritic cells were severly reduced. It was observed that spleens from Id2 $^{-/-}$ mice have lost their immature B-cell population while more mature B-cells were accumulated. Furthermore, the development of nasopharyngeal-associated lymphoid tissue was defective. T-cell development was not affected in these mice.[124-129] No hematopoietic abnormalities were found in *Id4*$^{-/-}$ mice. All combinations of Id double knockout mice are embryonic lethal, which indicates that, at least in mammals, ID functions are required for survival.[130;131] Overexpression studies also demonstrate the involvement of ID proteins in the regulation of cell fate choices in the hematopoietic system (Figure 6). Upregulation of ID1 in CD34$^+$ primary cells correlates with inhibition of erythrocyte differentiation while granulocyte differentiation was induced.[132] In line with this, overexpression of ID1 in the erythroleukemia cell line K562 blocks erythrocyte differentiation.[133;134] In contrast, ID3 overexpression promotes differentiation in K562 cells, which might be explained by different preferences for partner proteins of the two ID proteins.[135] Recently, it has been shown that ectopic expression of ID1 in CD34$^+$ cord blood cells enhances neutrophil differentiation whereas eosinophil development was inhibited. In contrast, ID2 overexpression enhances both neutrophil and eosinophil differentiation.[136] These data indicate a differential role of ID proteins during lineage commitment. Overexpression of ID3 in human CD34$^+$ fetal liver and thymic progenitor cells resulted in an inhibition of T-cell differentiation while natural killer cell development was promoted.[137;138] Furthermore, transgenic mice in which Id1 or Id2 are specifically overexpressed in T-cells also show a block in T-cell development. Similar to the E2A-deficient mice and as would be expected given the opposing functions of *ID* and *E2A* gene products, Id1 and Id2 transgenic mice develop T-cell

lymphomas.[139;140] Overexpression of Id1 in early B-cells, strongly impaired mouse B-cell development[141] and when ID3 was introduced into uncommitted fetal liver cells, their capacity to develop into B-cells and lymphoid dendritic cells was inhibited while myeloid dendritic cell development was not affected.[142;143] The latter has also been shown for ID2. Finally, constitutive expression of Id1 in a murine myeloid precursor cell line inhibited the ability to differentiate upon growth factor stimulation, which is in line with the observed increase in myeloid commitment of HSCs from Id1-deficient mice.[144] Evidence for a role of ID proteins in cell fate decisions also comes from expression profiling studies. Analysis of ID expression in human hematopoietic cell lines revealed that, in general, ID levels decline with increasing maturity. One exception is ID2, which shows upregulation during differentiation along monocyte-macrophage, granulocytic and erythroid lineages.[92;145-147] Together, these data suggest a role for ID proteins in regulating specific hematopoietic lineage choices.

	Phenotype	Reference
Id1 $^{-/-}$	- loss of HSC self-renewal capacity - HSCs show increased tendency towards myeloid differentiation	121
Id2 $^{-/-}$	**General** - high neonatal morbidity and retarded growth **Hematopoietic** - disturbed development of peripheral lymph nodes and Peyer's patches - defective NK cell differentiation - lack of Langerhans cells - reduction in the number of splenic CD8α+ DCs - loss of immature B-cell population in the spleen - accumulation of mature B-cells in the spleen - defective NALT tissue	128,126,125, 129,127,124
Id3 $^{-/-}$	- defective B-cell proliferation - block in thymocyte maturation	122,133
Id4 $^{-/-}$	- no hematopoietic abnormalities	

Table 1. Hematopoietic abnormalities in Id knockout mice. Hematopoietic stem cell (HSC); natural killer (NK) cell; dendritic cell (DC); nasopharyngeal-associated lymphoid tissue (NALT).

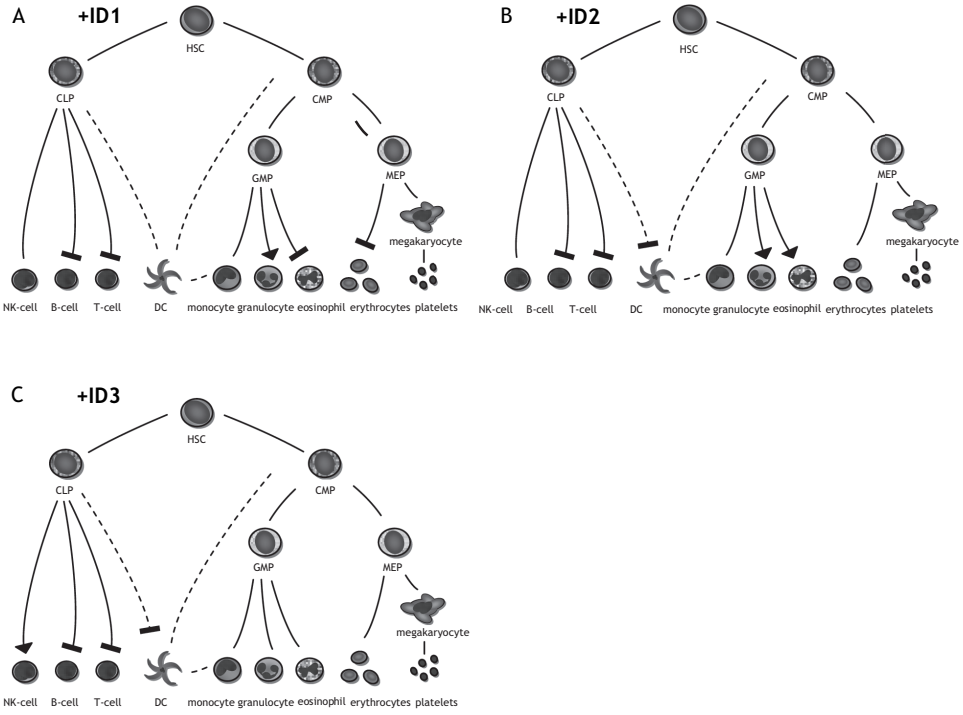

Figure 6. Schematic overview of ID proteins and their effect on lineage specific differentiation. (**A**) Effect of overexpression of ID1 (+ ID1) on differentiation towards a specific lineage. (**B**) Effect of overexpression of ID2 (+ ID2) on differentiation towards a specific lineage. (**C**) Effect of overexpression of ID3 (+ ID3) on differentiation towards a specific lineage. The black bars represent defective or blocked differentiation; the black arrows represent enhancement of differentiation.

6.2 ID proteins and cell cycle regulation

Since ID proteins have been shown to affect the balance between cell growth and differentiation and since, in many cases, cellular differentiation is accompanied by cell cycle arrest, it was postulated that ID proteins might also be involved in cell cycle regulation. Indeed, several lines of evidence suggest a role for ID proteins in cell cycle control. Since both positive and negative actions on cell cycle progression have been reported for the different *ID* genes, their role appears to be context-dependent.

For example, following serum stimulation of quiescent fibroblasts, the expression of Id1, Id2 and Id3 is induced and inhibition of Id protein synthesis inhibits cell cycle re-entry of G0-arrested fibroblasts.[89;148;149] In contrast, ID2 is highly expressed in human $CD34^+$ progenitor cells following growth arrest by cytokine starvation and the expression is decreased in the first hours after re-stimulation with cytokines. Therefore, ID2 expression seems to correlate with a state of quiescence rather than cell growth.[145] Comparable, loss of Id1 in murine bone marrow HSCs induces an increase in HSC turnover.[121] ID levels fluctuate throughout the cell cycle but whether this reflects an important role as modulator of cell cycle transitions remains to be clarified.

The molecular mechanisms by which ID proteins are able to modulate the cell cycle are not well understood. Two distinct pathways have been described coupling ID function directly to promotion of cell cycle progression. One pathway involves the interaction between the Rb proteins and ID2 and ID4 that leads to the expression of genes required for S-phase transition.[101;102;150] The second pathway, which is described for ID1, involves the inhibition of the E2A-mediated activation of the cyclin-dependent kinase inhibitor $p21^{waf1/Cip1}$.[151]

6.3 ID proteins and tumorigenesis

The ability of ID proteins to affect lineage commitment and to control cell cycling suggests that these proteins could have a role in tumorigenesis. Perhaps the best example that links ID overexpression to transformation of hematopoietic cells is the fact that Id1 and Id2 transgenic mice develop T-cell lymphomas.[139;140] Since *E2A*-deficient mice show a high incidence of T-cell tumours it might be possible that the inhibition of E2A by Id proteins is involved during tumorigenesis. Although the precise molecular mechanism remains unclear, these observations suggest an important role for ID and bHLH proteins in tumorigenesis within the hematopoietic system *in vivo*. Besides E-proteins, other ID interacting proteins such as Rb and Ets-family proteins are implicated in tumorigenesis. Rb proteins act as tumour suppressors and are functionally inactivated in the majority of human tumours. The lethality observed in $Rb^{-/-}$ mice at E14.5 is accompanied by widespread proliferation, defective differentiation and apoptosis in the nervous system and hematopoietic lineages. Loss of *Id2* partially rescues the $Rb^{-/-}$ phenotype, suggesting that Rb is restraining the

activity of Id2.[101;102;150;152] The data indicate that an increase in the available pool of Id2, which may also be achieved by hyperphosphorylated or inactive Rb protein, leads to cell cycle progression and defective differentiation. Concerning the Ets-family proteins, binding of ID1 targets Ets-mediated activation of the tumour suppressor p16^{Ink4a}. It has been demonstrated that p16 ^{Ink4a} expression levels are controlled by a balance between Ets and ID1.[153] It may well be possible that deregulated ID protein expression levels are involved in the inhibition of p16 ^{Ink4a} expression, which occurs frequently in a wide range of human cancers, including leukemia.

One case has been described in which two ID4 short splice variants were found to be overexpressed due to a t(6;14)(p22;q32) chromosomal translocation in a B-cell lineage ALL.[154] No other *ID* gene mutations were found in various tumour types, indicating that *ID* genes are not common oncogenes. However, since the *ID* genes have not been examined for genetic alterations in many studies, more studies are required to investigate this in more detail. In contrast to the observed overexpression of ID4 splice variants, *ID4* was also found to be silenced by methylation in murine and human leukemias, indicating a possible role in tumour suppression.[155] Further studies should clarify the possible roles of ID4 and its aberrant splice variants in distinct types of leukemia.

Although ID genes may not be common oncogenes, elevated levels of ID mRNA and protein expression has been reported in diverse human tumour types, including leukemia, and it has been demonstrated that upregulation may be mediated by well-established oncoproteins as RAS,[156;157] MYC [150;158;159] and the chimera Ewing's sarcoma (EWS)-ETS.[160;161]

A feature of some oncoproteins, such as MYC and E2F1, is the ability to drive apoptosis when ectopically overexpressed. Depending on the cell context, these proteins can either function as anti-apoptotic or as pro-apoptotic molecules. Several groups have demonstrated that ID proteins also have pro-apoptotic properties in cell line and primary cell models.[162-166] In addition, enforced expression of *Id* genes *in vivo* also induces apoptosis. For example, the T-cell block and the development of T-cell lymphomas in transgenic mice in which Id1 and Id2 are specifically overexpressed in T lymphocytes, is accompanied by widespread apoptosis.[140] Together, these data indicate that, although ID family members are not common oncoproteins, at least they share some features of oncoproteins.

Box 3. ID proteins and tumour angiogenesis

In addition to the involvement of ID proteins in lineage commitment and cell cycle regulation, an essential role of these proteins have been demonstrated in neovascularization of tumours, which allows rapid growth.[171] This property of ID proteins was first discovered when studying the growth of tumours in $Id1^{-/-}/Id3^{+/-}$ mice. In subcutaneous tumour models, the mice with reduced Id levels showed a marked decrease in primary tumour growth and/or metastasis due to poor vascularization with concomitant necrosis of the tumour cells.[130] Bone marrow transplantation of these $Id1^{-/-}/Id3^{+/-}$ mice with cells from a wild-type donor restores tumour angiogenesis and, conversely, introduction of $Id1^{+/-}/Id3^{-/-}$ bone marrow cells into a wild-type host results in a profound delay in the appearance of subcutaneous tumours. It has been found that bone marrow-derived endothelial precursor cells and hematopoietic precursor cells play an important role in the formation of new vessels and that disruption of Id1 and Id3 results in the failure to mobilize these bone marrow precursor cells from the marrow to the sites of tumour vasculature.[172] Thus, by targeting ID proteins it might be possible to inhibit tumour angiogenesis.

7. Concluding remarks

As illustrated by the above overview of ID proteins and their roles during normal and malignant hematopoiesis, ID protein functions have been implicated in a wide range of biological processes. Since the expression pattern of the four mammalian ID proteins is cell lineage and developmental stage-specific, the different ID proteins have different binding specificities and their activity is regulated in several distinct ways, it is likely that their mode of action is cell type specific. Therefore, it is doubtful whether their biological functions and molecular mechanisms as found in a particular cell lineage can be extrapolated to other mammalian cell lineages. It has been proposed that ID proteins are good candidates for developing targeted therapies, since ID proteins have been demonstrated to mediate the activity of several important genes that are involved in carcinogenic processes. To target the functional activities of these intracellular proteins in myeloid malignancies, it will be important to further establish the role of ID proteins in normal and malignant myeloid development. The significance of the interactions between ID proteins and its various bHLH and non-bHLH binding partners, the identification of target genes whose expression is regulated by ID proteins, the transcriptional control of ID

protein expression and the posttranslational regulation of ID protein activity during myelopoiesis remains to be clarified in order to be able to use the ID proteins to target myeloid malignancies. In this thesis we investigated myeloid hematopoiesis by using APL as a model.

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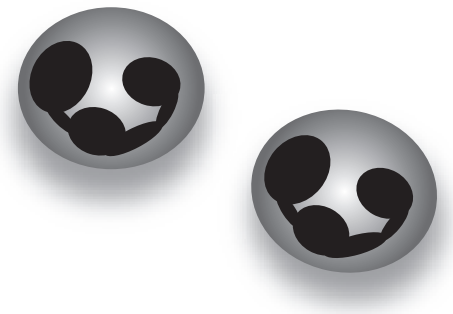
Chapter 2

ID1 and ID2 are retinoic acid responsive genes and induce a G0/G1 accumulation in Acute Promyelocytic Leukemia cells



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Abstract

Acute promyelocytic leukemia (APL) is uniquely sensitive to treatment with all-*trans* retinoic acid (ATRA), which results in the expression of genes that induce the terminal granulocytic differentiation of the leukemic blasts. Here we report the identification of two ATRA responsive genes in APL cells, *ID1* and *ID2*. These proteins act as antagonists of basic helix-loop-helix (bHLH) transcription factors. ATRA induced a rapid increase in ID1 and ID2, both in the APL cell line NB4 as well as in primary patient cells. In addition, a strong downregulation of E2A was observed. E2A acts as a general heterodimerization partner for many bHLH proteins that are involved in differentiation control in various tissues. The simultaneous upregulation of ID1 and ID2, and the downregulation of E2A suggest a role for bHLH proteins in the induction of differentiation of APL cells following ATRA treatment. To test the relevance of this upregulation, ID1 and ID2 were overexpressed in NB4 cells. Overexpression inhibited proliferation and induced a G0/G1 accumulation. These results indicate that *ID1* and *ID2* are important retinoic acid responsive genes in APL, and suggest that the inhibition of specific bHLH transcription factor complexes may play a role in the therapeutic effect of ATRA in APL.

Introduction

In 98% of the cases of acute promyelocytic leukemia (APL), the promyelocytic (*PML*) gene on chromosome 15 is fused to the retinoic acid receptor α (*RARA*) gene on chromosome 17 resulting in a PML-RARα fusion protein. PML-RARα may interfere with the normal function of the retinoic acid receptor, a nuclear hormone receptor that acts as a ligand-dependent transcription factor.[1] Several important RARα target genes have been identified that are deregulated by PML-RARα in APL cells including *p21$^{waf1/Cip1}$* [2-4] and the transcription factors CCAAT/enhancer-binding-protein-β and -ε (*C/EBP*β and *C/EBP*ε).[5;6] p21$^{waf1/Cip1}$ is a cyclin-dependent kinase inhibitor that is involved in the regulation of the activity of several cyclin-dependent kinases and in the cell cycle. The C/EBP transcription factors are involved in the regulation of granulocytic differentiation.

Hematopoietic differentiation is controlled by specific combinations of transcription factors. Some of these are expressed in stem cells, such as AML1, whereas others have a more lineage-specific expression pattern. GATA1, PU.1, C/EBPα and C/EBPε are lineage-specific transcription factors that are important in myeloid development. The results of both knockout and expression studies have shown that GATA1 participates in the differentiation of common myeloid progenitors to megakaryocyte/erythroid progenitors, PU.1 is involved in the development of monocytes/macrophages and C/EBPα and C/EBPε have a role in granulopoiesis. These transcription factors may lock lineage commitment decisions irreversibly by autostimulation of their own expression. In addition, they may inhibit the expression of transcription factors that are involved in alternate differentiation routes, as has been described for PU.1 and GATA1.[7]

Members of the bHLH family of transcription factors have been implicated in cell fate decisions in many different tissues. For example, the bHLH transcription factors MyoD and myogenin are involved in the development of muscle tissue and during neurogenesis the bHLH protein MASH-1 plays an essential role.[8] As a common theme, tissue-specific bHLH factors form heterodimers with members of the more ubiquitously expressed E-protein bHLH family members E12/47, HEB and E2-2, which are generally considered as promiscuous heterodimerization partners.

ID proteins constitute a separate class of helix-loop-helix (HLH) proteins lacking the basic DNA-binding domain and acting as dominant-negative regulators of bHLH

transcription factors by trapping them in heterodimeric complexes which are unable to bind DNA.[8] In addition, ID proteins have been implicated in cell cycle control through direct interaction with the retinoblastoma protein,[9] and by interaction with the centrosome.[10] Recent data indicate the involvement of ID proteins in angiogenesis, metastasis and tumorigenesis.[11-14] In hematopoiesis, bHLH transcription factors and their dominant-negative regulators, the ID proteins, have been implicated in lymphopoiesis. Constitutive expression of Id1 targeted to pro-B cells in transgenic mice revealed impairment of B lymphocyte development.[15] In addition, several mutations affecting bHLH proteins have been implicated in lymphoid leukemia. Three bHLH family members (*SCL/TAL-1*, *LYL-1* and *E2A*) are located at the sites of recurrent chromosomal translocation breakpoints in leukemia. The t(1;14) in patients with T-cell acute lymphoblastic leukemia (T-ALL) transposes the *SCL* gene to the *TCR* α/δ locus on chromosome 14.[16] The t(7;19) in T-ALL involves the *LYL-1* gene that is translocated to the *TCR* β locus.[17] *E2A* is involved in the t(1;19) that occurs in approximately 25% of the cases of childhood pre-B-ALL and results in the generation of an E2A-PBX fusion protein.[18] In this paper, we describe the involvement of ID1 and ID2 in the response of APL cells to retinoic acid treatment.

Materials and methods

Cell culture

Bone marrow mononuclear cells of APL patients were isolated at diagnosis by lysis of erythrocytes in 0.14 M NH_4Cl buffer for 30 min on ice. Indirect immunostaining with a monoclonal anti-PML antibody (PG-M3, Santa Cruz Biotechnology, Santa Cruz, CA, USA) showed a microspeckled pattern characteristic for the PML-RARα fusion protein.[19-21] Cytogenetic analysis showed that all patients were positive for the t(15;17)(q22;q21) chromosome translocation. Patients and NB4 cells[22] were cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (Life Technologies). The amphotrophic Phoenix cell line (Φ-NX-A) (kindly provided by Dr. G.P. Nolan, Stanford University Medical Center, Stanford, CA) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% FCS and 2mM glutamine. Cultures were incubated at 37°C in a 5% CO_2 completely humidified atmosphere.

Northern blotting

NB4 cells were induced with all-*trans* retinoic acid (ATRA) (dissolved in ethanol to a stock solution of 10^{-3} M, Sigma, St Louis, MO, USA) at a final concentration of 10^{-6} M. Total cellular RNA was isolated by guanidinium-isothiocyanate lysis and centrifugation on a 5.7 M CsCl cushion. PolyA$^+$ RNA was isolated from total RNA by using a mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). In total, 10 μg of total RNA or 1 μg of PolyA$^+$ RNA was size-fractionated by 1% agarose-formaldehyde gel electrophoresis and transferred onto Hybond N$^+$ nylon membranes (Amersham, Buckinghamshire, UK). The filters were pre-hybridized and hybridized at 65°C overnight in phosphate buffer (2N NaH_2PO_4, 2N Na_2HPO_4), 7% sodium dodecyl sulphate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA) pH=8.0 and 1% bovine serum albumine (BSA). DNA probes were labeled with [^{32}P]α-dATP by random primed labeling (Boehringer, Mannheim, Germany). After hybridization, the filters were washed in 0.2 x SSC/0.2% SDS for 15 min at 65°C. Northern blots were hybridized to radiolabeled human ID1 (kindly provided by Dr. S. Stegman, NKI, Amsterdam, The Netherlands), murine Id2 (kind gift from Dr. X.H. Sun, NYU, New York, NY, USA), human ID3 (kindly provided by Dr. J.D. Norton, Christie CRC Research Centre, Manchester, UK), human ID4 (kindly provided by Dr. L. Lania, University of Naples 'Federico II', Naples, Italy) and E2A (kindly provided by Dr. S. Stegman). As a control for equal loading, filters were stripped and hybridized to a 777bp HindIII-EcoRI human GAPDH fragment.

Western blotting

Non-stimulated and ATRA-stimulated NB4 cells were lysed in RIPA-buffer containing 1% Nonidet P-40 (NP-40), 0.5% natrium-deoxycholaat, 0.1% SDS and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Total protein lysates (50 μg) and molecular weight markers were separated on 12% (ID1 and ID2) or 7% (E2A) SDS-poly-acrylamide gels and electrophoretically transferred onto polyvinylidenefluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Blots were probed with antibodies to ID1 (Z-8), ID2 (C-20) (Santa Cruz Biotechnology) and with anti-human E2A (E12/E47) antibody (BD Biosciences, San Diego, CA, USA). As a control for equal loading, blots were probed with antibodies to Cdc2 p34 (H-297) or to Actin (I-19) (Santa Cruz Biotechnology).

Construction of retroviral vectors

The pLZRS retroviral vector in which the multiple cloning site is linked to the downstream internal ribosomal entry sequence (IRES) and a truncated form of the nerve growth factor receptor (ΔNGFR) were kindly provided by Dr. G.P. Nolan and Dr. M.H.M. Heemskerk, LUMC, Leiden, The Netherlands, respectively. The pLZRS-ID1-IRES-ΔNGFR was constructed by cloning

the human ID1 cDNA sequence into the BamH1/Xho1 site of pLZRS-ΔNGFR and pLZRS-ID2-IRES-ΔNGFR was constructed by cloning the human ID2 cDNA sequence into the BamH1 site of pLZRS-ΔNGFR. The pLZRS-ΔNGFR and pLZRS-EGFP-IRES-ΔNGFR were used as control vectors in the experiments. The pLZRS-EGFP-IRES-ΔNGFR was constructed as follows: pEGFP-C1 was purchased from Clontech Laboratories (Palo Alto, CA, USA) and the EGFP fragment was isolated by BamH1/Not1 digestion. This EGFP fragment was cloned into the BamH1/Not1 site of pcDNA3, isolated by BamH1/Xho1 digestion and subsequently cloned into the BamH1/Xho1 site of pLZRS-ΔNGFR.

Retroviral supernatants, transduction of NB4 cells and magnetic cell sorting

Retroviral constructs were transfected into Φ-NX-A cells using the calcium phosphate precipitation method. At 24 hours prior to transfection, Φ-NX-A cells were seeded at 2.8 x 10^6 cells/75cm^2. At 16 hours after transfection, the medium was replaced with fresh medium. Supernatants were harvested, centrifuged and frozen in aliquots at -80°C. For transduction, cells were cultured on retronectin (Takara Bio Inc., Otsu, Shiga 520-2193, Japan) -coated 35 mm Petri dishes together with 2 ml of thawed supernatant for 22 hours and then washed with phosphate-buffered saline (PBS) (B. Braun Melsungen AG, Melsungen, Germany). The transduction efficiencies, measured by the expression of the marker ΔNGFR, were analyzed by flow cytometry 1 day after transduction on a Beckman Coulter Cytomics FC 500 (Beckman Coulter, Miami, FL, USA). Transduction efficiencies were between 17 and 21% for all different transduced cells. Expression levels of the ΔNGFR were followed during a culture period of at least 22 days and revealed that levels did not change during culture. ΔNGFR expression was detected by using the supernatant of the 20.4 hybridoma (American Type Culture Collection) producing anti-human NGFR antibody. Transduced NB4 cells were isolated by magnetic cell sorting (MACS) on the basis of ΔNGFR positivity. Flow cytometric analysis of ΔNGFR expression of the MACS-sorted cells showed a purity of more than 90% in all experiments. Expression of ID1 and ID2 in isolated transduced cells was confirmed by Western blotting.

Colony-forming assay

Clonogenic growth of tranduced and MACS-sorted NB4 cells was measured by plating 800 cells/ml in semisolid (1.2% methylcellulose; Methocel, Fluka Chemie, Buchs, Switzerland) culture medium (Iscove's modified Dulbecco's medium, IMDM, Life Technologies) supplemented with 20% FCS. Where appropriate, ATRA was added to the semisolid medium at final concentrations as indicated in the figure legends. Colonies were counted after 6 days of culture.

Cellular differentiation and cell cycle characteristics

Differentiation of transduced and MACS-sorted NB4 cells was assessed on May-Grünwald-Giemsa stained cytospin slides. In addition, the nitroblue-tetrazolium (NBT, Sigma) reduction test was performed as described.[23] The expression of the differentiation-related surface marker CD11c was measured with a phycoerythrin (PE)-conjugated monoclonal antibody (Immunotech, Marseille, France), apoptosis was measured using Annexin-V-Alexa Fluor 647 (Molecular Probes, Eugene, OR, USA). Results were analyzed on a Beckman Coulter Cytomics FC 500 (Beckman Coulter). More than 5 days after transduction and MACS-sorting NB4 cells were stained for DNA by incubation with a hypotonic propidium iodide solution. Bivariate staining for BrdUrd incorporation and DNA content and calculation of the cell cycle time was performed as described previously.[24] Flow cytometric analysis of the results was performed on a Coulter Epics Elite flowcytometer (Beckman Coulter, Miami, FL, USA).

Results

ID1 and ID2 are rapidly induced upon ATRA treatment of APL cells

bHLH transcription factors and their inhibitors, ID proteins, play an important role in the regulation of proliferation and differentiation of various cell types. We assessed the expression of all four *ID* gene family members (*ID1-ID4*) in APL cells before and after incubation with 10^{-6} M ATRA. Total RNA was isolated from the PML-RARα positive APL cell line NB4 that can be induced to differentiate with ATRA. ID1 and ID2 mRNA levels strongly increased after exposure to ATRA (Figure 1A and B). ID1 mRNA expression was upregulated within 2 hours and returned to undetectable levels at 48 hours after ATRA stimulation. ID2 mRNA expression was also upregulated within 2 hours but remained elevated and further increased after 48 to 96 hours after stimulation. In contrast, ID3 and ID4 mRNA could not be detected on Northern blots using total RNA (not shown) or polyA+ mRNA (Figure 1C and D, respectively).

The expression pattern of ID1 and ID2 was confirmed in APL patient cells (Figure 1E and F). Leukemic cells were freshly isolated from bone marrow of newly diagnosed patients with previously untreated APL and incubated in the presence or absence of ATRA. After culture for various times RNA was isolated. Both ID1 and ID2 mRNA were rapidly induced by ATRA. Figure 1E shows that ID1 was induced within 2 hours and

declined to undetectable levels after 24 hours. ID2 levels remained elevated at 24 hours of incubation (Figure 1F).

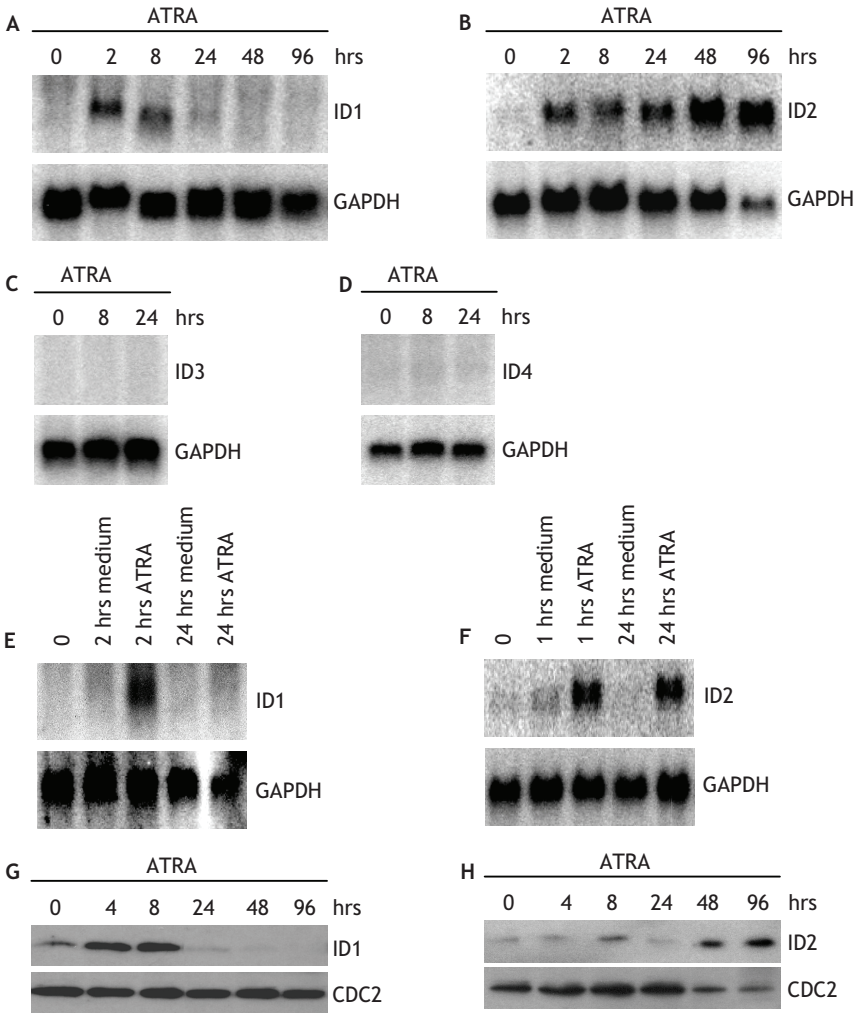

Figure 1. ID1 and ID2 mRNA and protein are upregulated upon ATRA treatment in both NB4 and fresh APL patient cells. Northern blot analysis was performed with total RNA from NB4 cells (**A,B**) after exposure to 10-6 M ATRA for 0, 2, 8, 24, 48 and 96 hours. (**C,D**) ID3 and ID4 mRNA was not detectable in APL cells. PolyA$^+$ RNA was extracted from NB4 cells after exposure to 10^{-6} M ATRA for 0, 8 and 24 hours and blots were hybridized with radiolabeled ID3 (**C**) and ID4 (**D**) probes. Analysis of ID1 and ID2 mRNA expression in patient cells (**E,F**) was performed by Northern blot analysis with total RNA derived from APL patient cells after ATRA treatment. The

blots were hybridized with radiolabeled ID1 (**A,E**) and ID2 (**B,F**) probes. A GAPDH probe was used as a control for equal loading of RNA in all Northern blot experiments. (**G,H**) Total cell lysates (50 μg) from non-stimulated and ATRA-stimulated NB4 cells (10^{-6} M ATRA was added for 0, 4, 8, 24, 48 and 96 hours) were subjected to immunoblot analysis using antibodies to ID1 (**G**) and ID2 (**H**). The same blots were reprobed with a CDC2 antibody to demonstrate equal loading.

In response to ATRA, ID1 and ID2 were also clearly induced at the protein level (Figure 1G and H). Western blotting demonstrated that ID1 protein levels were increased at 4 and 8 hours after ATRA incubation to diminish to almost undetectable levels at 24 hours after incubation with ATRA (Figure 1G). ID2 protein was induced at 48 to 96 hours after ATRA incubation (Figure 1H). Thus, ID1 and ID2 mRNA and protein are rapidly upregulated by ATRA in both NB4 as well as fresh APL cells.

E2A is downregulated during ATRA-induced APL cell differentiation

Since ID proteins act as general inhibitors of bHLH transcription factors by inhibiting their binding to DNA, we measured the expression of E2A, a ubiquitously expressed bHLH protein that serves as a promiscuous heterodimerization partner for many, more tissue-restricted bHLH proteins. Myeloid specific bHLH proteins are not known yet. E2A mRNA was expressed in non-stimulated NB4 cells but was downregulated within 48 hours after exposure to ATRA (Figure 2A). Also at the protein level, E2A expression strongly decreased upon ATRA-induced differentiation (Figure 2B): within 48 hours, E2A protein expression dropped to undetectable levels. The simultaneous downregulation of E2A and upregulation of ID1 and ID2 suggest a strong repression of bHLH transcription factor activity upon differentiation induction of APL cells by ATRA.

ID1 and ID2 inhibit the clonogenic growth of NB4 cells

To determine the biological relevance of this induction pattern, we tested the effect of forced ID1 and ID2 overexpression in NB4 cells. Cells were retrovirally transduced using a pLZRS retroviral vector that contains the ID1 or ID2 coding sequence in front of an IRES-ΔNGFR cassette, allowing the isolation of transduced cells with anti-NGFR coated immunobeads. As controls, cells were transduced with a vector containing the enhanced green-fluorescent protein (EGFP) coding sequence in front of the IRES-ΔNGFR cassette and with a vector that contained the IRES-ΔNGFR cassette alone (empty vector). Immunobead selection resulted in more than 90% purity in all

experiments. Stable enhanced expression of ID1 and ID2 in these cells was confirmed by Western blotting (Figure 3A). The levels of ID1 and ID2 expression in the transduced cells were comparable to the endogenous levels in NB4 cells after induction with ATRA as determined by Western blotting (data not shown). The effect of ID1 and ID2 expression was analyzed in a colony-forming assay (Figure 3B). Compared to control-transduced cells, expression of ID1 and ID2 resulted in inhibition of clonogenic growth by 70 and 50% respectively.

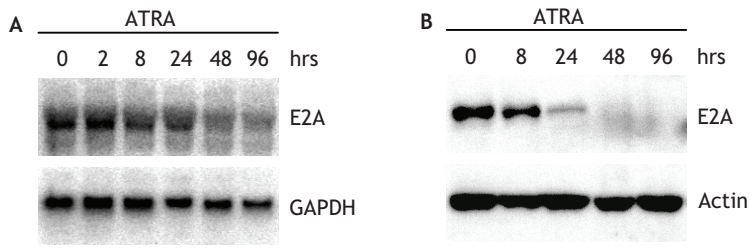

Figure 2. E2A mRNA and protein is downregulated upon ATRA treatment of NB4 cells. (**A**) Northern blot analysis was performed with total RNA extracted from NB4 cells after exposure to 10^{-6} M ATRA for 0, 2, 8, 24, 48 and 96 hours. The blot was hybridized with a radiolabeled E2A probe. A GAPDH probe was used as a control for equal loading of RNA. (**B**) Total cell lysates (50 μg) from non-stimulated and ATRA-stimulated NB4 cells (10^{-6} M ATRA was added for 0, 8, 24, 48 and 96 hours) were subjected to immunoblot analysis using an anti-human E2A antibody. As a control for equal loading, the same blot was reprobed with an Actin antibody.

Since PML-RARα requires higher concentrations of ATRA to become active as a transcriptional activator than the normal, unrearranged RARα,[1] we tested whether overexpression of ID1 and ID2 would be sufficient to restore responsiveness of the cells at lower concentrations. Colony-forming assays were performed in the presence of increasing concentrations of ATRA (Figure 3B). Apart from the reduction of colony numbers, overexpression of ID1 or ID2 did not alter the concentration at which inhibition of colony formation was observed, which remained at between 10^{-8} and 3 x 10^{-8} M ATRA for both control and ID1 or ID2 expressing cells.

ID1 and ID2 inhibit the proliferation of APL cells

To explain the inhibitory effect of ID1 and ID2 on colony formation, we compared the growth characteristics and differentiation of ID1-, ID2- and vector-transduced

NB4 cells in liquid cultures. To ensure exactly comparable growth conditions, non-transduced and transduced NB4 cells were mixed 1:1, seeded into culture flasks and analyzed at various time intervals afterwards. Proliferation of the transduced and non-transduced cells was analyzed using ΔNGFR expression to distinguish between the transduced and non-transduced cells (Figure 4). When empty vector- or EGFP-transduced cells were seeded, the proportion of transduced cells remained constant over a 3-week period, indicating comparable growth rates of transduced and non-transduced NB4 cells. When ID1- or ID2-transduced cells were seeded, a steady decrease of transduced cells was observed (from 50 to 16 and 22%, respectively) during a 3-week culture period. As the transduced cells represent a population of cells with different viral integration sites, and the number of empty vector- and EGFP-transduced cells did remain stable during culture, silencing of the expression from the viral construct can not explain this result. Therefore, we concluded that ID1 and ID2 inhibited the growth of NB4 cells, consistent with the inhibition of colony formation. To determine whether ID1 and ID2 induced the cells to differentiate towards granulocytes, we measured various differentiation parameters in isolated ID1- and ID2-transduced cells. Overexpression of ID1 and ID2 did not induce CD11c expression (Table 1) and did not stain positive in the NBT reduction assay (not shown). Furthermore, apoptosis (as measured by Annexin-V positivity) was not different in ID1- or ID2-transduced cells compared to vector-transduced or non-transduced cells (Table 1). Together, this indicates that ID1 and ID2 did not induce differentiation or apoptosis of the NB4 cells. In order to analyze cell proliferation parameters in more detail, cells were pulse-labeled with BrdUrd. BrdUrd is incorporated into the DNA during the S phase. Dividing cells are labeled and the transition of these cells through the cell cycle can be monitored. At 0 and 7 hours after labeling, BrdUrd/DNA flow cytometry was performed (Figure 5A). In five independent experiments, no significant difference in the duration of one complete cell cycle between the ID1- (25,6 hrs ± 3,3), ID2- (22,5 hrs ± 1,2) and vector- (22,8 hrs ± 2,4) transduced cells was observed. However, calculation of the percentage of cells in the different phases of the cell cycle indicated that both ID1 and ID2 caused an increase of cells in the G0/G1 phase, from 34,7% (± 0,9) in the vector-transduced cell populations to 41,6% (± 1,4) and 38,9% (± 1,9) in the ID1 and ID2 overexpressing cell populations, respectively (Figure 5B). Therefore, what we show is that once cells divide to enter mitosis, the cell cycle time of ID1- and ID2-transduced and vector-transduced cells is comparable,

whereas the number of cells that are cycling is different. We conclude that ID1 and ID2 increased accumulation of cells in G0/G1 in NB4 cells, without inducing apoptosis or enhancing differentiation.

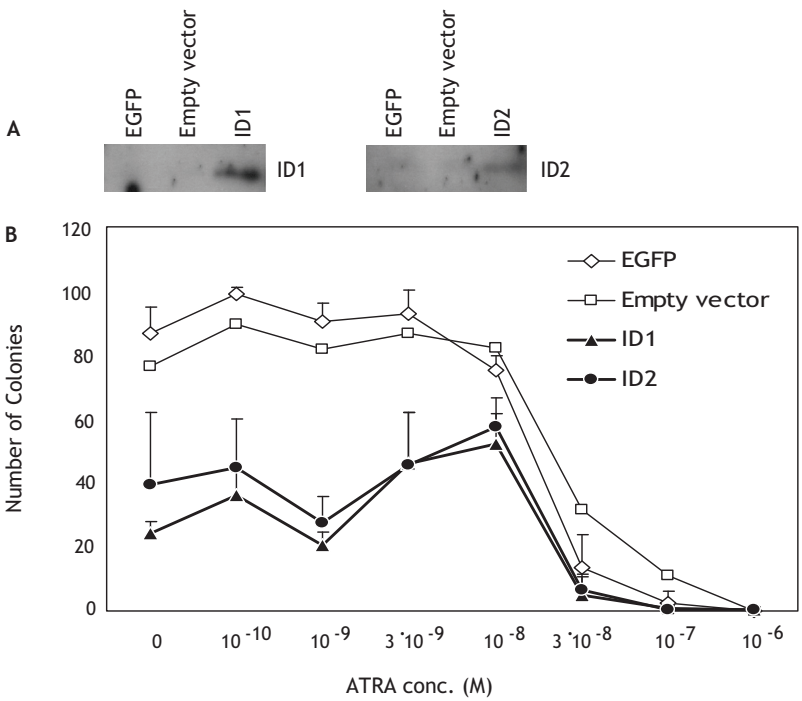

Figure 3. ID1 and ID2 inhibit the clonogenic growth of NB4 cells. NB4 cells were retrovirally transduced with pLZRS-ID1-ΔNGFR (ID1) and pLZRS-ID2-ΔNGFR (ID2) and as a control with pLZRS-EGFP-ΔNGFR (EGFP) and pLZRS-ΔNGFR (empty vector). (**A**) Total cell lysates from ID1-, ID2-, EGFP-, and empty vector-transduced cells were subjected to immunoblot analysis using antibodies to ID1 (left panel) and ID2 (right panel) after a culture period of 39 days. (**B**) Colony-forming assays were performed on transduced and MACS-sorted ΔNGFR-positive cells (n=4). Transduced cells were plated per 800 cells in semisolid culture medium supplemented with ATRA at final concentrations (conc.) as indicated in the figure and colonies were counted after 6 days. pLZRS-EGFP-ΔNGFR and pLZRS-ΔNGFR showed a mean (± SD) of 86 (± 8) and 76 colonies, respectively. Overexpression of ID1 and ID2 resulted in only 24 (± 3) and 39 (± 22) colonies per 800 cells, respectively. The concentration of ATRA at which colony growth decreased was similar for pLZRS-ID1-ΔNGFR, pLZRS-ID2-ΔNGFR and control-transduced cells.

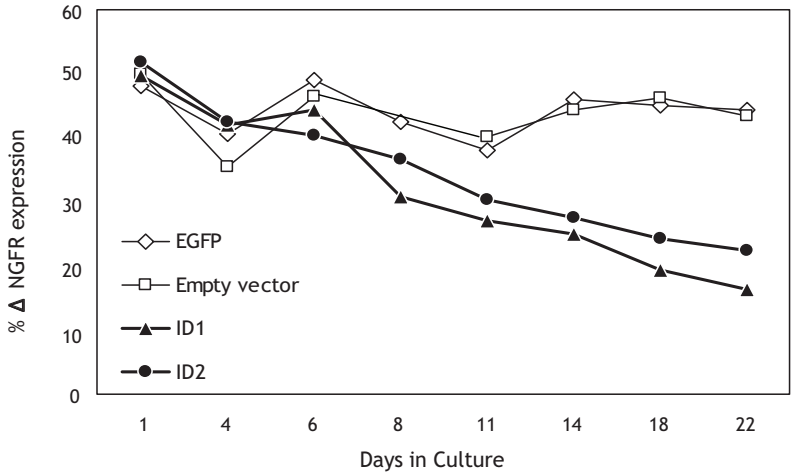

Figure 4. ID1 and ID2 inhibit proliferation of NB4 cells. pLZRS-ID1-ΔNGFR (ID1), pLZRS-ID2-ΔNGFR (ID2) and as a control pLZRS-EGFP-ΔNGFR (EGFP) and pLZRS-ΔNGFR (empty vector) were retrovirally transduced to NB4 cells. A mixed population of 50% ΔNGFR-positive transduced and sorted cells and 50% non-transduced NB4 cells were flow cytometrically analyzed for ΔNGFR expression during 22 days of liquid culture in at least two independent experiments. ID1 and ID2 overexpression demonstrated a decrease in ΔNGFR-positive cells from 50 to 16 and 22%, respectively. Percentages ΔNGFR-positive cells of the control (empty vector- or EGFP-transduced cells) remained constant.

	% CD11c positive cells				% Annexin-V positive cells			
Days in culture	Non-transduced	Vector	ID1	ID2	Non-transduced	Vector	ID1	ID2
1	0.3	0.4	0.2	0.4	1.7	2.1	2.5	2.7
2	0.2	0.5	0.3	0.3	1.3	1.6	2.1	3.1
3	0.5	0.5	0.5	0.4	1.6	1.3	4.6	3.2
4	0.4	0.3	0.7	1.0	1.6	1.1	4.0	2.8
14	0.6	0.3	0.5	0.7	2.7	4.5	5.5	3.8

Table 1. ID1 and ID2 do not induce CD11c expression or Annexin-V positivity. pLZRS-ID1-ΔNGFR (ID1), pLZRS-ID2-ΔNGFR (ID2) and as a vector control pLZRS-EGFP-ΔNGFR and pLZRS-ΔNGFR were retrovirally transduced to NB4 cells. The populations of the different transduced and MACS-sorted ΔNGFR-positive cells and non-transduced cells were flow cytometrically analyzed for CD11c expression and Annexin-V positivity at different time points after transduction. No differences between non-transduced, vector-transduced and ID1- or ID2-transduced cells were observed.

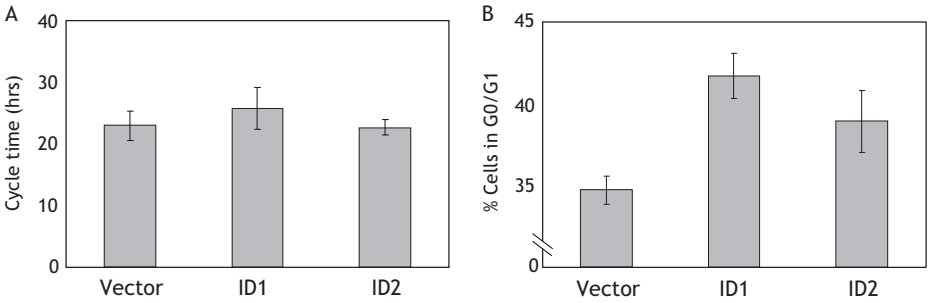

Figure 5. ID1 and ID2 cause an increase of cells in G0/G1 without affecting the cell cycle time. pLZRS-ID1-ΔNGFR (ID1), pLZRS-ID2-ΔNGFR (ID2) and as a vector control pLZRS-EGFP-ΔNGFR and pLZRS-ΔNGFR were retrovirally transduced to NB4 cells. (**A**) ID1 and ID2 overexpression does not alter the cell cycle time of NB4 cells as compared to vector-transduced and sorted cells, as calculated from the results of five independent BrdUrd incorporation experiments done at 7, 8 16 or 25 days after transduction (mean values ± SD). (**B**) ID1 and ID2 overexpression in NB4 cells cause an increase of cells in G0/G1 phase of the cell cycle as compared to vector-transduced and sorted cells as measured at different time points after transduction, either 7, 8 or 23 days. DNA histograms of five independent experiments showed a mean (± SD) of 41,6% (± 1,4) and 38,9% (± 1,9) cells in G0/G1 phase for ID1 and ID2 overexpressing NB4 cells, respectively, compared to 34,7% (± 0,9) for the vector-transduced and sorted cells.

Discussion

We identified two genes, *ID1* and *ID2*, which are rapidly induced by ATRA in APL cells. In both the NB4 cell line and in freshly isolated patient cells, ATRA induced the expression of these genes within 2 hours. ID2 expression remained high and was more enhanced after 48 hours of stimulation. In contrast, the expression of ID1 was transient, being downregulated again within 24 hours after stimulation. The rapid induction of ID1 and ID2 mRNA expression in NB4 cells by ATRA was not affected by cycloheximide indicating that ID1 and ID2 are direct ATRA-responsive genes (Van Wageningen et al., see Chapter 3 of this thesis).

One of the main biological actions of ID proteins is their capacity to inhibit the DNA binding by bHLH transcription factor complexes. They bind and inactivate various lineage-specific bHLH proteins as well as their more ubiquitously expressed heterodimerization partners (E12/47, HEB, E2-2). The four different ID proteins

(ID1-ID4) contain a highly conserved HLH domain and are capable of binding to various common partner proteins.[8] In line with this, they were shown to exert similar or redundant biological functions in various experimental models. However, the differential expression of different ID proteins in various tissues indicate different biological functions for each of the ID proteins under normal physiological conditions.[9;25] Various knockout mice were generated carrying null mutations in the different Id proteins. While no gross abnormalities were observed in *Id1*$^{-/-}$ mice,[26] *Id2*$^{-/-}$ mice showed retarded growth and high neonatal morbidity. Surviving *Id2*$^{-/-}$ mice displayed severely disturbed natural killer cell production and defective peripheral lymphoid organ development.[27] Redundancy of Id protein function was illustrated by the observation that breeding of various single *Id* gene knockout mice did not produce viable offspring carrying null mutations in two different Id proteins.[25]

One of the important bHLH proteins that is inactivated by ID proteins is E2A. E2A is important for lymphoid development and *E2A*$^{-/-}$ mice show severe defects in early B cell development.[28] In addition, a high incidence of T-cell tumours was observed in these mice.[26] In line with this, inhibition of bHLH trancription factors by the ectopic overexpression of Id2 in developing thymocytes led to the rapid development of lymphomas.[29] Further evidence for the antagonistic action of Id and E2A proteins came from studies with *E2A*/*Id1* double knockout mice. Compared to single *E2A* knockout mice, the double knockout mice survived significantly better.[26] The biological role of E2A may be dependent on the differentiation stage and cell type in which it is expressed. It has been shown to inhibit G1/S progression in various cell types including fibroblasts and early thymocytes.[30;31] However, E2A was also reported to be highly expressed in proliferating centroblastic B-cells[32] and to promote cell cycle progression in other cells as well.[33] This difference might be explained by the presence of different bHLH heterodimerization partners for E12 and E47 in different tissues.

The simultaneous upregulation of ID proteins and downregulation of E2A may represent a dual mechanism to ensure that E2A-dependent transcription is shut-off during differentiation. Alternatively, as various bHLH proteins were shown to be able to bind to DNA in a homodimeric configuration as well,[8] the primary function of ID1 and ID2 induction may be to inhibit E2A-independent homodimeric DNA-binding by more lineage-restricted bHLH transcription factor complexes. The decreased expression of E2A in maturing APL cells may be consistent with the extinguished

E2A expression in differentiated myeloid cells.[34] The induction of ID proteins during neutrophilic differentiation may be consistent with the reported expression of ID1 and ID2 in various myeloid cell lines, and in primary granulocytes and monocytes.[35-37] The rapid downregulation of ID1 at later stages after the initial upregulation is striking (Figure 1). However, our data suggest that downregulation of ID1 is not a prerequisite for terminal neutrophilic differentiation, as ID1 overexpressing cells did not become resistant to ATRA. This was shown by the inhibition of colony formation (Figure 3B), and also by the induction of the differentiation marker CD11c and NBT positivity in ID1-transduced NB4 cells after treatment with ATRA, which was comparable to non-transduced cells (data not shown). In various cell types, ID proteins have been characterized as proteins that inhibit differentiation, and overexpression of these proteins has been reported in many different types of tumours.[38] Two distinct pathways have been described coupling ID function directly to promotion of cell cycle progression. One pathway involves the interaction between the retinoblastoma proteins and ID2 and ID4 that leads to the expression of genes required for S phase progression. The second pathway, which is described for ID1, involves the inhibition of the E2A-mediated activation of the cyclin-dependent kinase inhibitor $p21^{waf1/Cip1}$.[9] Our data suggest that during neutrophilic differentiation, ID1 and ID2 have a different role, being associated with the induction of differentiation and accumulation of cells in G0/G1. The combined effects of ATRA on E2A and ID1 and ID2 suggest that the inactivation of specific bHLH transcription factor complexes in immature APL cells is important to allow neutrophilic differentiation. Different models by which such bHLH proteins could act may be suggested. Upregulation of ID proteins would effectively insulate the cell against any bHLH transcription factor, including those that are involved in other (non-neutrophilic) pathways of differentiation. One candidate could be the bHLH protein SCL/TAL-1 that stimulates erythroid differentiation.[39;40] This would be in line with the observation that ectopic expression of C/EBPα in human CD34 positive cells induced granulocytic differentiation and inhibited erythrocytic differentiation, while strongly inducing ID1 expression in these cells.[41] Potentially, a similar mode of action might be operational at other differentiation pathway crossroads, for instance at the neutrophil-eosinophil branchpoint, as ectopic expression of ID1 inhibited the growth of CD34 positive cord blood cells that were stimulated with IL-5, which induces eosinophilic rather than neutrophilic differentiation. (PJ Coffer et al., Blood 2003, 102(11): abstract 49)

Our data show that the mere overexpression of ID1 or ID2 is not sufficient to induce the differentiation of the cells. However, the increased accumulation of cells in G0/G1 might render the cells more sensitive for other differentiation stimuli, similar to what has been suggested for the differentiation of HL-60 cells in response to retinoic acid.[42]

We conclude that ID1 and ID2 are two retinoic acid-induced proteins in acute promyelocytic leukemia cells and suggest that the increased accumulation of the leukemic cells in G0/G1 may contribute to the beneficial effect of retinoic acid in the treatment of APL. Furthermore, we suggest that ID and bHLH proteins may have an important role in differentiation branchpoint decisions during myelopoiesis.

Acknowledgements

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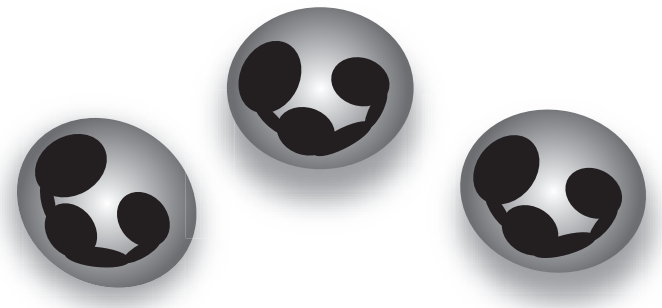
Chapter 3

Gene transactivation without direct DNA-binding defines a novel gain-of-function for PML-RARα



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Abstract

PML-RARα is the causative oncogene in 5-10% of the cases of acute myeloid leukemia. At physiological concentrations of retinoic acid PML-RARα silences RARα target genes, blocking differentiation of the cells. At high concentrations of ligand, it (re) activates the transcription of target genes, forcing terminal differentiation. The study of RARα target genes that mediate this differentiation has identified several genes that are important for proliferation and differentiation control in normal and malignant hematopoietic cells. In this paper we show that the PML-RARα fusion protein not only interferes with the transcription of regular RARα target genes. We show that the *ID1* and *ID2* promoters are activated by PML-RARα but, unexpectedly, not by wild type RARα/RXR. Our data support a model in which the PML-RARα fusion protein regulates a novel class of target genes by interaction with the Sp1 and NF-Y transcription factors, without directly binding to the DNA, defining a gain-of-function for the oncoprotein.

Introduction

Acute promyelocytic leukemia (APL) is characterized by an excess of immature promyelocytes in the bone marrow that fail to differentiate towards mature granulocytes. In approximately 98% of the cases, the retinoic acid receptor-α (RARα) gene is fused to the promyelocytic leukemia (PML) gene resulting in a PML-RARα fusion protein. The PML-RARα chimeric protein contains most of the PML sequence and a large part of RARα, including its DNA- and nuclear hormone-binding domains. APL blasts can be forced to terminally differentiate using pharmacological doses of all-*trans* retinoic acid (ATRA). When treated with chemotherapy, APL patients can be cured in approximately 40% of the cases. The combination of ATRA with chemotherapy leads to a remarkably high cure rate of approximately 90%,[1;2] and APL currently represents the best prognostic group amongst the different forms of leukemia. This treatment constitutes one of the first examples of successful induction of differentiation of malignant cells yielding significant clinical results.

The role of PML-RARα in transformation and terminal differentiation has been studied intensively in the past decade. PML-RARα was shown to act as a dominant oncogene, interfering with the normal function of the unrearranged PML as well as the unrearranged RARα protein. Expression of the fusion protein in immature hematopoietic cells induced a maturation block at the promyelocytic stage. Inoculation of PML-RARα-transduced bone marrow cells into irradiated syngenic mice resulted in the development of retinoic acid sensitive leukemia.[3;4] Furthermore, PML-RARα transgenic mice developed a myeloproliferative syndrome which progressed to overt leukemia in 30% to 90% of the animals after 6-12 months, depending on the promoter that was used.[5-7]

PML is a ubiquitously expressed protein that localizes to nuclear substructures termed nuclear bodies. More than 50 protein partners with various biological functions colocalize with PML.[8] In APL cells these nuclear bodies are disrupted and dispersed into numerous small microspeckles.[9] PML has multiple tumor-suppressor functions and is involved in growth control, replicative senescence and apoptosis.[10] PML -/- mice are prone to develop tumors in response to various forms of stress. In addition, PML-RARα transgenic mice develop leukemia much faster in a PML -/- background.[11]

Retinoic acid receptors are transcription factors that activate genes in a ligand-dependent manner. RARα binds to DNA as a heterodimer with RXR proteins. In the absence of ligand, both RARα/RXR and PML-RARα bind corepressors like N-Cor and SMRT and recruit histone deacetylases leading to gene silencing. In the presence of ligand, the corepressors are replaced by coactivators, leading to transcriptional activation. However, PML-RARα releases the corepressors at much higher concentrations of ligand compared to the unrearranged receptors. Since PML-RARα competes with unrearranged receptors for the same DNA-binding sites, the presence of the fusion protein results in dominant silencing of retinoic acid receptor target genes at physiological concentrations of ligand. At higher, supra-physiological concentrations the fusion protein can still function as a transcriptional activator releasing the corepressor complex and allowing the transcription of genes that are important for granulocytic differentiation.[12-15] The release of the differentiation block by high concentrations of retinoic acid leads to terminal granulocytic differentiation of the leukemic cells and the induction of hematological remissions in the patients. RARα target genes are not restricted to genes that contain a consensus retinoic acid receptor binding-site in their promoter. Liganded RARα is able to repress AP-1 mediated transcription.[16-18] PML-RARα abnormally regulates AP-1 activity as it stimulates AP-1-dependent transcription in the presence of ligand, whereas unliganded PML-RARα inhibits AP-1 dependent transcription.[19] In addition, RARα may interfere with GATA-2-dependent transcription. Liganded RARα enhances GATA-2-dependent gene transcription via direct protein-protein interaction.[20] This activity is retained in the PML-RARα fusion protein.[21] Furthermore, interaction of normal retinoid receptors with the transcription factor Sp1 has been shown.[22;23] Together, the data support a model in which PML-RARα interferes with RARα target gene expression in a dominant fashion, and that restoration of target gene expression by high concentrations of ligand is important for the induction of differentiation.

Apart from interference with the function of both parental proteins in a dominant-negative manner, a gain-of-function for PML-RARα is suggested by various observations. PML-RARα may bind to DNA as a heterodimer with RXR, but also independently from RXR as a homodimer.[24] It may bind to regular retinoic acid receptor binding sites consisting of a repeated consensus [(A/G)G(T/G)TCA] sequence, but the required spacing between the two half-sites is less stringent for the fusion protein. This allows the fusion protein to bind to a wider range of DNA-target sequences compared to

normal receptors.[25;26] The importance of PML-RARα as a transcriptional activator of differentiation-inducing genes was shown by *in vitro* experiments. Expression of PML-RARα in the myeloid cell line U937 (that also expresses normal retinoic acid receptors) enhanced their sensitivity to the induction of differentiation by ATRA.[27] In addition, forced expression of PML-RARα, but not RARα, in an ATRA resistant APL cell line with constitutive degradation of the chimeric protein restored ATRA sensitivity.[28] Importantly, in APL patients that became resistant to differentiation induction with ATRA during therapy, additional mutations were found in the ligand-binding domain of the RARα part of the PML-RARα fusion protein, indicating an important role for the fusion protein during the retinoic acid-induced differentiation of the leukemic cells.[29] Finally, experimental mouse models in which various natural and artificial RARα fusion proteins were expressed also support a model in which interference with the function of RARα and PML is important, but in addition, they suggest that the PML-RARα fusion protein exhibits gain-of function characteristics, unique to the fusion protein.[7;30] So far, the mechanisms behind these observations remain largely unclear.

We have previously shown that the transcription factor inhibitors ID1 and ID2 are upregulated upon treatment with ATRA and play a role in cell cycle arrest during APL differentiation.[31] In this study we investigated the mechanism by which these genes are regulated in APL cells. We found that *ID1* and *ID2* are regulated by PML-RARα through a novel mechanism, which is not shared with normal RARα, defining an as yet unrecognized class of retinoic acid-induced genes in APL.

Materials and Methods

Cell culture

NB4 cells, U937 and U937-PR9 cells (kindly provided by Dr. P.G. Pelicci and Dr. F. Grignani) were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA), Hep3b cells in IMDM (Gibco) and HEK293 cells in DMEM + 2 mM L-glutamine. Media were supplemented with 10% FCS (Gibco). ATRA was used at a final concentration of 10^{-6} M (Sigma, St Louis, MO, USA) and $ZnSO_4$ at 100 μM. Cycloheximide (ICN, Costa Mesa, CA, USA) was added 30 min prior to ATRA at 4 μg/ml.

DNA constructs and antibodies

The human *ID1* promoter was obtained from J. Campisi (University of California, USA). The *ID2* promoter[32] was cloned into the XhoI-HindIII sites of the pGL3-basic vector (Promega, Madison WI, USA) after amplification with the following primers: sense 5'-GTACGGTACCTCGAGTTGGGCATGGTTTGCAATA-3' and anti-sense 5'-GTACAGATCTAAGCTTGAAGCCCGAGCCCGGC-3'. $RARE_3$-tk-luc,[33] PML-RARαΔR[34] and PML-RARαΔCC, PML-RARα, RARα and RXR expression constructs[25] were as described. FLAG-PML-RARα was from A. Tomita[35] and was recloned into a CMV-expression vector. *ID1* promoter deletion/mutation fragments were constructed by PCR and cloned into the XhoI-HindIII sides of PGL3-basic (Promega). All constructs were sequence verified. The dominant-negative NF-YA (YAm29) construct and anti-NF-YB polyclonal antiserum were from R. Mantovani (University of Milan, Italy). pGEX-Sp1 was from H. Rotheneder (University of Vienna, Austria). Anti-Sp1 (PEP-2), anti-NF-YA (H-209), anti-RARα (C-20) and anti-ID1 (Z-8) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PML rabbit antibody was as described.[9] Anti-FLAG (M2) antibody was from Sigma. As a control in the ChIP experiments, human IgG was used (Ivegam, Sanquin, The Netherlands)

Northern blotting

Total cellular RNA was isolated by guanidium-isothiocyanate lysis and centrifugation on a 5.7 M cesium chloride cushion. 10 μg of total RNA was size-fractionated by 1% agarose-formaldehyde gel electrophoresis and transferred onto Hybond N^+ nylon membranes (Amersham, Buckinghamshire, UK). Filters were hybridized at 65°C overnight in phosphate buffer (2N NaH_2PO_4, 7% sodium dodecyl sulphate (SDS), 1 mM EDTA pH 8 and 1% BSA). DNA probes were labeled with [^{32}P]α-dATP by random primed labeling (Boehringer, Mannheim, Germany). After hybridization, filters were washed in 0.2xSSC/0.2% SDS for 15 min at 65°C. Northern blots were hybridized to radiolabeled human *ID1* or *ID2* probes (kindly provided by Dr. S. Stegman). As a control for equal loading, filters were stripped and hybridized to a 777bp HindIII-EcoRI human GAPDH fragment.

Transactivation studies

Cells were transfected using calcium-phosphate precipitates with 0.25 μg pGL3-*ID1* or -*ID2* promoter, 0.05 μg nuclear receptor expression vector, 0.1 μg of Renilla vector (pRL-CMV, Promega) and 1 μg YAm29 expression vector. The total amount of DNA was normalized to 1.4 μg for all transfections using empty vectors. Cells were harvested 16 hours after ATRA treatment

using 100 µl Passive Lysis Buffer (Promega). Firefly luciferase and renilla luciferase activities were measured on a luminometer (Lumat LB 9507, Perkin-Elmer/Applied Biosystems, CA, USA) using Dual-Luciferase Reporter Assay System reagents (Promega).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described.[36] 5 µg was incubated in a total volume of 15 µl containing 1 µg of double stranded poly(dI)-poly(dC), 10 mM Hepes, 50 mM KCL, 1 mM DTT, 1 mM PMSF, 2.5 mM $MgCl_2$, 10% (w/v) glycerol, 300 µg/ml BSA and 0.5 ng of a labeled double stranded oligonucleotide probe: *ID1* 5'-CCGCCCATTGGCTGCTTTTGAACGT. To show specificity of binding, non-labeled 100 fold excess of double-stranded oligonucleotides were added to compete for binding with the labeled probe. For this, either the *ID1* probe (self competition), a sequence not containing any CCAAT box 5'- TCAGAGTTCAAGGTTCTAGTCGCTGCGGC, or a NF-Y binding site containing oligonucleotide from the *CD10* gene was used 5'-ATCCCGACCAATGAGCGCACGGGGCCGGGT.[37] DNA-Protein complexes were resolved on a 5% non-denaturing polyacrylamide gel in 0.5xTBE buffer.

GST pull-down

GST-fusion proteins were produced in E.coli BL21 which were induced at A_{600} = 0.5 with 300 µM IPTG for 4 hours. Proteins were released by sonication and loaded onto glutathion-agarose (Sigma) by incubation at 4ºC for 2 hours in Lysis buffer (0.15 M NaCl, 50 mM Tris [pH 8.3], 10 mM EDTA, 0.5% NP40 and protease inhibitors). Pull-down was performed using *in vitro* translated PML-RARα and PML-RARαΔcc (reticulocyte lysate, Promega) or rHSp1 (Promega), by 2 hours incubation at 4°C. Beads were washed in lysis buffer and subsequently resuspended in SDS-loading buffer.

Chromatin immunoprecipitation

DNA-protein cross-linking was done for 30 min at room temperature by adding formaldehyde at a final concentration of 1% directly to the culture medium. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM. Cells were washed with ice-cold phosphate-buffered saline, buffer B (10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, 20 mM HEPES [pH 7.6]), buffer C (1 mM EDTA, 0.5 mM EGTA, 0.15 M NaCl, 50 mM HEPES [pH 7.6]) and resuspended in incubation buffer (0.15% SDS, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6] and protease inhibitors) at $33x10^6$ cells/ml. Chromatin was sonicated using the Bioruptor (Cosmo Bio, Tokyo, Japan), at high intensity for 15 min with

0.5 min intervals. Insoluble material was removed by centrifugation at 4°C for 15 min 120 μl supernatant was incubated with 30 μl precoated protein A/G plus agarose beads 50% v/v (Santa Cruz), 0.1% BSA, 36 μl 5x incubation buffer, protease inhibitors and 2-5 μg antibody (anti-NF-YA, anti-PML, anti-FLAG or non-specific IgG from human serum) and rocked at 4°C for 16 hours. Beads were harvested by centrifugation and washed twice with buffer 1 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES [pH 7.6]), once with buffer 2 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES [pH 7.6]), once with buffer 3 (0.25 M LiCL, 0.5% NaDOC, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]), and twice with buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]). Chromatin antibody complexes were eluted by the addition of 1% SDS and 0.1 M NaHCO3 to the pellet and incubated for 20 min at room temperature. Cross-linking was reversed by the addition of NaCl (0.44 M final conc.) and incubation of the eluted samples for at least 4 h. at 65°C. DNA was recovered by phenol-chloroform-isoamylalcohol extraction followed by chloroform-isoamylalcohol extraction and precipitated by the addition of 0.1 volume of 1 M sodium acetate (pH 5.2), and 2.5 volumes of ethanol. Precipitated DNA was dissolved in water, and input as well as immunoprecipitated DNA was analyzed using quantitative PCR for genomic sequences from the *ID1*, *ID2*, *RAR*β and *p21* promoter regions.[38]

Quantitative PCR

Quantitative PCR to measure ID1 and ID2 mRNA expression was performed with the ABI/PRISM 7700 Sequence Detection system (ABI/PE, Foster City, CA, USA). As a reference gene *PBGD* was used. The primer/probe sequences for the *ID1* gene were: sense 5'-GTTACTCACGCCTCAAGGAGCT, anti-sense 5'-GAGAATCTCCACCTTGCTCACC, probe FAM 5'-CCCACCCTGCCCCAGAACCG; for *ID2*: sense 5'-GACTGCTACTCCAAGCTC-AAGGA, anti-sense 5'-CGTGCTGCAGGATTTCCAT, probe FAM 5'-CCCAGCATCCCCCAGAA-CAAGAAGG. PCRs were done in a 50 μl reaction mixture (1.25 U AmpliTaq Gold, 1xbuffer A (both Perkin-Elmer), 250 mM dNTPs (Pharmacia) and 5 mM $MgCl_2$, for 10 min at 95°C followed by 45 cycles of 15 sec at 95°C and 1 min at 62°C. Quantitative PCR following ChIP was done using Sybr Green PCR (Applied Biosystems). Primer sequences for the *ID1* promoter were; sense 5'- CACTGCGAGCAGGCACTAGAC, anti-sense 5'-AGCCACAGCTTGTCTTT; for the *ID2* promoter; sense 5'-CTGTAC-TCTATTTACCACCCCAGCTG, anti-sense 5'-GGCGTGGGCTTGGTTCTT; for the *RAR*β promoter; sense 5'-TTGGGTCATTTGAAGGTTAGCA, anti-sense 5'-CACACAGAATG-AAAG-ATTGAATTGC, for the *p21* gene sense 5'-GGCGGGGCGGTTGTAT, anti-sense 5'-AAGGAA-CTGACTTCGGCAGC.

Results

ID1 and *ID2* are direct retinoic acid-responsive genes in APL cells

Basic helix-loop-helix (bHLH) transcription factors and their inhibitors, ID proteins, play crucial roles in the regulation of differentiation in various cell types. We have shown that ID1 and ID2 are upregulated in APL cells upon exposure to ATRA.[31] Upregulation of ID1 and ID2 mRNA was confirmed with qPCR (Figure 1A and B), showing clear upregulation within 4 hours after the addition of ATRA. To investigate whether ID1 and ID2 were directly upregulated by ATRA, cells were treated with cycloheximide prior to the addition of ATRA to inhibit protein translation. Both ID1 and ID2 mRNAs were upregulated within 0.5 hours by ATRA, regardless of the addition of cycloheximide (Figure 1C and D). This indicates that both genes were directly upregulated, without intermediate protein production.

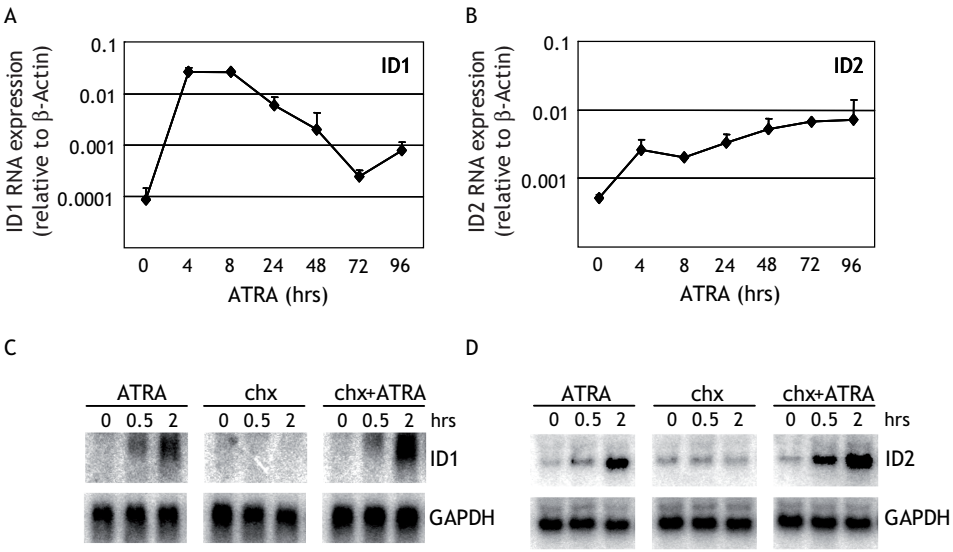


Figure 1. *ID1* and *ID2* are direct retinoic acid target genes in NB4 cells. Quantitative PCR and Northern blot analysis of NB4 cells treated with ATRA. mRNA was isolated from NB4 cells and ID1 (**A**) and ID2 expression (**B**) were determined using specific primers and probes by quantitative PCR (n=4). Quantities were normalized based on β-actin expression. To investigate whether the induction of ID1 and ID2 was dependent on intermediate protein production, cells were treated with cycloheximide alone (4 μg/ml) or with the combination of cycloheximide and ATRA (10^{-6}

M). Blots were hybridized using radiolabeled ID1- (**C**) and ID2-specific (**D**) probes. As a control for equal loading blots were stripped and rehybridized with GAPDH specific probes.

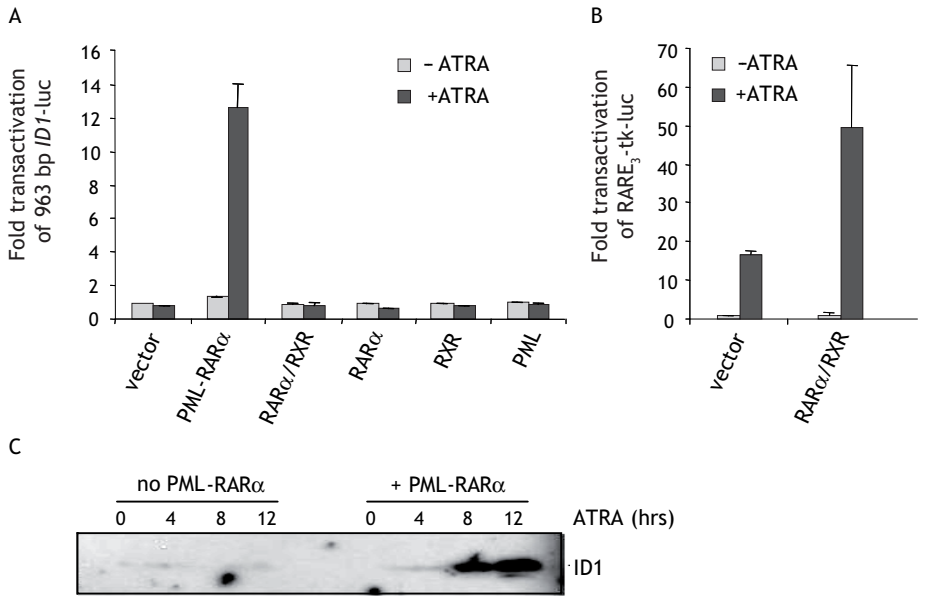


Figure 2. The *ID1* promoter is transactivated by PML-RARα but not by RARα/RXR. (**A**) Hep3B cells were transfected with the 963bp ID1 promoter-luciferase reporter construct (*ID1-luc*) together with a control vector expressing Renilla-luciferase. In addition, vectors coding for the various proteins indicated in the figure were transfected. Transactivation is expressed as arbitrary units and is corrected for transfection efficiency measured by Renilla-luciferase. Background luminescence of the cells transfected with only the reporter construct without nuclear receptors and in the absence of ATRA was set at 1. Cells were cultured for 16 hrs without (light gray bars) and with ATRA (dark gray bars). Mean values and standard deviations from three independent experiments ± SD are shown. (**B**) To show transactivation by unrearranged retinoic acid receptors, cells were transfected with the RARE3-tk-luc vector, containing three bona-fide RAREs. Mean values of three independent experiments ± SD are shown. (**C**) ID1 expression is increased by PML-RARα in the presence of ATRA. The zinc-inducible PML-RARα cell line U937-PR9 was grown in the absence (left panel) or presence (right panel) of zinc for 16 hours. Cells were treated with ATRA and harvested at the indicated time points. Proteins were size-fractionated by SDS-PAGE. Immunostaining was done with anti-ID1 antibody.

The *ID1* upstream promoter is transactivated by PML-RARα but not by RARα/RXR

To identify the regulatory DNA sequences through which the induction of *ID1* by retinoic acid was mediated, we analyzed the 5' upstream promoter sequence in transactivation assays. A 963 bp fragment of the promoter, including the putative TATA box, was cloned into a luciferase reporter construct. When expressed alone, RARα, RXR and PML were not able to transactivate the *ID1* promoter both in the absence and presence of ATRA (Figure 2A). In contrast, PML-RARα transactivated the promoter more than 12-fold in an ATRA-dependent fashion. Surprisingly, the combination of RARα/RXR did not transactivate the promoter, both in the absence and presence of ATRA. To verify that RARα and RXR were expressed and functional, a control luciferase construct was used containing three bona-fide RAREs from the RARβ promoter [$RARE_3$-tk-luc[39]]. This construct was strongly transactivated in the presence of ATRA through endogenous retinoic acid receptors (Figure 2B, left bars). This was further enhanced when RARα and RXR were co-transfected (Figure 2B, right bars). Together this shows that PML-RARα may regulate *ID1* expression through the upstream 963 bp promoter whereas RARα/RXR cannot.

To test whether PML-RARα expression would result in ATRA-dependent induction of the endogenous *ID1* gene, we used the U937-PR9 cell line that is stably transfected with a Zn^{2+}-inducible PML-RARα expression cassette.[34] In PML-RARα expressing U937 cells, ATRA strongly induced ID1 expression (Figure 2C, right panel), in contrast to U937 cells that did not express PML-RARα (Figure 2C, left panel).

Transactivation of the *ID1* promoter is dependent on GC- and CCAAT box motifs

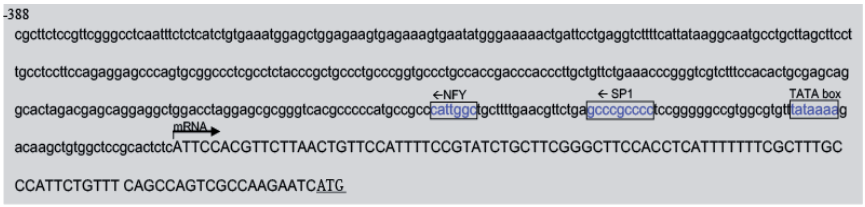
To determine the DNA sequences that are relevant for the observed PML-RARα-dependent transactivation, we analyzed the 963 bp upstream promoter construct of the *ID1* gene further. The consensus RAR/RXR binding sequence (RARE) consists of a repeated (A/G)G(T/G)TCA sequence that is separated by two or five base pairs. However, in the 963 bp *ID1* promoter, no consensus RARE was found. As PML-RARα may bind to a much wider variety of DNA sequences,[25;26] we made luciferase constructs with different promoter truncations. All the truncation mutants could still be transactivated by PML-RARα (Figure 3B), including the smallest, 121bp promoter construct. This construct does not contain any sequence that even remotely resembles

a retinoic acid receptor-binding site (Figure 3A). Analysis of the 121 bp promoter sequence for putative binding sites for other transcription factors showed a perfect consensus-binding site for the transcription factors NF-Y (CCAAT box), and Sp1 (GC box). To test their relevance, we mutated these sites individually and in combination in the context of the 963 bp promoter fragment. Mutation of the GC box or the CCAAT box alone partially impaired transactivation by PML-RARα, while deletion of the GC box and the CCAAT box in combination almost completely abolished transactivation (Figure 3C, raw Renilla and Firefly luciferase data are given in Supplemental figure 1). This indicated that transactivation of the *ID1* promoter by PML-RARα was dependent on the GC and CCAAT motifs.

Functional NF-Y is necessary for transactivation of the *ID1* promoter by PML-RARα

Sp1 and NF-Y are ubiquitously expressed transcription factors. NF-Y is a trimeric protein complex consisting of the subunits NF-YA, NF-YB and NF-YC. All three subunits are necessary for the complex to bind to the DNA.[40] Using real-time PCR, we confirmed that Sp1 and all three NF-Y subunits were expressed in primary APL cells (Supplemental figure 2). Binding of Sp1 to the promoter of *ID1* was shown previously.[41] Therefore, we tested whether NF-Y was able to bind to the CCAAT site from the *ID1* promoter. Incubation of a radioactively labeled DNA-probe containing the CCAAT box with cellular protein extracts resulted in a clear shifted complex (Figure 4A, lane 1). This shift was competed by a 100-fold excess of non-labeled probe (lane 2) and by an excess of an oligo containing the NF-Y binding site of the *CD10* promoter (lane 5[42]), but not by an excess of probe lacking a NF-Y binding site (lane 3). When anti-NF-YB antiserum was added (lane 4) the protein-DNA complex was supershifted, identifying the DNA binding protein complex as NF-Y. To further test whether NF-Y is present on the endogenous *ID1* promoter, we performed chromatin immunoprecipitation (ChIP) assays. Using anti-NF-YA antibody, recovery of the *ID1* promoter sequences from U937 cells was over 70 times higher than recovery with non-specific IgGs (Figure 4B, right bars). In the same experiment, no enrichment was seen for the *RAR*β promoter (Figure 4B, left bars), indicating that NF-Y is present on the endogenous *ID1* promoter but not on the *RAR*β promoter.

A



B

ID1 promoter fragment	vector	RARα/RXR	PML-RARα
823bp tataa LUC	1.4±0.1	1.8 ±0.1	11.1±1.7
397bp LUC	1.5±0.4	1.9 ±0.7	7.7 ±1.7
355bp LUC	1.2±0.4	1.6 ±0.6	7.8±0.3
325bp LUC	1.6±0.1	2.2±1.1	6.7±1.6
298bp LUC	1.6±0.4	2.0±1.0	8.9±4.5
121 bp LUC	1.6±0.4	1.4 ±0.6	9.5±4.7

C

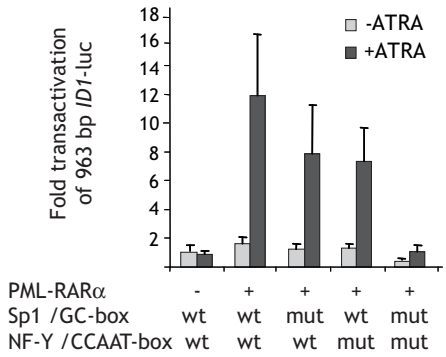


Figure 3. The CCAAT- and GC-box in the *ID1* promoter are required for PML-RARα-mediated transactivation. (**A**) *ID1* promoter region showing the presence of consensus Sp1 and NF-Y binding sites. (**B**) Transactivation of *ID1* promoter-luciferase constructs. Several deletion constructs were generated and transfected in combination with RARα plus RXR, or with PML-RARα. Transactivation assays were performed as described in figure 2. Mean values from three independent experiments ± SD are shown. (**C**) To investigate the importance of the putative Sp1 and NF-Y binding sites in the -121 bp upstream promoter sequence of the *ID1* gene for transactivation by PML-RARα, these sites were mutated in the context of the –963 bp promoter fragment. Mutations were introduced either alone, or in combination. Transactivation by PML-RARα was performed as described in figure 1. Sequences were mutated as follows; GC

box: CCGCCC was replaced by CTATCC and for the NF-Y site: ATTGG was replaced by ACACG. Transactivation was significantly lower in the promoter fragments with one mutated binding site in comparison to the wt promoter fragment ($p<0.01$).

A dominant-negative form of NF-YA has been described (YAm29[43]). This mutated NF-YA subunit can still bind to the NF-YB and NF-YC subunits, but is not able to bind to DNA, preventing the formation of a functional trimeric NF-Y complex. To investigate whether functional NF-Y was necessary for the transactivation of *ID1*, the YAm29 mutant was tested in a transactivation assay. Transactivation of *ID1* by PML-RARα upon ATRA treatment was severely decreased in the presence of dominant-negative NF-YA (Figure 4C). This effect was promoter-specific as YAm29 did not influence transactivation of the *RAR*β promoter construct ($RARE_3$-tk-luc) by PML-RARα or RARα/RXR (data not shown).

Upregulation of *ID1* is dependent on PML-RARα, but does not require the DNA-binding domain of the fusion protein

To test whether PML-RARα might transactivate the *ID1* promoter without directly binding to the DNA, we used a PML-RARα construct from which the DNA binding domain was deleted (PML-RAR/ΔR[34]). PML-RAR/ΔR has been shown to be unable to transactivate a promoter containing a bona-fide RARE. In contrast, PML-RAR/ΔR retained the ability to transactivate the *ID1* promoter (Figure 4D). Similar results were found when the transactivation assay was repeated in the myeloid cell line HL-60 (Supplemental figure 3). This indicated that transactivation of the *ID1* promoter by PML-RARα does not require direct binding of PML-RARα to the DNA.

PLZF-RARα is generated by a t(11;17) translocation that is found in approximately 2% of the patients with APL. It contains the same part of the retinoic acid-receptor as PML-RARα. Similar to PML-RARα, this fusion protein may form homodimers with DNA-binding capacity.[44] Interestingly, PLZF-RARα was not able to transactivate the *ID1* promoter, suggesting that the PML part of PML-RARα is necessary for transactivation of the *ID1* promoter (Figure 4D). When the coiled-coil domain of PML-RARα was deleted (PML-RAR/ΔCC[25;34]) it was no longer able to transactivate the *ID1* promoter (Figure 4D). As this domain is involved in protein-protein interactions, this suggested that PML-RARα homodimerization or interaction of PML-RARα with another protein was necessary for the transactivation of *ID1*.

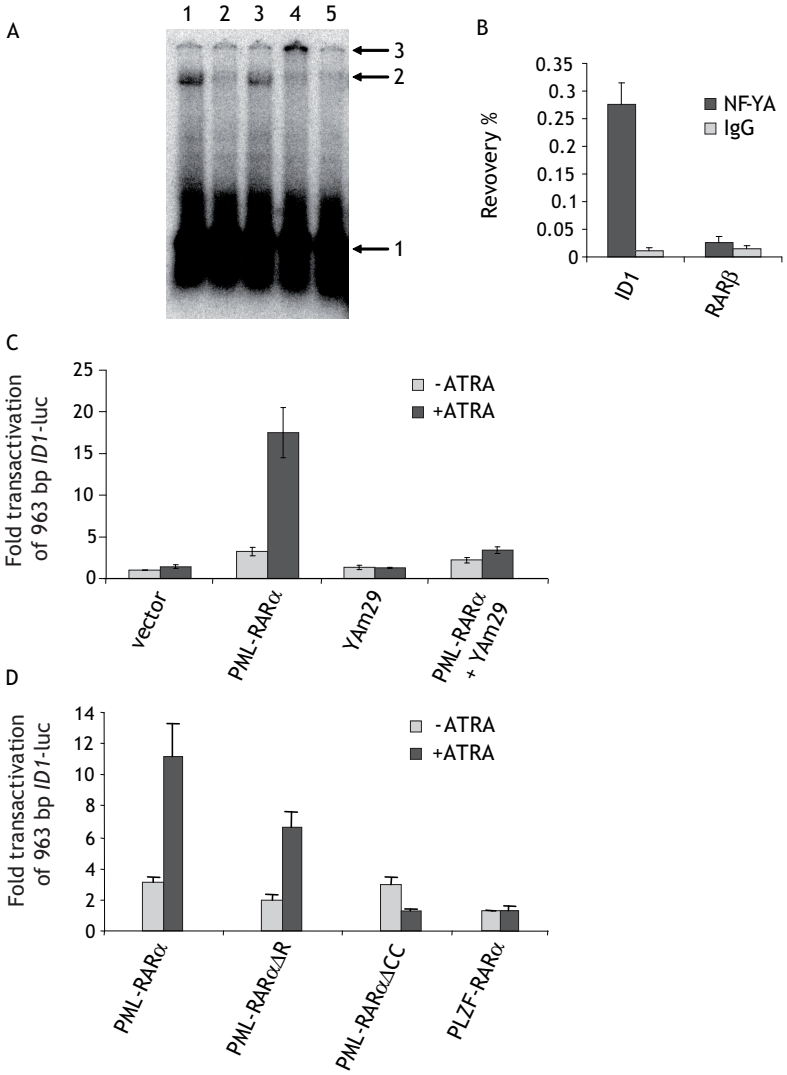


Figure 4. NF-Y binds to the CCAAT-box element from the *ID1* promoter and is required for PML-RARα mediated transactivation. (**A**) EMSA showing binding of NF-Y to the putative NF-Y binding site from the *ID1* promoter. Hep3B-nuclear extracts were incubated with a labeled DNA probe containing the NF-Y-box from the *ID1* promoter region. A clearly shifted protein-DNA complex was seen (lane 1). Competition experiments were done with 100x cold ID1 probe (lane 2) and with an unlabeled probe containing the confirmed NF-Y binding site from the *CD10* gene promoter (lane 5). In addition, a sequence without any recognizable NF-Y binding site was used for competition (lane 3). Addition of anti-NF-YB antibody shifted the complex to a higher molecular weight complex (lane 4). Arrows indicate free probe (1), shifted DNA-

protein complex (2) and the supershifted DNA-protein-antibody complex (3). (**B**) To detect DNA-binding of NF-Y in intact cells, ChIP assays were performed using anti NF-YA antibodies. As a control, the non-specific IgG fraction from human serum was used. The Y-axis shows the recovery (%) of *ID1* or RARβ sequences relative to the input In U937 cells, NF-Y clearly bound to the *ID1* promoter (right bars), but not to the *RAR*β gene (left bars). Mean values from three independent experiments ± SD are shown. (**C**) To further show the importance of NF-Y for the PML-RARα mediated transcriptional activation of *ID1*, transfections (as described in figure 2) were performed using the dominant-negative NF-YA subunit (YAm29) and the 963 ID1 promoter construct. In the presence of YAm29 PML-RARα mediated transcription was severely diminished (right bars). (**D**) To investigate the importance of different domains of PML-RARα, vectors encoding the DNA binding-defective PML-RARαΔR mutant, and a mutant lacking the coiled-coil protein-protein interaction domain of PML (PML-RARαΔCC) were used. In addition, a PLZF-RARα expression construct was used. Transfections were performed as in figure 2 using the 963 ID1 promoter construct, mean values of three independent experiments ±SD are shown.

The *ID2* promoter is also transactivated by PML-RARα but not by RARα/RXR

To investigate whether *ID2* was regulated in a similar manner as *ID1*, we cloned the upstream promoter of *ID2* (812bp) into a luciferase reporter plasmid. Similar to *ID1*, no consensus retinoic acid receptor-binding site could be found in the *ID2* promoter sequence. Also comparable to *ID1*, RARα, RXR, PML, PLZF-RARα and RARα/RXR did not transactivate the *ID2* promoter whereas PML-RARα did (Figure 5A). Furthermore, transactivation of *ID2* by PML-RARα was abolished in the presence of dominant-negative NF-YA (Figure 5B). Inspection of the *ID2* promoter sequence revealed 3 NF-Y and 5 Sp1 consensus binding sites (Figure 5C). Together, these data show that like *ID*1, *ID2* may also be transactivated by PML-RARα without direct DNA binding of the fusion protein.

PML-RARα directly interacts with Sp1

NF-Y and Sp1 have been shown to work in concert on many promoters. Direct physical interaction between Sp1 and NF-YA has been shown, indicating that these proteins may bind to adjacent DNA binding sites and form a complex that regulates transcription.[45] We investigated whether PML-RARα could physically interact with Sp1 or NF-YA. GST-tagged, bacterially produced NF-YA, and Sp1 proteins were made, as well as in vitro translated (reticulocyte lysate) PML-RARα for GST pull-down experiments. rhSp1 was

commercially available. Whereas a clear interaction was observed between GST-NF-YA and recombinant Sp1 (Figure 6A), in none of the conditions tested we could show a direct interaction between NF-YA and PML-RARα. In contrast, an interaction between PML-RARα and Sp1 was readily observed, whereas no interaction was seen with non-loaded or GST-loaded beads (Figure 6A). GST-Sp1 was also able to capture PML-RAR/ΔCC although with a much lower efficiency than PML-RARα. This suggests that the binding of PML-RARα is partly through the coiled-coil region and partly through the RARα part of the fusion protein. This is in agreement with earlier publications that have shown binding of both PML and RARα to Sp1.[23;46]

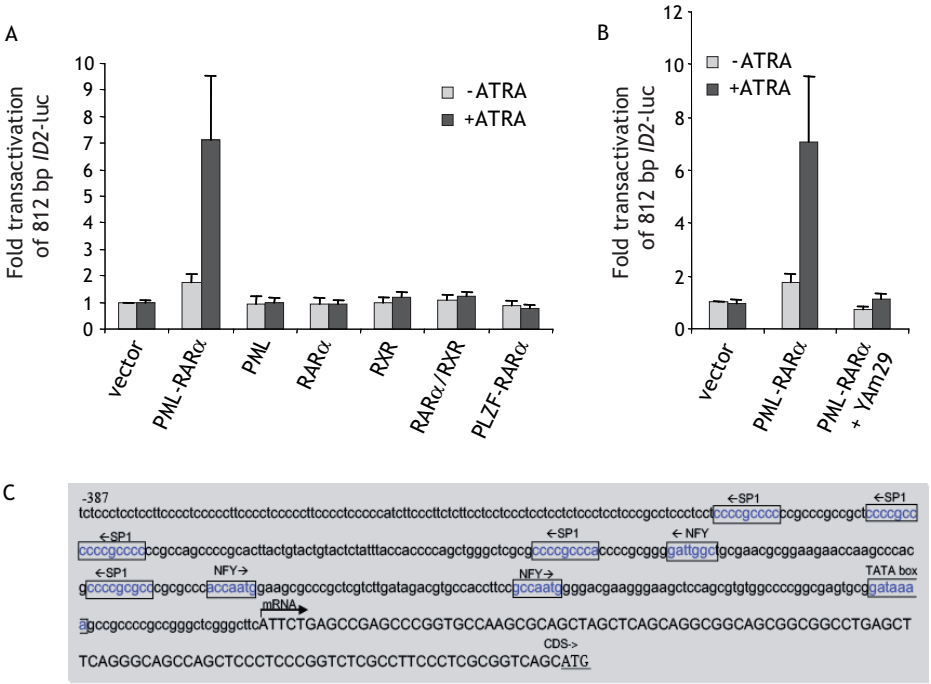


Figure 5. The *ID2* promoter is transactivated by PML-RARα but not by RARα/RXR. (**A**) Cells were transiently transfected with an 812 bp *ID2*-luciferase reporter construct (*ID2-luc*). Transfections and controls were as described in figure 2. Background luminescence of the cells transfected with only a reporter construct (no nuclear receptor and no ATRA) was set at 1. Transactivation was measured after treatment without (light gray bars) and with (dark gray bars) 10^{-6} M ATRA. Mean values from at least three independent experiments ± SD are shown. (**B**) Dominant negative NF-Y (YAm29) inhibits the transactivation of the *ID2* promoter construct by

PML-RARα. Transfections were done as described in figure 2, mean values of three independent experiments ± SD are shown. (**C**) Promoter region of the *ID2* gene with putative Sp1 and NF-Y binding sites.

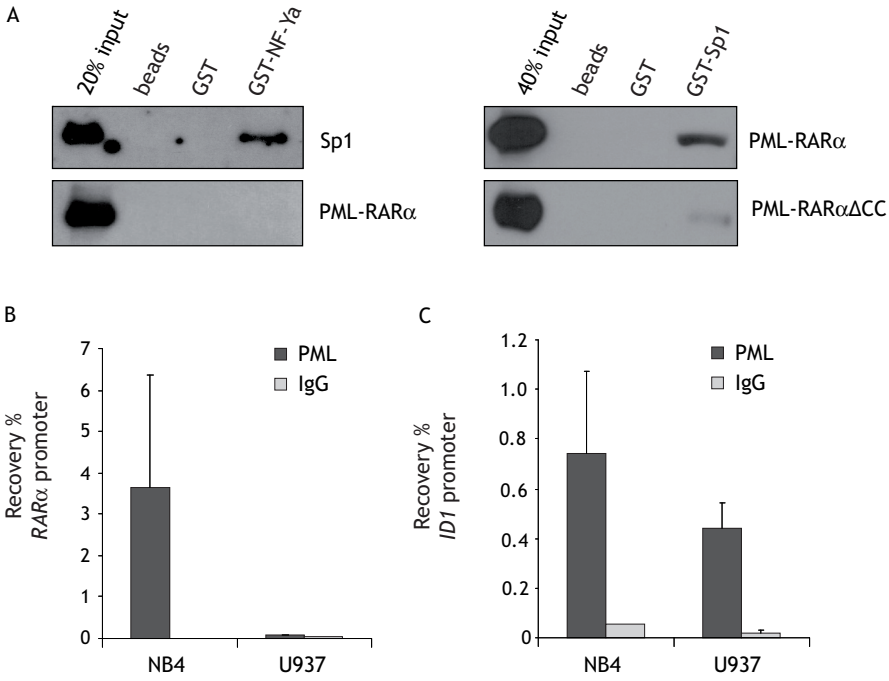


Figure 6. PML-RARα binds Sp1 and is present on the endogenous *ID1* promoter. (**A**) To test binding of PML-RARα to Sp1 and NF-Y, GST pull-down experiments were performed. Empty beads and beads loaded with GST or GST-NF-YA (panels to the left) were incubated with *in vitro* translated Sp1 (positive control) or *in vitro* translated PML-RARα. GST-Sp1 (panels to the right) was incubated with *in vitro* translated PML-RARα or PML-RARαΔCC. Beads were washed and subsequently resuspended in loading buffer. Protein was resolved on SDS-PAGE. Immunostaining was performed with anti-Sp1 or anti-RARα antibody. Clear interactions between NF-YA and Sp1 and between PML-RARα and Sp1 were observed. To show binding of PML-RARα to the endogenous *RAR*β (**B**) and *ID1* (**C**) genes, ChIP assays were performed in PML-RARα positive NB4 cells and in PML-RARα negative U937 cells. Cells were treated with ATRA for 30 min. ChIP was done with anti-PML antiserum. As a control, the non-specific IgG fraction from human serum was used. The Y-axis shows the recovery (%) of *ID1* or *RAR*β sequences relative to the input. Mean values ± SD of 4 independent experiments are shown.

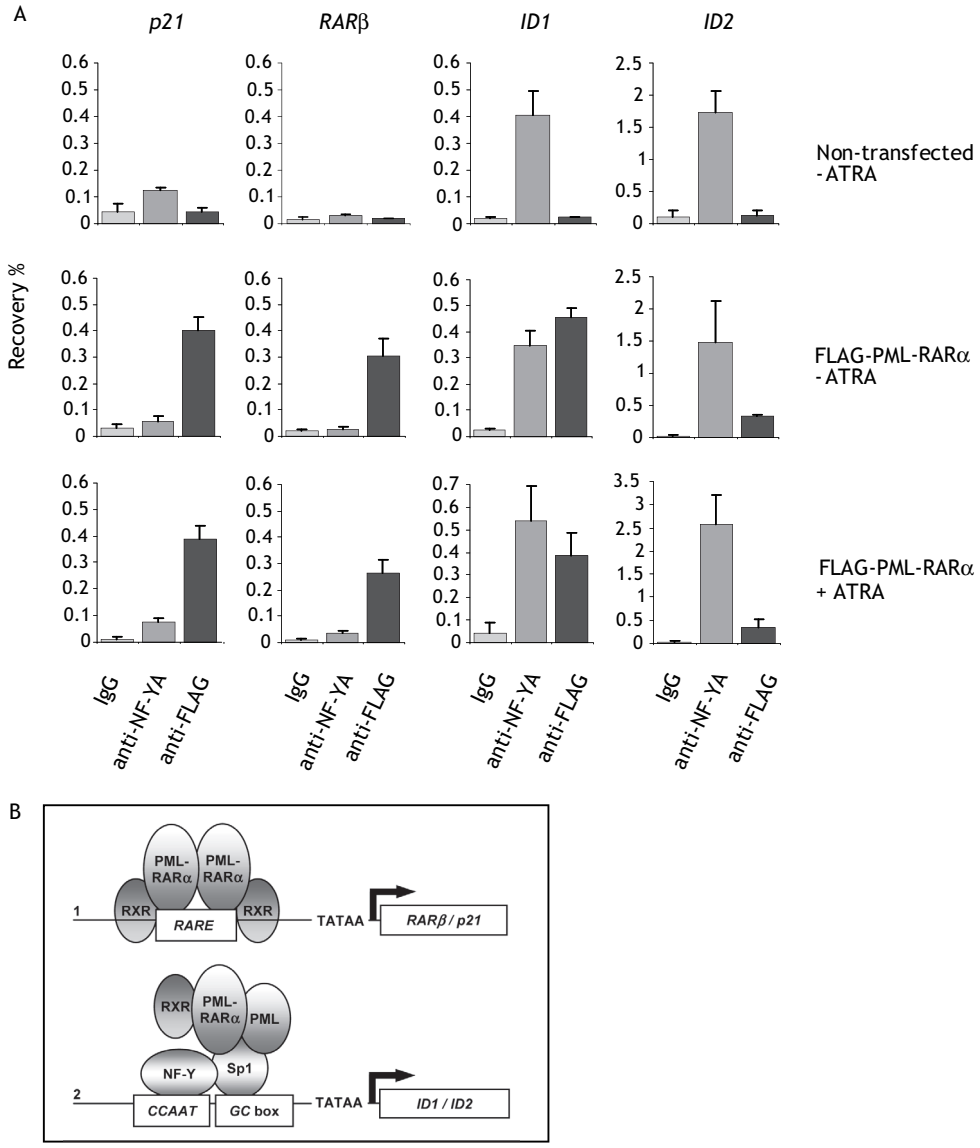


Figure 7. Binding of PML-RARα to the endogenous *p21*, *RAR*β, *ID1* and *ID2* genes. (**A**) To show binding of PML-RARα and NF-Y to the endogenous *p21*, *RAR*β, *ID1* and *ID2* gene ChIP assays were performed. As anti-PML antibodies do not discriminate between the unrearranged PML protein and the PML-RARα fusion protein, FLAG-tagged PML-RARα was used. Cells were transfected with or without FLAG-PML-RARα and cultured in the presence or absence of 10^{-6} M ATRA. ChIP was performed using anti-FLAG-antibodies, anti-NF-YA antibodies and control IgGs. The Y-axis

shows the recovery (%) of *ID1*/*ID2*/*p21* or *RAR*β sequences relative to the input. Mean values of three independent experiments ± SD are shown. (**B**) Dominant negative and gain-of-function model for PML-RARα. Genes that are regulated through a retinoic acid responsive element (RARE) may be bound by PML-RARα (1). Competition with normal, unrearranged retinoid receptors results in a dominant-negative silencing of the gene by PML-RARα in the absence of ligand. Addition of high dose retinoic acid may reverse the silencing, allowing transcription. Sp1 and NF-Y regulated genes may be targeted by PML-RARα through interaction with Sp1 (2). Tethering of PML-RARα to these promoters renders them responsive to retinoic acid, representing a gain-of-function for the PML-RARα fusion protein.

PML and PML-RARα are present on the endogenous *ID1* promoter

To show the physical interaction of PML-RARα with the endogenous *ID1* and *ID2* promoters in intact cells, we performed ChIP assays using PML-specific antibodies. The *RAR*β promoter, that contains a well defined RARE, served as a positive control. In NB4 cells, we found a clear enrichment for the *RAR*β promoter (Figure 6B, right panel). In contrast, in U937 cells which are PML-RARα negative, no enrichment for the *RAR*β promoter was seen (Figure 6B, left panel), indicating that PML-RARα binds to the *RAR*β promoter in NB4 cells. The *ID1* gene was also precipitated with anti-PML antibody in NB4 cells (Figure 6C, right panel). However, this was not only observed in the PML-RARα positive NB4 cells, but also in the PML-RARα negative U937 cells (Figure 6C, left panel). This indicates that in U937 cells, unrearranged PML protein was present on the *ID1* promoter. As the anti-PML does not discriminate between PML and PML-RARα, we could not determine which of these two proteins was bound to the *ID1* promoter in NB4 cells. To investigate this further we used a FLAG-tagged PML-RARα expression construct, allowing us to unequivocally identify binding by the PML-RARα fusion protein. HEK-293 cells were transfected with FLAG-PML-RARα (Figure 7A). As a negative control, ChIP was performed using non-specific IgGs. We compared the presence of (FLAG-tagged) PML-RARα and NF-Y on the two classical ATRA target genes *RAR*β and *p21*, and on *ID1* and *ID2*. Enrichment of all 4 promoters was found using anti-FLAG antibody, indicating that PML-RARα was bound to these four genes in intact cells (Figure 7A, compare upper and middle rows). Using anti-NF-Y antibody, enrichment of the *ID1* and *ID2* promoters was found but no enrichment of *RAR*β and *p21*, indicating that NF-Y was present on *ID1* and *ID2*, but not on the two classical ATRA target genes *RAR*β and *p21*. To see whether the presence of PML-RARα and NF-Y

on the different promoters would be altered by the presence of retinoic acid, ChIP assays were performed in the presence and the absence of ATRA (Figure 7A, middle and bottom rows). ATRA did not influence the binding of NF-Y or PML-RARα to these promoters showing that their binding is ligand-independent.

Together, these data show that there are two different mechanisms by which PML-RARα may regulate the transcription of target genes (Figure 7B). The first class of target genes consists of genes that contain a retinoic acid response element in their promoter. These genes are normally regulated by unrearranged retinoic acid receptors and may be deregulated by PML-RARα in a dominant-negative manner in the absence of ligand. In the presence of high concentrations of ligand, their expression is restored. The second class of target genes consists of genes that are not normally regulated by retinoic acid receptors. Fusion of the RARα moiety to PML renders these genes responsive to retinoic acid, which defines a novel gain-of-function for the PML-RARα fusion protein.

Discussion

The successful treatment of APL with high dose retinoic acid has shown that the differentiation block of the malignant cells can be overcome, leading to terminal differentiation of the leukemic cells and disappearance of the disease. As this is one of the first examples of successful differentiation-induction therapy in cancer, many studies focused on the molecular mechanisms that contribute to the transformation to leukemia, and on the mechanisms that mediate the retinoic acid-induced differentiation of the cells. PML-RARα was shown to interfere with the expression of normal RARα target genes. RARα is an important modulator of granulopoiesis and acts either by direct binding to the DNA, or through interaction with other transcription factors. In this report we show that PML-RARα interacts with Sp1 and may interfere with the expression of genes that are not normally regulated by retinoic acid receptors. Previously, it was shown that both unrearranged PML[46] and unrearranged RAR/RXR complexes[22;23] are able to interact with Sp1. Therefore, the interaction of PML-RARα with Sp1 could be mediated by the RARα part as well as the PML part of the fusion protein. Here we show that PML-RARα binds Sp1 and that this binding is significantly less efficient when the coiled-coil domain is deleted. The

lack of response of the *ID1* and the *ID2* promoters to RARα/RXR, PLZF-RARα and PML-RARαΔCC (Figures 2, 4 and 5) suggests that the PML part of the fusion protein is essential for regulating transcription.

We show that PML-RARα physically interacts with Sp1 in the absence of DNA (Figure 6). Roder et al. have shown that NF-Y and Sp1 interact physically in the absence of DNA and we obtained the same result (Figure 6). This suggests that the PML-RARα/Sp1/NF-Y complex may form before binding to DNA. Figure 3C suggests that the PML-RARα/Sp1/NF-Y complex still binds, although less efficiently, to the ID1 promoter when one transcription factor binding site is mutated. Figure 4C shows that the presence of a dominant-negative form of NF-Y abolishes transactivation of the *ID1* promoter by PML-RARα. We hypothesize that this dominant-negative form of NF-Y disrupts the PML-RARα/Sp1/NF-Y complex and thereby impairs binding to and transativation of the *ID1* promoter. We propose a model in which PML-RARα binds to a DNA-bound Sp1-NF-Y complex, rendering the expression of these genes sensitive to ATRA, and defining a novel gain-of-function for the fusion protein (Figure 7B). In the ChIP experiments, we show that unrearranged PML is recruited to the *ID1* gene (Figure 6C). The physiological meaning of this remains unclear as no effect of PML was observed in the transactivation assays.

As Sp1 is involved in the transcriptional control of various myeloid-specific genes[47;48] deregulation of its target genes may be relevant in APL. For *ID1* and *ID2*, a role in myelopoiesis and in APL was described previously. Overexpression of ID1 or ID2 in NB4 cells inhibits their proliferation and induces a G0/G1 arrest.[31] In addition, ectopic expression of ID1 in CD34+ cells inhibited eosinophil development, whereas neutrophilic differentiation was enhanced. Expression of ID2 accelerated the definitive maturation of myeloid cells.[49] Other genes may be targeted by a similar mechanism as well. The promoter of the important retinoic acid responsive gene *C/EBP*β was transactivated by PML-RARα, but also lacks a consensus RARE within the tested region.[50;51] Possibly, also this gene is (de)regulated by tethering of PML-RARα to the promoter through protein-protein interactions rather than by direct DNA-binding.

Apart from the DNA-binding domain from RARα, the coiled-coil domain of PML was shown to be important for optimal induction of terminal differentiation by the fusion protein.[52] This suggested that homodimerization of PML-RARα is an important feature of the fusion protein, but might also indicate that other protein-protein interactions mediated through the PML part are involved. Several alternative RARα

fusion proteins occur at low frequency (2% of the cases) in APL patients, in which the same part of RARα is fused to other proteins than PML (reviewed in[44]). Common to the various RARα partner proteins is the presence of a dimerization domain, suggesting that homodimerization is an important property of these fusion proteins. However, depending on the fusion partner, the sensitivity to retinoic acid differs suggesting a broader role for the RARα partner protein than just the provision of a dimerization domain. Specifically, PLZF-RARα positive leukemia appears to be more resistant to ATRA, which has been explained by the recruitment of corepressors by the PLZF-part of the fusion protein that are not released upon treatment with ATRA.[15;53] This insensitivity could be reversed, as treatment with a combination of ATRA and G-CSF induced granulocytic differentiation in a synergistic manner.[54] Also in transgenic mice, different types of disease with different sensitivities to ATRA developed for the various fusion proteins.[7;30] Furthermore, when RARα fusion proteins were made by the coupling of RARα to artificial dimerization domains, several characteristics of PML-RARα were recapitulated *in vitro*, but in vivo these proteins induced leukemia with very low efficiency.[55] Interestingly, when these fusion proteins were expressed in a PML -/- background, the animals did still not develop leukemia efficiently, leading to the conclusion that the mere combination of disruption of PML and dimerization of RARα does not recapitulate the full oncogenic potential of PML-RARα, and that the fusion protein has gain-of-function characteristics. Together, this showed that dimerization of RARα is important but not sufficient to reproduce the complete phenotype of PML-RARα.

It remains to be investigated whether PML-RARα recruits co-repressors to the promoters of Sp1 and NF-Y target genes. This would lead to repression of gene expression in the absence of ATRA, possibly contributing to transformation of the cells, similar to the effect of PML-RARα on regular retinoic acid receptor target genes. So far, we did not observe important downregulation of ID1 and ID2 mRNA in freshly isolated leukemia cells from APL patient cells compared to non-APL acute myeloid leukemia cells, suggesting that this is not the case (data not shown). However, as *ID1* and *ID2* expression in most leukemic samples was low, further studies are required.

Potentially, RXR may also be present in the PML-RARα/Sp1/NF-Y complex. RXR is an essential part of the PML-RARα complex during transformation and synergy has been shown between ATRA and RXR specific agonist on transcriptional activation.[24-26;56] Therefore, RXR specific agonist may also affect transcription of Sp1 and NF-Y target genes. In summary, we define a novel, Sp1 and NF-Y-dependent mechanism by which

the PML-RARα fusion protein interferes with gene transcription. This implicates that PML-RARα (de)regulates an additional class of genes that are normally not regulated by retinoid receptors and defines a gain-of-function for the PML-RARα fusion protein.

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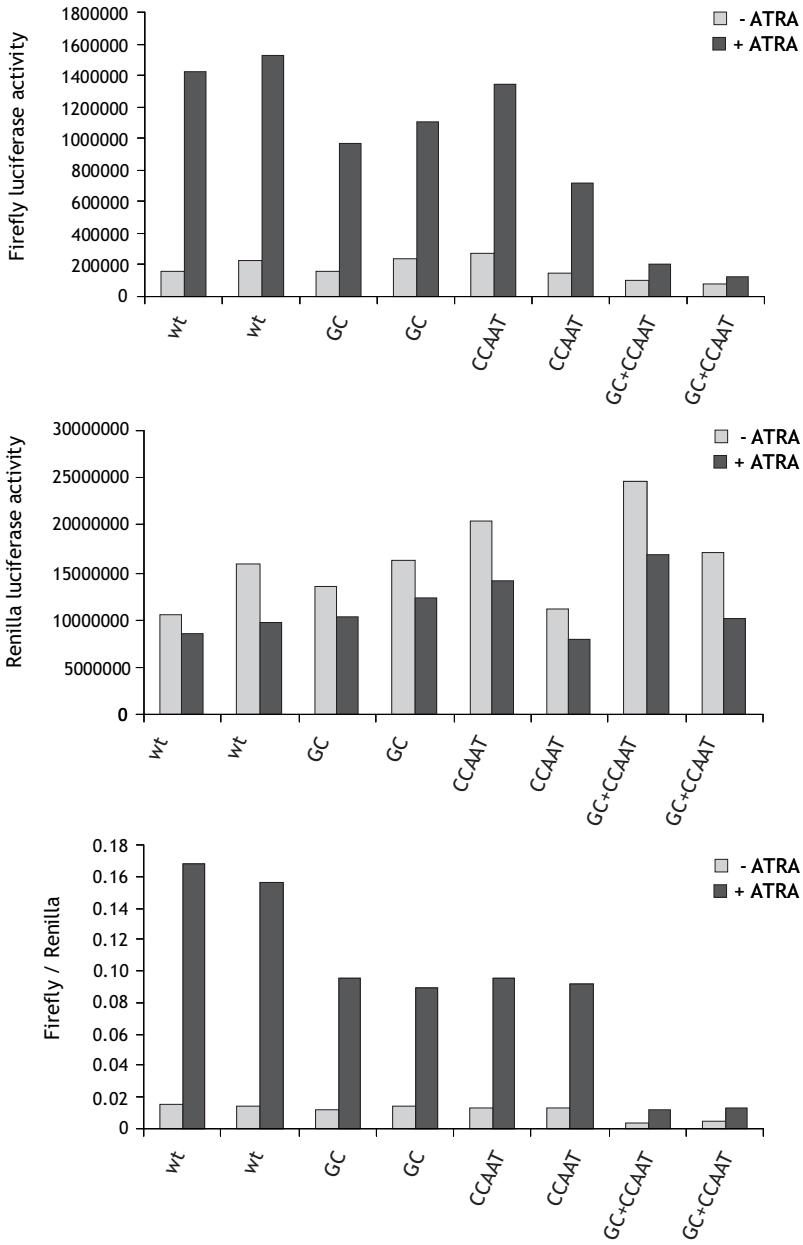
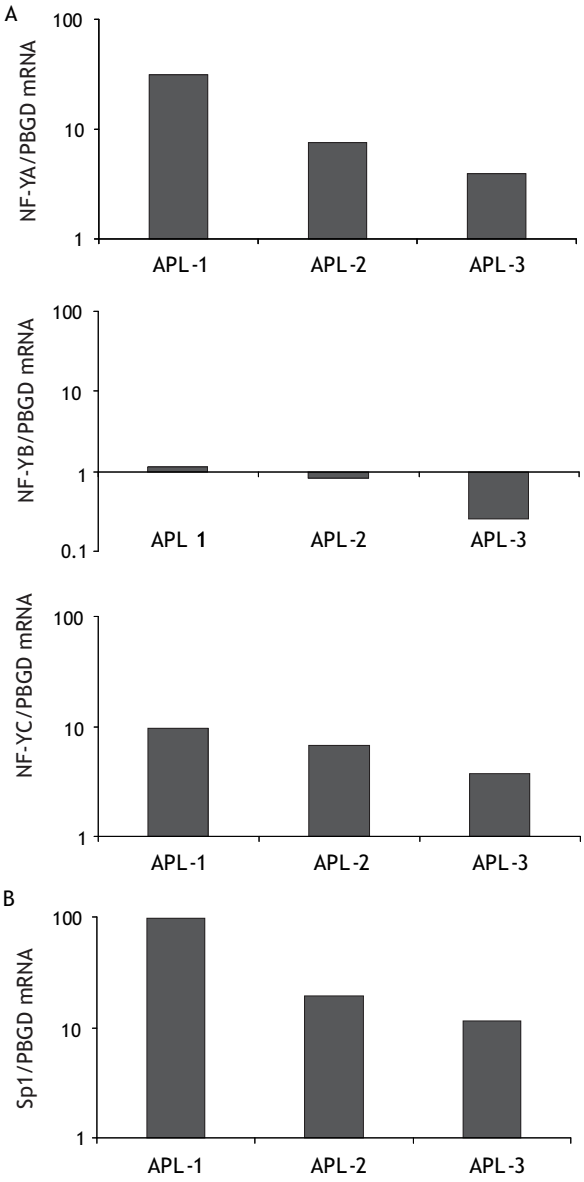
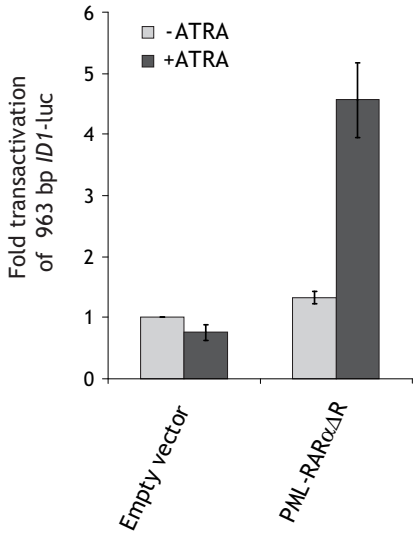


Supplemental figure 1. Transactivation data before correction. Raw luciferase activaties are shown for one transactivation experiment from figure 3C. The *ID1* promoter, wt or with a mutated GC box and/or CCAAT box, was cloned in front of the *firefly* gene. A renilla expression construct was co-transfected for correction. Note that the correction does not qualitatively change the results.



Supplemental figure 2. NF-Y and Sp1 are expressed in APL cells. mRNA expression levels of NF-Y (**A**) and Sp1 (**B**) were determined in mononuclear cells from 3 APL patients. Bone marrow samples were obtained from t(15;17) APL patients following informed consent. Mononuclear cells were isolated by density gradient centrifugation using ficoll (1.077 g/ml, Sigma). Cells were taken up in RNA-bee (ISO-TEX Diagnostics, Friendswood, USA). RNA was used as template in a RT-DNA reaction as described before (de Vries; br j cancer 1999). Quantitative PCR was performed with the ABI/PRISM 7700 Sequence Detection system (ABI/PE, Foster City, Ca, USA). As a reference gene we used PBGD (de Vries; BJC 1999). The primer/probe sets for detecting NF-Y and Sp1 were from Applied biosystems (Foster City, Ca, USA). PCRs were performed in universal master mix (Roche). Conditions were as follows: 10 min 95°C followed by 45 cycles of 15 sec 95°C and 1 min 62°C.



Supplemental figure 3. Transactivation of the *ID1* promoter in myeloid cells. HL60 cells were electroporated with the 963bp *ID1* promoter-luciferase reporter construct (*ID1-luc*) together with a control vector expressing Renilla-luciferase. In addition an empty vector or a vector coding for PML-RARαΔR was transfected. Transactivation is expressed as arbitrary units and is corrected for transfection efficiency measured by Renilla-luciferase. Back-ground luminescence of the cells trans-fected with only the reporter construct without nuclear receptors and in the absence of ATRA was set at 1. Cells were cultured for 8 hrs without (light gray bars) and with (dark gray bars) ATRA.

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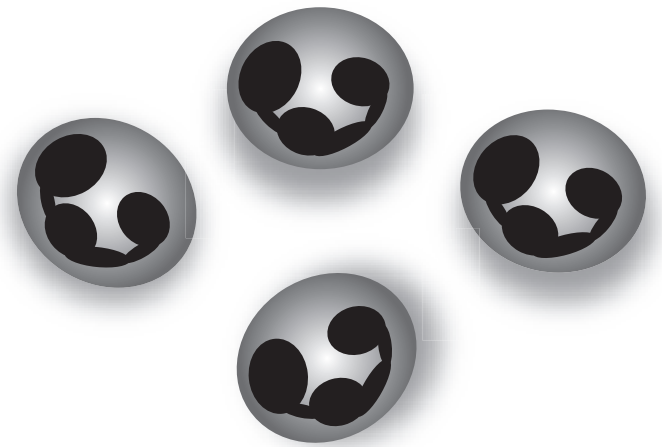
Chapter 4

HES1 gene expression is directly regulated by PML-RARα in a NF-Y-dependent manner



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Abstract

The oncogenic fusion protein PML-RARα is expressed in 98% of the cases of acute promyelocytic leukemia (APL). PML-RARα interferes with the expression of normal retinoic acid receptor target genes, blocking the differentiation of the malignant cells at physiological concentrations of retinoids. Restoration of the expression of these PML-RARα target genes using high concentrations of all-*trans* retinoic acid (ATRA), forces the leukemic cells into terminal granulocytic differentiation. Here we report the identification of a novel direct ATRA-responsive gene in APL: *Hairy-Enhancer of Split 1* (*HES1*). ATRA induced a rapid, transient increase in HES1 expression, followed by a clear decrease, both in PML-RARα positive cell lines as well as in primary APL patient cells. We show that PML-RARα is directly involved in the transcriptional regulation of *HES1*, while wild type RARα/RXR is not. We demonstrate that transactivation of *HES1* by PML-RARα does not require the DNA-binding domain of the fusion protein but depends on NF-Y transcription factors. These results indicate that PML-RARα not only interferes with the transcription of regular retinoic acid receptor target genes, but may also interfere with the expression of NF-Y-regulated genes.

Introduction

Acute promyelocytic leukemia (APL) accounts for 5-10% of all acute myeloid leukemias (AMLs) and is classified as AML-M3, according to the French-American-British (FAB) classification system based on morphological criteria. APL is chacarterized by a block in differentiation at the promyelocytic stage of hematopoietic development and by mutations that affect the retinoic acid receptor α (RARα). In 98% of the cases of APL, the promyelocytic leukemia (*PML*) gene on chromosome 15 is fused to the retinoic acid receptor α (*RARA*) gene on chromosome 17 resulting in a PML-RARα fusion protein. This chimeric protein acts as a dominant oncogene, interfering with the normal function of the unrearranged PML as well as the unrearranged RARα protein. PML-RARα may silence normal RARα target genes at physiological concentrations of the RARα ligand, all-*trans* retinoic acid (ATRA). It has been postulated that these target genes mediate PML-RARα-induced transformation. At high concentrations of ligand, PML-RARα (re)activates the transcription of genes, forcing the leukemic cells into terminal granulocytic differentiation.[1] The importance of PML-RARα as a transcriptional activator of differentiation-inducing genes has been illustrated by in vitro experiments. Forced expression of PML-RARα, but not RARα, in an ATRA resistant APL cell line with constitutive degradation of the chimeric protein restored ATRA sensitivity.[2] In addition, in APL patients that became resistant to differentiation induction with ATRA during therapy, additional mutations were found in the ligand-binding domain of the PML-RARα fusion protein, indicating an important role for the fusion protein in retinoic acid-induced differentiation of APL cells.[3] To date, several important RARα target genes have been identified that are deregulated by PML-RARα in APL cells including *p21$^{waf1/Cip1}$* [4-6] and *CCAAT/enhancer-binding-protein*-β and ε (*C/EBP*β and *C/EBP*ε).[7;8] Previously, we described the involvement of Inhibitor of DNA binding 1 and 2 (ID1 and ID2) proteins in the response of APL cells to ATRA treatment and we showed that ectopic expression of these genes induced an accumulation of APL cells in the G0/G1 phase of the cell cycle.[9] Recently, we found that *ID1* and *ID2* are direct retinoic acid responsive genes that are transcriptionally regulated by PML-RARα. Interestingly, we found that PML-RARα transcriptionally regulates *ID1* and *ID2* expression via a novel mechanism, which is not shared with normal RARα. *ID1* and *ID2* are transcriptionally activated by PML-RARα independently of its DNA-binding domain and transcriptional activation by PML-RARα requires the presence of the NF-Y

transcription factor complex and Sp1 binding sequences. These results indicate that PML-RARα not only interferes with the transcriptional regulation of regular retinoic acid receptor target genes, but also with the expression of an additional class of genes that are normally not regulated by retinoic acid receptors (Van Wageningen et al., manuscript accepted for publication in Blood). Whether this is restricted to *ID1* and *ID2* gene expression or represents a more common mechanism remains unknown.

ID1 and ID2 belong to the family of helix-loop-helix (HLH) proteins that act as negative regulators of basic-HLH (bHLH) transcription factors. Many developmental processes are positively or negatively controlled by bHLH genes and their inhibitors and members of this family have been implicated in cell fate decisions in many different tissues.[10] In hematopoiesis, bHLH transcription factors and their inhibitors have been implicated in lymphopoiesis and three bHLH family members (*SCL/TAL-1*, *LYL-1* and *E2A*) are located at the sites of recurrent chromosomal translocation breakpoints in leukemia.[11-14] Besides the ID proteins, Hairy-Enhancer of Split (HES) proteins (HES1-7) also act as transcriptional repressors of bHLH-regulated genes. Like ID proteins, HES1 proteins act by sequestration of bHLH transcription factors into non-functional heterodimeric complexes.[15;16] In addition to this passive repression mechanism, HES1 proteins may function as active repressors by binding to DNA. HES1 proteins contain a DNA binding domain that preferentially recognizes the consensus sequences CACNAG (or N-box), unlike most other bHLH factors, which bind to the consensus sequences CANNTG (or E-box).[15] HES1 homodimers bind to N-box sequences and the corepressor TLE/Groucho is subsequently recruited to mediate repression by interaction with histone deacetylases. Furthermore, the orange domain/helix3-helix4 of HES1 contributes to transcriptional repression, but the exact underlying mechanism is not known.[17] Transcriptional repression by HES1 of its own promoter is mediated through this latter mechanism.[18] Apart from repression of its own promoter, *human achaete-scute homolog-1* (*hASH1*)[19], *CD4*,[20] *acid a-glucosidase* (*GAA*)[21] and *p21$^{waf1/Cip1}$* [22] have been proposed as candidate HES1 target genes. A dual function has been described for HES1 both as a transcriptional activator and as a transcriptional repressor of *GAA* depending on the cell type.[23]

In the present study we investigated whether transcriptional regulation of inhibitors of bHLH transcription factors by PML-RARα during ATRA-induced APL cell differentiation is a more general mechanism. We investigated whether besides the ID proteins, HES1 is also regulated in APL cells. We show that *HES1* is a direct

ATRA-responsive gene, which is transcriptionally regulated by PML-RARα via a NF-Y-dependent mechanism.

Materials and methods

Cell culture

Leukemic cells were isolated from bone marrow of patients at diagnosis after informed consent. Mononuclear cells were isolated by means of a Ficoll-1077 (Amersham, Buckinghamshire, UK) gradient and cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 20% fetal calf serum (FCS). NB4 and U937-PR9 cells (kindly provided by Dr. P.G. Pelicci) were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS. HEK 293 cells were cultured in DMEM (Life Technologies) and Hep3B cells were cultured in IMDM (Life Technologies) both supplemented with 10% FCS and 2 mM glutamine (Life Technologies). Cultures were incubated at 37°C in a 5% CO_2 completely humidified atmosphere. ATRA (Sigma, St Louis, MO, USA) was used at a concentration of 10^{-6} M, and where indicated 100 μM $ZnSO_4$ was added 16 hours prior to ATRA. Cycloheximide (ICN, Costa Mesa, CA, USA) was used at 4 μg/ml, and was added 30 min prior to ATRA.

Quantitative PCR

RNA from cell lines was isolated using RNA-Bee (Iso-Tex Diagnostics, Friendswood, TX, USA) and RNA from primary cells was isolated using the mini RNA isolation II kit (Zymo Research, Orange, CA, USA). Bone marrow mononuclear cells of AML patients were isolated by lysis of erythrocytes in 0.14 M NH_4Cl buffer for 30 min on ice, after which RNA was isolated using RNA-Bee (Iso-Tex Diagnostics). cDNA was generated in a reverse transcription reaction as previously described.[24] HES1 mRNA levels were measured with real-time quantitative PCR using the primers: sense 5'-GAAGG-CGGACATTCTGGAAA-3', anti-sense 5'-GCGGGTCACCTCGTTCAT-3'. Quantitative PCR was done using SYBR green PCR (Applied Biosystems, Foster City, CA, USA). β-actin or PBGD was used as a reference gene. Primer sequences for the *HES1* promoter were as follows: sense 5'-AGTCAAAGCAGCTCTGTTACATATGAG-3', anti-sense 5'-TTTGGTCTTGATCTTGTCTATTTCTTTT-3'. Primers for the *RAR*β promoter were: sense 5'-TTGGGTCATTTGAAGGTTAGCA-3', anti-sense 5'-CACACAGAATGAAAGATTGAATTGC-3'. Quantitative PCR for the *ALBUMIN* promoter was performed using probe ALB-VIC 5'-TGCTGAAACATTCACCTTCCATGCAGA-3' with the primers, sense 5'-TGAAACAT-

ACGTTCCCAAAGAGTTT-3', anti-sense 5'-CTCTCCTTCTCAGAAAGTGTGC-ATAT-3'. Primers and probe were all located to one exon of the *ALBUMIN* gene. Quantitative PCR reactions were performed on the ABI/PRISM 7700 Sequence Detection system (ABI/PE, Foster City, CA, USA).

Western blotting

Cells were lysed in RIPA-buffer containing 1% Nonidet P-40 (NP-40), 0.5% natrium-deoxycholaat, 0.1% SDS and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Total protein lysates (75 μg) and molecular weight markers were separated on a 10% SDS-polyacrylamide gel and electrophoretically transferred onto polyvinylidenefluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Blots were probed with anti-murine HES1 rabbit polyclonal antibody (a kind gift from Dr. Tetsuo Sudo, Toray Industries, Inc., Japan). As a control for equal loading, blots were probed with an antibody to Actin (I-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Chromatin immunoprecipitation (ChIP)

HEK 293 cells were transiently transfected with FLAG-PML-RARα (kindly provided by A. Tomita[25] which was re-cloned into a pCMV expression vector) using the calcium phosphate precipitation method. ChIP assays were performed as described previously.[26] At 24 hours after transfection DNA-protein cross-linking was done for 30 min at room temperature by adding formaldehyde at a final concentration of 1% directly to the culture medium. Where indicated, ATRA was added 30 min before cross-linking. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM for 5 min at room temperature. Cells were scraped and washed with ice-cold phosphate-buffered saline, buffer B (10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, 20 mM HEPES [pH 7.6]), buffer C (1 mM EDTA, 0.5 mM EGTA, 0.15 M NaCl, 50 mM HEPES [pH 7.6]) and resuspended in incubation buffer (0.15% SDS, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6] and protease inhibitors) at $33x10^6$ cells/ml. Chromatin was sonicated using the Bioruptor (Cosmo Bio, Tokyo, Japan), at high intensity for 15 min with 0.5 min intervals. Insoluble material was removed by centrifugation at 4°C for 15 min. A total of 120 μl supernatant was incubated with 30 μl pre-coated protein A/G plus agarose beads 50% v/v (Santa Cruz), 0.1% BSA, 36 μl 5x incubation buffer, protease inhibitors and 2-5 μg antibody (anti-NF-YA (H-209, Santa Cruz), anti-FLAG (M2, Sigma) or non-specific IgG from human serum) and rocked at 4°C for 16 hours. Beads were harvested by centrifugation and washed twice with buffer 1 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES [pH 7.6]), once with buffer 2 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES [pH 7.6]), once with buffer 3 (0.25 M LiCL, 0.5%

NaDOC, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]), and twice with buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]). Chromatin antibody complexes were eluted by the addition of 1% SDS and 0.1 M NaHCO3 to the pellet and incubated for 20 min at room temperature. Cross-linking was reversed by the addition of NaCl (0.44 M final concentration) and incubation of the eluted samples for at least 4 hours at 65°C. DNA was recovered by phenol-chloroform-isoamylalcohol extraction followed by chloroform-isoamylalcohol extraction and precipitated by the addition of 0.1 volume of 1 M sodium acetate (pH 5.2), and 2.5 volumes of ethanol. Precipitated DNA was dissolved in water, and input as well as immunoprecipitated DNA was analyzed using quantitative PCR for genomic sequences of the *HES1* and *RAR*β promoter regions. For calculation of the fold enrichment, quantitative PCR was performed for *ALBUMIN* genomic sequences before and after the immunoprecipitation. Quantitative PCR following ChIP was done using SYBR green PCR (Applied Biosystems).

Transactivation studies

Hep3B cells were transfected using the calcium phosphate precipitation method with an equal amount of reporter plasmid (0.25 μg pGL3-*HES1*) and different amounts of effector plasmids (0.25 μg nuclear receptor expression vector, 1.0 μg YAm29 expression vector, 0.1 μg Renilla vector (pRL-CMV, Promega, Madison, WI, USA). The total amount of plasmid was normalised by the addition of empty vector as the carrier in each transfection. The *HES1* promoter (containing the –252 to +31 promoter fragment of *HES1*) was cloned into the XhoI-HindIII site of the pGL3-basic vector (Promega) after amplification with the following primers: sense 5'-GTACCTCGAGGAGAGTAGCAAAGGGTTAAAATCCT-3', anti-sense 5'-GTACAAGCTTCTGTTATCAGCACCAGCTCCG-3'. $RARE_3$-tk-luc,[27] PML-RARαΔR,[28] PML-RARαΔCC, PML-RARα, PLZF-RARα, RARα and RXR[29] expression vectors were as described and dominant-negative NF-YA (YAm29) was kindly provided by R. Mantovani (University of Milan, Italy). Where indicated ATRA (Sigma) was added 24 hours after transfection. Cells were lysed 40 hours after transfection using Passive Lysis Buffer (Promega). Firefly and Renilla activity was measured on a luminometer (Lumat LB 9507, ABI/PE) using Luciferase Assay reagents (Promega).

Results

HES1 is rapidly and directly induced in APL cells upon ATRA treatment

To investigate whether, besides the ID proteins, other inhibitors of bHLH transcription factors are regulated in APL cells, we assessed the expression level of HES1 during

ATRA-induced granulocytic differentiation of APL cells. Total RNA and protein was isolated from the PML-RARα positive APL cell line NB4, cultured in the absence or presence of ATRA for several time intervals, and HES1 expression levels were measured by RT-PCR (Figure 1A) and Western blotting (Figure 1B). HES1 mRNA and protein expression levels were induced within half an hour of ATRA stimulation and reached maximum levels (> 5-fold induced) at 1 hour of ATRA stimulation. Subsequently, from 1 to 24 hours following ATRA stimulation, HES1 mRNA expression levels decreased about 100-fold and remained at this level during further differentiation. HES1 protein levels diminished to undetectable levels at 4 hours of ATRA stimulation. The expression pattern of HES1 was confirmed in primary APL patient cells (Figure 1C). Cells were cultured in the presence or absence of ATRA for several time intervals, after which RNA was isolated. In all cases we observed a clear upregulation of HES1 mRNA levels within 4 hours of ATRA stimulation after which expression levels declined again. Thus, HES1 expression levels are rapidly upregulated by ATRA both in the NB4 cell line as well as in primary APL patient cells. To investigate whether HES1 was directly regulated by retinoic acid receptors, NB4 cells were treated with cycloheximide to inhibit protein translation. Upregulation of HES1 mRNA expression by ATRA was not affected by the presence of cycloheximide, indicating that HES1 is directly induced, without intermediate protein production (Figure 1D).

PML-RARα regulates HES1 expression

APL is characterized by a block in granulocytic differentiation and PML-RARα is involved in this process by deregulating expression of retinoic acid response genes. To investigate whether HES1 expression levels are regulated by PML-RARα, we used the U937-PR9 cell line, which is stably transfected with a Zn^{2+} -inducible PML-RARα expression cassette. This cell line was cultured in the presence or absence of zinc, and the expression level of HES1 was measured by RT-PCR. Expression of PML-RARα in U937 cells resulted in slightly enhanced HES1 expression levels in the absence of ATRA (Figure 2).

To study the effect of PML-RARα on HES1 expression during ATRA-induced differentiation, U937-PR9 cells were cultured with or without zinc followed by ATRA stimulation for several time intervals. Figure 2 shows that the presence of PML-RARα caused an upregulation of HES1 mRNA expression at 4 hours of ATRA stimulation. Comparable to NB4 cells, this induction was transient. Unlike in NB4 and primary APL patient cells, no suppression of HES1 was observed at later time points. Perhaps this

suppression in NB4 and APL patient cells is due to degradation of PML-RARα following ATRA treatment of these cells.

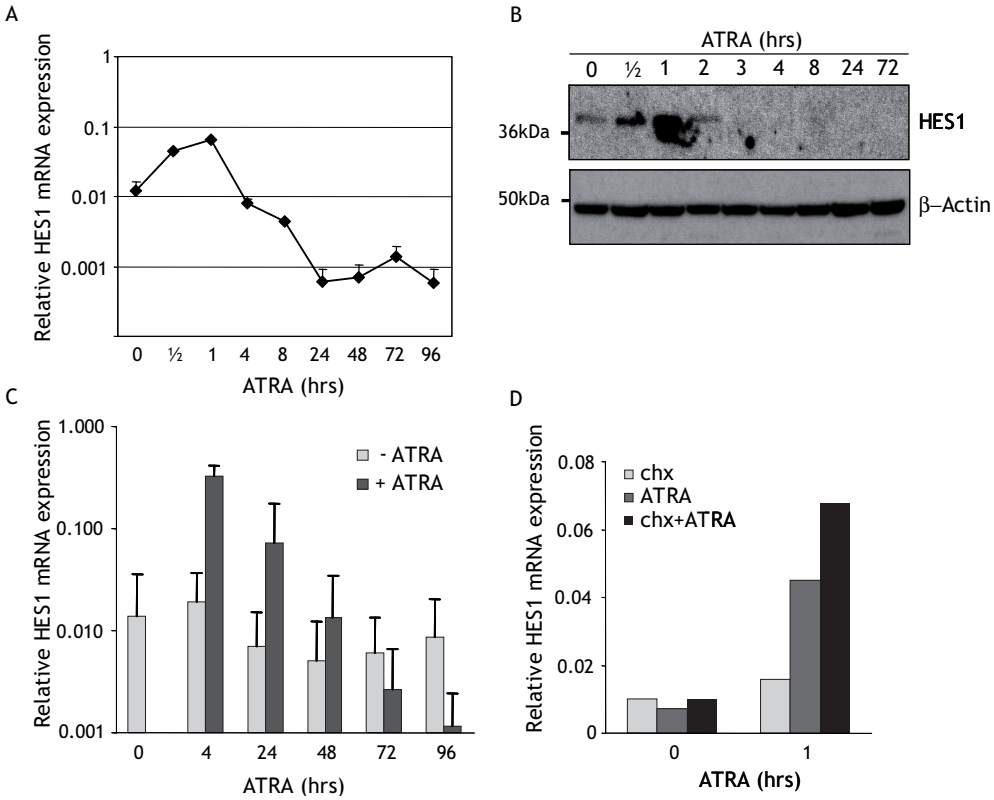

Figure 1. HES1 is a direct ATRA-responsive gene in APL cells. (**A**) Total RNA samples derived from non-stimulated and ATRA-stimulated NB4 cells (10^{-6} M ATRA was added for indicated time intervals) were subjected to RT-PCR using specific primers for the detection of HES1 mRNA. Mean values and standard deviations of at least two independent experiments are shown. (**B**) Total cell lysates (75 mg) from non-stimulated and ATRA-stimulated NB4 cells (10^{-6} M ATRA was added for indicated time intervals) were subjected to immunoblot analysis using HES1 antibody. The same blot was reprobed with a β-actin antibody to demonstrate equal loading. The molecular weight (kDa) marker is indicated at the left. (**C**) RT-PCR was performed with total RNA from bone marrow derived mononuclear cells from five APL patients after *in vitro* exposure to 10^{-6} M ATRA for indicated time points (dark gray bars). As a control cells were cultured in the absence of ATRA (light gray bars). (**D**) NB4 cells were treated with cycloheximide (light gray bars), ATRA (dark gray bars) or cycloheximide and ATRA (black bars). Total RNA was derived from these samples and subjected to RT-PCR using specific primers for the detection of HES1 mRNA. Values were normalised for β-actin expression in all experiments.

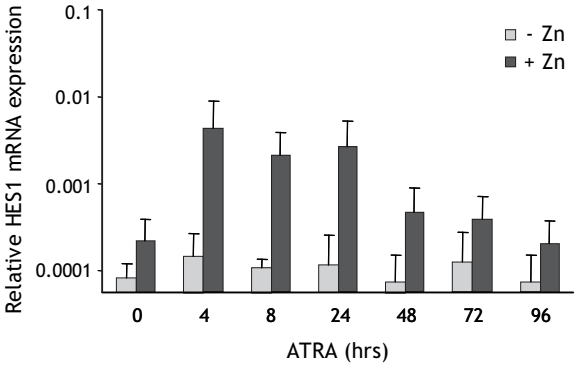

Figure 2. PML-RARα is involved in the transcriptional regulation of HES1. RT-PCR was performed with total RNA extracted from U937-PR9 after culturing the cells in the presence (dark gray bars) or absence (light gray bars) of Zn^{2+} and ATRA for indicated time points. β-actin was used for normalisation.

To investigate whether PML-RARα is associated with deregulated HES1 expression in primary patient cells, we analysed the expression level of HES1 in cells from patients diagnosed with AML subtypes M0 to M7, and compared these with the level of expression in cells from patients with AML-M3 (Figure 3). As in U937 cells, also in primary leukemic cells expression of PML-RARα resulted in a somewhat higher expression level of HES1. These data indicate that PML-RARα-mediated leukemic transformation does not involve active downregulation of HES1.

PML-RARα transactivates the *HES1* promoter without direct DNA binding

Since we demonstrated that expression of HES1 is directly upregulated in APL cells upon stimulation with ATRA and that expression of PML-RARα contributes to this ATRA-induced upregulation of HES1 expression in U937 cells, we investigated the mechanism that results in the transcriptional activation of HES1 by PML-RARα. We cloned a 283 bp fragment of the *HES1* promoter, including the putative TATA-box, into a luciferase reporter construct. When expressed alone, RARα, RXR and PML were not able to transactivate this *HES1* promoter construct both in the absence and presence of ATRA. The combination RARα/RXR also did not transactivate the promoter. In contrast, PML-RARα transactivated the promoter more than 6-fold in an ATRA-dependent fashion and almost 2-fold in the absence of ligand (Figure 4A). To verify

that RARα and RXR were expressed and functional, a control luciferase construct was used containing three bona-fide retinoic acid response elements (RAREs) from the RARβ promoter ($RARE_3$-tk-luc[30]). This construct was strongly transactivated in the presence of ATRA through endogenous retinoic acid receptors and transactivation was further enhanced when RARα and RXR were co-transfected (Figure 4B).

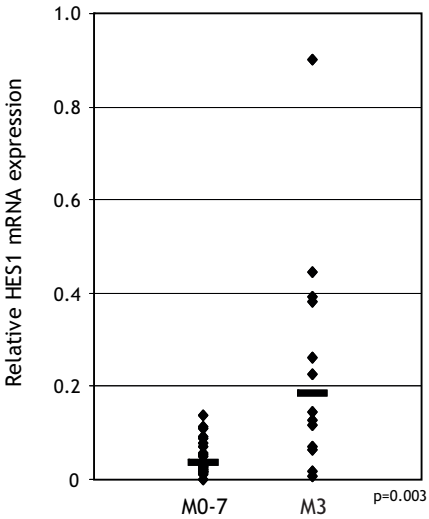

Figure 3. HES1 expression levels in APL cells compared to non-APL AML cells. RNA was isolated from bone marrow mononuclear cells of newly diagnosed AML patients (M0 n=2, M1 n=7, M2 n=6, M3 n=14, M4 n=5, M5 n=5, M6 n=1 and M7 n=2). RT-PCR for HES1 was performed and PBGD was used as a reference gene. Compared are the expression levels of HES1 in AML-M3 (M3, n=14) versus AML-M0 to M7 (M0-7, n=28). The black horizontal bar indicates the median. The significance level (p-value) between the two groups was calculated using Student's t-test and was set at 0.05.

To investigate the importance of the different domains of PML-RARα, we tested whether vectors encoding the DNA-binding-defective PML-RARα mutant (PML-RARαΔR) and a mutant lacking the coiled-coil domain (PML-RARαΔCC) were able to transactivate the HES1 promoter. In figure 4C we show that PML-RARαΔR is able to transactivate the HES1 promoter, indicating that transactivation of the HES1 promoter by PML-RARα does not require direct binding of PML-RARα to the DNA. However, PML-RARαΔCC did not transactivate the HES1 promoter, suggesting that

PML-RARα homodimerisation or interaction with another protein is necessary for transactivation. When PLZF-RARα, which contains the same part of the retinoic acid receptor as PML-RARα, was used no transactivation could be observed (Figure 4C).

NF-YA is necessary for PML-RARα-dependent transactivation of the *HES1* promoter

Recently, we have found that PML-RARα-dependent transactivation of the *ID1* and *ID2* promoter is dependent on NF-Y (CCAAT box) and on Sp1 (GC box) binding sequences in the 5' upstream promoter region (Van Wageningen et al., manuscript accepted for publication in Blood). We investigated whether PML-RARα-dependent transactivation of the *HES1* promoter might also be regulated via NF-Y. When analysing the sequence of a 283 bp fragment of the *HES1* 5' promoter to determine the DNA sequences that are relevant for the observed PML-RARα-dependent transactivation, no consensus RARE was found. However, we observed perfect consensus binding sites for the NF-Y transcription factor and Sp1 (Figure 5A). NF-Y is a trimeric protein complex consisting of the subunits NF-YA, NF-YB and NF-YC. All three subunits are necessary for the complex to bind to DNA. A dominant-negative form of NF-YA has been described (YAm29[31]). This mutated NF-YA subunit can still bind to the NF-YB and NF-YC subunits, but is not able to bind to DNA, preventing the formation of a functional trimeric NF-Y complex. To investigate whether functional NF-Y was necessary for transactivation of the *HES1* promoter the YAm29 mutant was used in a transactivation assay. Figure 5B shows that transactivation of *HES1* by PML-RARα was strongly decreased in the presence of dominant-negative NF-YA. This indicates that PML-RARα-dependent transactivation of the *HES1* promoter is dependent on a functional NF-Y complex.

PML-RARα and NF-YA bind to the *HES1* promoter

To study whether PML-RARα is present on the *HES1* promoter we performed ChIP assays. HEK293 cells were transfected with FLAG-PML-RARα and immunoprecipitation was performed using chromatin from these cells and an antibody directed against FLAG or non-specific IgG as a control. Quantitative PCR was used to measure the amount of immunoprecipitated *HES1* promoter, which was compared to the control genomic sequences of *ALBUMIN*. ChIP assays using chromatin from PML-RARα transfected cells revealed a clear increase in *HES1* promoter recovery when compared to untransfected cells, indicating that PML-RARα is present on the *HES1* promoter

(Figure 6, compare upper and middle rows). ChIP assays were performed both in the presence and absence of ATRA and we found that ATRA did not influence the binding of PML-RARα to the *HES1* promoter, suggesting that binding of PML-RARα is ligand-independent (Figure 6, middle and bottom rows). The *RARB* promoter, which contains a well-defined RARE served as a positive control.

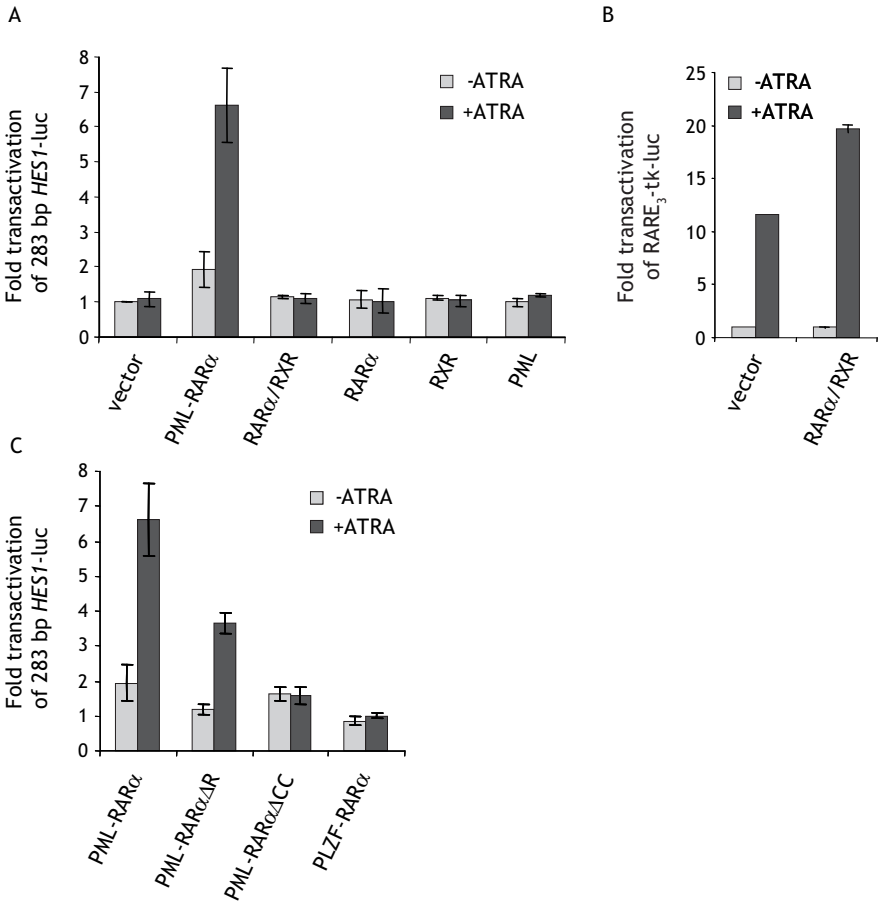

Figure 4. Transactivation of the *HES1* promoter by PML-RARα. (**A** and **C**) Hep3B cells were transfected with a *HES1* promoter-luciferase reporter construct (pGL3-*HES1*), a control vector expressing Renilla-luciferase and vectors coding for the various proteins indicated in the figure. Background luminescence of the cells transfected with only the reporter construct in the absence of ATRA was set to 1. Transactivation was corrected for transfection efficiency measured by Renilla activity. Cells were cultured with (dark gray bars) or without (light gray

bars) ATRA. Mean values and standard deviations from three independent experiments are shown. (**B**) To show transactivation by unrearranged retinoic acid receptors, Hep3B cells were transfected with the luciferase construct $RARE_3$-tk-luc, containing three bona-fide RAREs. Mean values and standard deviations from three independent experiments are shown.

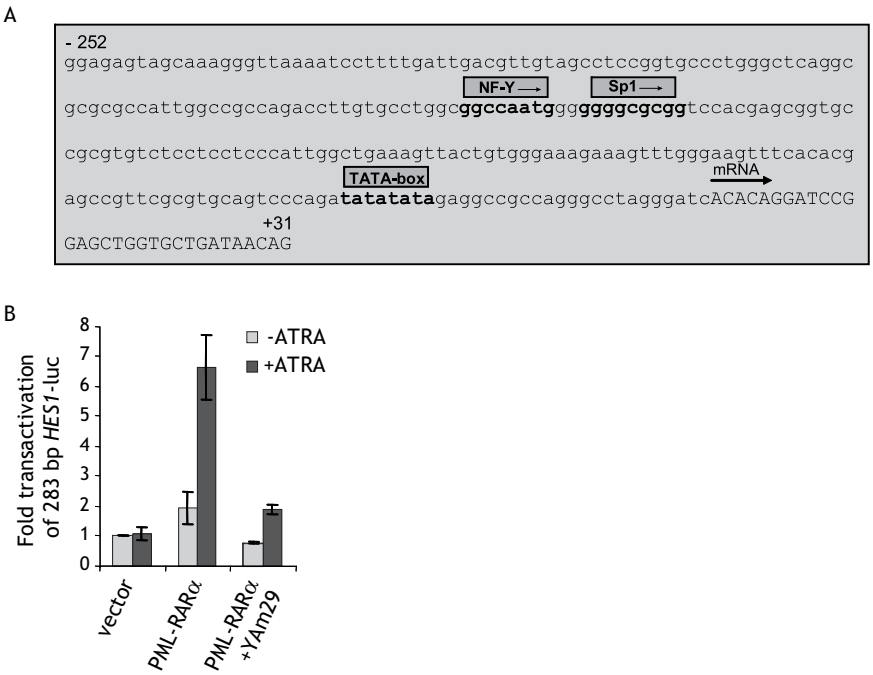

Figure 5. Functional NF-YA is necessary for PML-RARα-dependent transactivation of the *HES1* promoter. (**A**) The 283 bp promoter fragment of the *HES1* gene showing the consensus NF-Y and Sp1 binding sites. (**B**) Hep3B cells were transfected with a *HES1* promoter-luciferase reporter construct (pGL3-*HES1*), a control vector expressing Renilla-luciferase and vectors coding for the various proteins indicated in the figure. Background luminescence of the cells transfected with only the reporter construct in the absence of ATRA was set to 1. Transactivation was corrected for transfection efficiency measured by Renilla activity. Cells were cultured with (dark gray bars) or without (light gray bars) ATRA. Mean values and standard deviations from three independent experiments are shown.

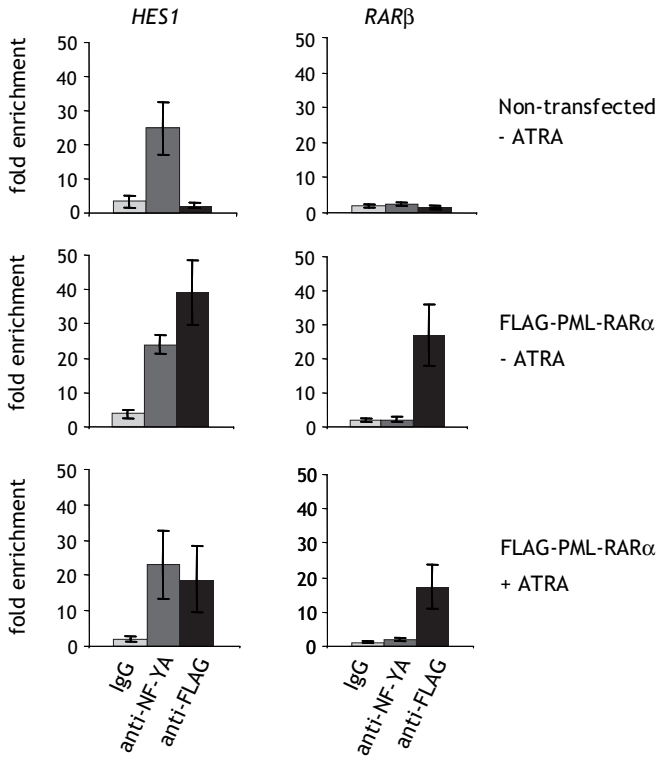

Figure 6. PML-RARα and NF-YA bind to the *HES1* promoter. ChIP assays were performed both for the *HES1* promoter and for the *RAR*β promoter. Sonicated chromatin of HEK 293 cells transfected with FLAG-PML-RARα or of non-transfected HEK 293 cells were used for ChIP analysis using antibodies against NF-YA or FLAG or non-specific IgG as a control (n=4). Where indicated, cells were cultured in the presence of ATRA. Quantitative PCR was used to measure the amount of immunoprecipitated DNA of the *HES1* and *RAR*β promoter, which was compared to the control genomic sequences of ALBUMIN. The recovery of *HES1* and *RAR*β was plotted as the fold enrichment over the recovery of *ALBUMIN*.

Since NF-YA is necessary for PML-RARα-dependent transactivation of the 283 bp HES1 promoter fragment, we tested whether NF-YA is present on the endogenous *HES1* promoter. ChIP assays were performed as described using an anti-NF-YA antibody. We found a more than 7-fold enrichment of NF-YA on the *HES1* promoter compared to the control (Figure 6, upper row). No enrichment was found for the *RAR*β promoter, indicating that NF-YA was present on the *HES1* promoter but not on the *RAR*β promoter. The addition of ATRA did not influence the binding of NF-YA to the *HES1*

promoter (Figure 6, compare upper and middle row with bottom row). In summary, these data show that besides ID1 and ID2 another repressor of bHLH transcription factors, HES1, is a direct ATRA-responsive gene in APL cells. HES1 is transcriptionally regulated by PML-RARα in a NF-Y-dependent manner.

Discussion

PML-RARα has been shown to silence normal retinoic acid receptor target genes at physiological concentrations of retinoic acid and it has been postulated that repression of these genes contributes to malignant transformation. By restoring the expression of these RARα target genes using high concentrations of retinoic acid, the leukemic cells will be forced into terminal differentiation. In the present study we demonstrate that both protein and RNA expression of HES1 is transiently upregulated and subsequently downregulated during ATRA-induced granulocytic differentiation of APL cells. Transactivation studies revealed a lack of response of the HES1 promoter to wild type retinoic acid receptors whereas PML-RARα transactivated the promoter in a ligand-dependent manner. These results indicate that PML-RARα not only interferes with the transcriptional regulation of regular retinoic acid receptor target genes via a consensus RARE, but also with the expression of an additional class of genes that are normally not regulated by retinoic acid receptors. We show that transactivation of the HES1 promoter by PML-RARα was independent of its DNA-binding domain, but that the coiled-coil domain of PML-RARα was important for transactivation. Furthermore, transactivation of HES1 by PML-RARα also required the presence of a functional NF-Y transcription factor complex. ChIP experiments revealed that both NF-YA and PML-RARα are associated with the HES1 promoter. Previously, we proposed a model in which PML-RARα binds to a DNA-bound Sp1-NF-Y complex, rendering the expression of the genes *ID1* and *ID2* sensitive to ATRA, thereby defining a novel gain-of-function for the fusion protein. Here we suggest that apart from *ID1* and *ID2*, *HES1* may be transcriptionally regulated by PML-RARα via a similar mechanism.

As indicated above, gene silencing mediated by PML-RARα has been implicated in leukemogenesis. Remarkably, unliganded PML-RARα upregulated the expression of HES1. Expression of PML-RARα both in U937 cells and in Heb3B cells resulted in slightly enhanced HES1 expression in the absence of ligand. Also in primary leukemic cells a

somewhat higher expression of HES1 was observed in APL patient cells compared to leukemic cells from patients with other FAB-subtypes lacking PML-RARα. These data are in line with a study by Alcalay et al. in which the mRNA expression level of HES1 in normal $CD34^+$ cells was compared with the level of expression in blasts bearing the t(15;17).[32] They found higher HES1 expression levels in the APL cells. These data indicate that PML-RARα does not target HES1 gene expression in a dominant-negative manner.

HES1 is an important gene functioning in stem cell biology by keeping hematopoietic cells in a proliferative and undifferentiated state. Overexpression of HES1 in murine early hematopoietic precursor cells caused suppression of the development of B-lineage cells *in vivo*. The myeloid compartment in these mice showed no abnormalities *in vivo*, but myeloid clonogenic assays revealed that HES1 expressing cells remained immature and retained proliferative capability *ex vivo*.[33] Furthermore, it has been demonstrated that murine Hes1 plays a role in the expansion of immature thymocytes.[34;35] In line with the effects of HES1 overexpression in murine bone marrow cells,[33] overexpression of HES1 in human $CD34^+$ cord blood cells inhibited B-cell development. In addition, when HES1 was overexpressed in $CD34^+$ cells they remained more immature than vector transduced controls.[36] Furthermore, preservation of long-term reconstituting activity of HES1-transduced hematopoietic stem cells (HSCs) *in vitro* was demonstrated in several reports.[37-39] As HES1 expression has been implicated in a block in differentiation it is tempting to speculate that the slight induction mediated by PML-RARα in the absence of ligand contributes to leukemogenesis. Because HES1 expression also correlates with proliferation, the further transient HES1 induction, observed after stimulation with ATRA, might be involved in the transient leukocytosis, which occurs in APL patients treated with ATRA.[40;41] Further studies are required to test this hypothesis. After 24 hours of ATRA stimulation a significant downmodulation of HES1 was observed. The shutdown of HES1 may be necessary to allow terminal granulocytic differentiation of the cells.

HES1 is known as a target gene of Notch signaling and by targeting Notch1, many of the HES1 phenotypes, induced by overexpression or knockdown, can be mimicked. Notch signaling is involved in many cellular processes, including the maintenance of stem cells, cell fate determination, differentiation, proliferation and apoptosis. Its role in these processes is very much cell type dependent. For example, Notch signaling induces HSCs to maintain their undifferentiated state, while Notch signaling directs

a common lymphoid progenitor towards T-lineage cells at the expense of B-lineage cells. Activating mutations in the *Notch1* gene have been involved in tumorigenesis, which has been illustrated by the development of T-cell leukemias.[42-44] Alcalay et al. reported that expression of PML-RARα resulted in activation of Notch signaling through Jagged1, which functions as a ligand for the Notch receptor, thereby activating expression of Notch target genes, including HES1.[32] Here we demonstrate that PML-RARα directly activates expression of HES1 by binding to its promoter region. The effects of Notch activation on ATRA-induced differentiation of NB4 cells has been investigated and revealed a reduction of ATRA-induced apoptosis and a partial alteration of ATRA-induced neutrophilic differentiation into monocytic.[45] Further studies using overexpression and knockdown of HES1 should help to clarify the exact role of HES1 in APL.

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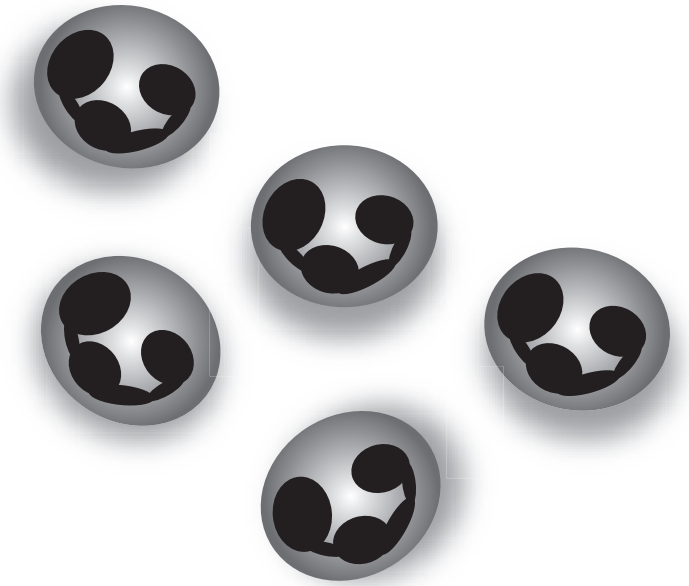
Chapter 5

The helix-loop-helix protein ID2 interacts with the transcriptional repressor MBD1



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Abstract

Inhibitor of DNA binding 2 (ID2) belongs to the family of helix-loop-helix (HLH) proteins and is involved in cell lineage commitment and proliferation- differentiation control in many mammalian tissues. ID proteins inhibit DNA binding of basic-HLH transcription factors by forming heterodimeric complexes. Previously, we demonstrated that ID2 is upregulated during retinoic acid induced granulocytic differentiation of acute promyelocytic leukemia (APL) cells and that forced expression of ID2 is involved in cell cycle arrest of APL cells. Since myeloid-specific binding partners of ID proteins have not been identified yet, we performed a yeast two-hybrid assay with ID2 and a bone marrow derived hematopoietic cDNA library to search for unknown ID2-interacting proteins. Here, we present all the known and novel ID2-interacting proteins we identified in this assay. One of the novel identified ID2-interacting proteins is methyl-CpG binding protein 1 (MBD1). We confirmed the interaction between ID2 and MBD1 using co-immunoprecipitation studies.

Introduction

ID2 is a member of the helix-loop-helix (HLH) family of proteins which is involved in many developmental processes, including hematopoiesis.[1] ID2 is essential for the generation of peripheral lymph nodes and Peyer's patches, for the differentiation of natural killer cells, and for the formation of Langerhans cells and splenic CD8α^+ dendritic cells. Furthermore, the development of nasopharyngeal-associated lymphoid tissue is defective in ID2 deficient mice.[2-4] Overexpression studies in chicken embryos implicated ID2 in the promotion of ectodermal cells towards neural rather than epidermal lineages.[5] The involvement of ID2 in cellular proliferation and differentiation depends on the specific cell lineages and developmental stages. ID2 expression levels increase during terminal differentiation of myeloid lineages,[6-8] during development of Langerhans cells[3] and following differentiation of mammary epithelial cells.[9] In B- and T-cell development and oligodendrocyte development the expression of ID2 is correlated with proliferation and decreases during differentiation.[10-12] ID2 may play a role in tumorigenesis as mRNA and protein levels of ID2 are elevated in many different tumour cell types.[13] ID2 is a direct target of Myc family members. Activation of N-Myc and c-Myc (in neuroblastoma and Ewing sarcoma) results in increased ID2 expression levels.[14-18] Furthermore, ID2 is a downstream target of the retinoblastoma (Rb) protein. By keeping control of ID2, Rb might prevent ID2-mediated inhibition of transcription factors that control normal development.[19;20] Overexpression of ID2 in thymocytes leads to development of lymphomas, suggesting a role for ID2 in transformation.[10] Interestingly, a tumour-inhibitory effect of ID2 in the intestinal epithelium was reported, since mice that lack ID2 develop intestinal tumours.[21]

Previously, we identified ID2 as a direct retinoic acid responsive gene in acute promyelocytic leukemia (APL) cells and we demonstrated that ectopic expression of ID2 in APL cells inhibits proliferation and induces cell cycle arrest. We suggested that the inhibition of specific bHLH transcription factor complexes by ID2 may play a role in the therapeutic effect of retinoic acid in APL. In addition, Buitenhuis et al. demonstrated that expression of ID2 in CD34$^+$ cells accelerated the definitive maturation of myeloid cells, indicating a more general role for ID2 in the regulation of myelopoiesis.[22] Since myeloid-specific binding partners of ID2 have not been identified yet, we performed a yeast two-hybrid assay to search for ID2-interacting proteins.

Materials and methods

Cell Culture

COS-1 cells were cultured in IMDM (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum and cultures were incubated at 37˚C in a 5% CO_2 completely humidified atmosphere.

Plasmids

The bait vector pGBDK-ID2 used in the yeast two-hybrid analysis was constructed by cloning the human ID2 cDNA sequence into the BamH1 site of the pGBDK-c1 vector. For the co-immunoprecipitation assay, the HA-tagged MBD1 cDNA insert was isolated from the pACT2 vector by BglII and cloned into the BamH1 site of a pCMV expression vector generating pCMV-HA-MBD1. pCMV-ID1 and pCMV-ID2, used in the co-immunoprecipitation assays, were constructed by cloning their human cDNA sequences into a pCMV expression vector.

Yeast two-hybrid analysis

For yeast two-hybrid analysis the yeast strain AH109 (Clontech, Palo Alto, CA, USA) transformed with the pGBDK-ID2 vector and a MATCHMAKER bone marrow derived hematopoietic cDNA library cloned in the pACT2 vector and pre-transformed in the yeast strain Y187 (Clontech) were used. Library screening and identification of genes coding for interacting proteins were performed as described in the 'Pre-transformed MATCHMAKER Libraries User Manual' from Clontech.

Co-immunoprecipitation and Immunoblotting assays

For co-immunoprecipitations, COS-1 cells were cultured in T75 flasks and transiently transfected with pCMV-HA-MBD1 and pCMV-ID2 or pCMV-ID1 using the calcium phosphate precipitation method. Cells were lysed 48 hours after transfection in TNI-buffer containing 0.1% Nonidet P-40 (NP-40), 50 mM Tris-HCl pH 8.0, 150 mM NaCl and protease inhibitor cocktail (Roche, Basel, Switzerland) and lysates were incubated with HA-antibody (12CA5, Roche) and Prot-G Sepharose beads (Amersham, Buckinghamshire, UK) for 4 hours at 4˚C with gentle rotation. Beads were washed 3 times with TNI-buffer. Bound proteins were eluted in loading buffer by heating for 3 min at 100˚C, separated on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and electrophoretically transferred onto polyvinylidenefluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Blots were probed with antibodies to ID1 (Z-8, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ID2 (C-20, Santa Cruz Biotechnology) and HA (12CA5, Roche).

Results and Discussion

Identification of ID2-interacting proteins

To understand the function of ID2 during myelopoiesis, we searched for ID2-interacting proteins using a yeast two-hybrid assay. For this experiment an ID2 construct, containing the entire human ID2 coding sequence, was screened against a bone marrow derived cDNA library. Plasmid DNA was isolated from more than 90 positive clones and all inserts were sequenced. We found several known ID2-interacting proteins, but in addition we identified putative novel ID2-interacting proteins (Table 1).

The generally accepted role of all four members of the ID family (ID1-4) is to inhibit basic-HLH (bHLH) transcription factors from forming functional homo- or heterodimers, thereby inhibiting transcriptional activation of bHLH target genes. ID proteins lack a basic DNA-binding domain and their dominant-negative function on bHLH transcription factors involves protein-protein interactions mediated via their HLH-domain. In many different cell types bHLH transcription factors activate genes that are necessary for diffentiation. Of the known ID2-interacting proteins, TCF3 (E2A), a widely expressed bHLH transcription factor, was found at high frequency (> 50%) in this yeast two-hybrid screening. In addition, the muscle-specific bHLH transcription factors MYOD and MYF6 were found.

The specificity and functional activity of the individual ID proteins is determined by their expression level and their cytoplasmic or nuclear localization.[23-25] In addition, posttranslational modifications may alter the activity of the proteins.[26;27] It has been described that ID2 is able to form homodimers and heterodimers with ID1.[28;29] We show that ID2 is able to bind to all other ID family members suggesting that the inhibitory function of the individual ID proteins may also be determined by mutual interactions, apart from posttranslational modifications or cellular localization.

The effects of ID2 are not only mediated via binding to members of the HLH family of proteins, as ID2 is also found to interact with non-HLH proteins. ID2 has been demonstrated to interact with non-HLH proteins like retinoblastoma,[30;31] Ets-family proteins,[32] Pax,[33] and PC2,[34] upon which the activity of these proteins is inhibited. We show that ID2 is able to interact with COPS5. An interaction between COPS5 and ID1 and ID3 has been decribed by Berse et al..[35] COPS5 is a subunit of a multifunctional protein complex called the COP9 signalosome (CSN). CSN is highly homologous to the 19S regulatory subunit of the 26S proteasome, and is postulated to play a role in

protein degradation.[36] It has been demonstrated that ID1 and ID3 are degraded in a CSN regulated manner. The interaction of ID2 with COP5S may suggest a similar mechanism for ID2.

ID2 preys	Accession number	Gene description	#
Known			
TCF3	NM_003200	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	48
ID1	NM_002165	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	5
ID2	NM_002166	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	1
ID3	NM_002167	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1
ID4	NM_001546	Inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	2
MYF6	NM_002469	Myogenic factor 6 (herculin)	4
MYOD1	NM_002478	Myogenic differentiation 1	2
Novel			
COPS5	NM_004236	COP9 constitutive photomorphogenic homolog subunit 5 (Arabidopsis)	2
DEFA1	NM_004084	Defensin, alpha 1	1
EEF1A1	NM_001402	Eukaryotic translation elongation factor 1 alpha 1	1
EEF1G	NM_001404	Eukaryotic translation elongation factor 1 gamma	1
EFEMP1	NM_018894	EGF-containing fibulin-like extracellular matrix protein 1	1
ELA2	NM_001972	Elastase 2, neutrophil	1
ERAF	NM_016633	Erythroid associated factor	1
FSCN1	NM_003088	Fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)	1
GADD45B	NM_015675	Growth arrest and DNA-damage-inducible, beta	1
KIF22	NM_007317	Kinesin family member 22	1
LGALS1	NM_002305	Lectin, galactoside-binding, soluble, 1 (galectin 1)	1
LRRC14	NM_014665	Leucine rich repeat containing 14	1
MBD1	NM_015846	Methyl-CpG binding domain protein 1	1
MYL1	NM_079420	Myosin, light polypeptide 1, alkali; skeletal, fast	1
MYLPF	NM_013292	Fast skeletal myosin light chain 2	1
NCF1	NM_000265	Neutrophil cytosolic factor 1 (chronic granulomatous disease, autosomal 1)	1
PKM2	NM_002654	Pyruvate kinase, muscle	1
PPP3CC	NM_005605	Protein phosphatase 3, catalytic subunit, gamma isoform (calcineurin A gamma)	1
PSMA4	NM_002789	Proteasome (prosome, macropain) subunit, alpha type, 4	2
PX19	NM_013237	Px19-like protein	1
RHAG	NM_000324	Rh-associated glycoprotein	1
RPL4	NM_000968	Ribosomal protein L4	1
RPL24	NM_000986	Ribosomal protein L24	1
TMSB4X	NM_021109	Thymosin, beta 4, X-linked	3
VAMP5	NM_006634	Vesicle-associated membrane protein 5 (myobrevin)	1
VPS28	NM_016208	Vacuolar protein sorting 28 homolog (S. cerevisiae)	1

Table 1. Known and novel ID2-interacting proteins as identified by yeast two-hybrid screening. Summary of cDNAs isolated in a yeast two-hybrid screen using ID2 as bait and a human bone marrow derived hematopoietic cDNA library as prey. # indicates the number of found clones.

To our knowledge, the other proteins that are listed in Table 1 have not been associated with ID proteins before. As we are interested in ID2-interacting proteins that are involved in proliferation and differentiation of hematopoietic cells we analysed the function of the putative novel ID2-interacting proteins. One potentially interesting protein partner that may be involved in the regulation of hematopoiesis, is methyl-CpG-binding protein 1 (MBD1). MBD1 is involved in transcriptional repression in association with DNA methylation.[37;38] Methylation of DNA, which occurs at cytosine residues in CpG dinucleotides, is an important epigenetic modification to modulate the expression of many genes. Most of the CpG dinucleotides throughout the genome are methylated in normal cells, however, CpG-rich areas (CpG islands) that are located in promoter regions of many genes, may be protected from methylation resulting in transcriptional activation. Altered methylation patterns of CpG islands have been associated with cancer cells.

ID2 interacts with MBD1 in mammalian cells

In this yeast two-hybrid screening we identified MBD1 as a putative ID2-interacting protein. A ~3000bp insert containing human full-length MBD1 (variant 1; capable of repressing transcription from both methylated and unmethylated promoters) was isolated from one of the positive clones. Because yeast two-hybrid assays can produce false positive results, we tested the interaction by co-immunoprecipitation analysis. HA-tagged MBD1, isolated from the yeast expression vector, was cloned into a mammalian expression vector and transfected into COS-1 cells together with a mammalian expression vector that contains the human full-length ID2 coding sequence. When we immunoprecipitated MBD1 with an HA-antibody (Figure 1A), we were able to co-immunoprecipitate ID2 (Figure 1B), indicating that also in mammalian cells these two proteins interact with each other. As ID2 is coexpressed with ID1 in myeloid cells,[8] and since both proteins show high sequence homology and share several common binding partners, we tested whether ID1 could interact with MBD1 as well. Co-immunoprecipitation analysis with HA-tagged MBD1 and ID1 did not show an interaction between these two proteins (Figure 1C and 1D).

In conclusion, in the present study we show that ID2 interacts with MBD1. The upregulation of ID2 during retinoic acid induced terminal granulocytic differentiation of APL cells suggests a role for ID2 in this process. We hypothesize that the binding of ID2 to MBD1 may alter the function of MBD1, which contributes to the (re)expression of genes that were silenced by MBD1.

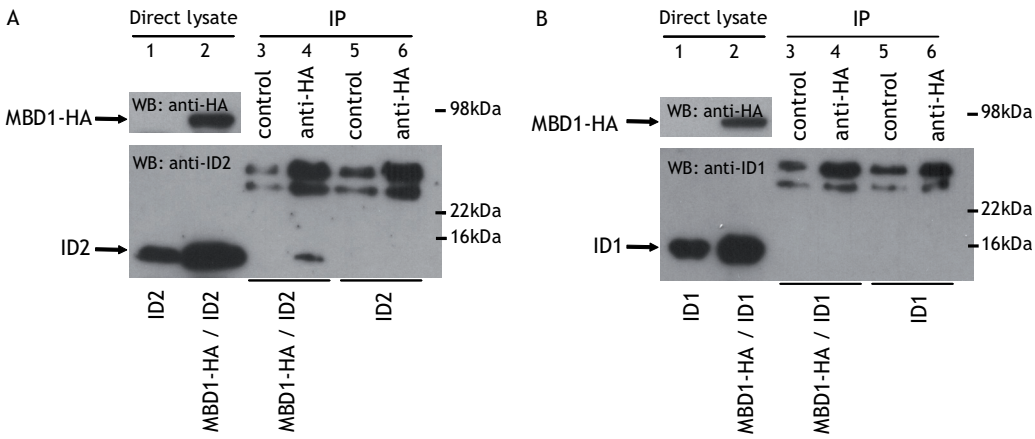

Figure 1. ID2 interacts with MBD1 in mammalian cells. (**A**) COS-1 cells were transfected with ID2 alone or in combination with HA-tagged MBD1 (HA-MBD1) and immunoprecipitated (IP) with anti-HA antibody and Prot-G Sepharose beads (anti-HA), or, as a control, with Prot-G Sepharose beads alone (control). Equal amounts of total cell lysates from transfected COS-1 cells were subjected to immunoblot analysis using anti-HA antibody (upper panel; lane 1 and 2) or using anti-ID2 antibody (lower panel; lane 1 and 2). ID2 was detected when co-transfecting HA-MBD1 and ID2 followed by immunoprecipitation using anti-HA antibody and immunoblot analysis using anti-ID2 antibody (lane 4). No ID2 was co-immunoprecipitated in the control experiments (lane 3 and 5) or when cells were transfected with ID2 alone (lane 6). (**B**) COS-1 cells were transfected with ID1 alone or in combination with HA-MBD1 and immunoprecipitated with anti-HA antibody and Prot-G Sepharose beads (anti-HA), or, as a control, with Prot-G Sepharose beads alone (control). Equal amounts of total cell lysates from transfected COS-1 cells were subjected to immunoblot analysis using anti-HA antibody (upper panel; lane 1 and 2) or using anti-ID1 antibody (lower panel; lane 1 and 2). ID1 was neither detected when co-transfecting HA-MBD1 and ID1 followed by immunoprecipitation using anti-HA antibody and immunoblot analysis using anti-ID1 antibody (lane 4) nor in the control experiments (lane 3, 5 and 6). The molecular weight (kDa) marker is indicated at the right.

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Chapter 6

Direct regulation of miR-132 and miR-212 by the PML-RARα oncogene

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Abstract

The oncogenic fusion protein PML-RARα plays a crucial role in the development of acute promyelocytic leukemia (APL). By interfering with the expression of retinoic acid receptor target genes, PML-RARα blocks the differentiation of the malignant cells. Treatment of APL with high doses of retinoic acid activates a transcriptional program resulting in terminal differentiation of the malignant blasts. Several genes that are important for the regulation of myeloid differentiation control were shown to be directly regulated by PML-RARα. We have analyzed the expression of mature microRNAs (miRNAs) before and after induction of differentiation in APL cells. We show that several miRNAs are significantly differentially expressed. To investigate whether the PML-RARα fusion protein might be directly involved in miRNA (de) regulation, we performed chromatin immunoprecipitation assays (CHIP). We found that two miRNAs (miR-132 and miR-212) are directly targeted by the PML-RARα fusion protein. As single miRNAs may regulate the expression of multiple target genes, direct deregulation of miRNAs by the PML-RARα fusion protein potentially deregulates multiple biological pathways, which may contribute to the transforming properties of the fusion protein.

Introduction

MicroRNAs (miRNAs) are small, non-coding, single stranded RNAs that have recently been uncovered as an important new class of gene regulatory molecules. Mature miRNAs are approximately 22 nucleotides in length and are processed from longer primary miRNA transcripts (pri-miRNAs) that are transcribed by RNA Polymerase II. Two RNase III enzymes, Drosha and Dicer process the mature miRNA from these pri-miRNA transcripts. As a final step towards functional activity, miRNAs are incorporated into a RNA induced silencing complex (RISC). The miRNA within a RISC complex binds its target mRNAs, which are then either degraded or translationally repressed.[1;2] It has been predicted that miRNAs regulate the expression of many human genes.[3] Since the sequence complementarity with their target mRNAs allows mismatches, one miRNA may regulate multiple genes and, vice versa, one gene may be targeted by several miRNAs.[4] This property makes it difficult to determine which mRNAs are targeted by a particular miRNA. Progress towards the unraveling of miRNA involvement in specific biological processes has been made by the identification of tissue- and cell type-specific miRNA expression patterns.[5;6] Strong evidence exists for a role of miRNAs during hematopoiesis and the functional involvement of several miRNAs during hematopoietic differentiation has been experimentally confirmed. The murine miRNAs miR-142s, miR-181a and miR-223 were the first to be identified as preferentially expressed in hematopoietic tissues, with miR-142s being expressed in B- and myeloid lineages, preferential expression of miR-181a in the B-lineage, and miR-223 preferentially expressed in myeloid lineages. When ectopically overexpressed in hematopoietic precursor cells, miR-181a induced an increase of B-lineage cells. Ectopic expression of miR-142s or miR-223 showed an increase in T-lineage cells.[7] Another miRNA that has been implicated in hematopoiesis is miR-155. MiR-155 negatively regulates normal myelopoiesis and erythropoiesis.[8] In addition, enhanced expression of miR-155 is associated with activation of B- and T-cells (reviewed in Kluiver et al.[9]). MiR-221 and miR-222 have been demonstrated to play a role in erythrocyte differentiation.[10] Downregulation of both miRNAs is correlated with increased expression of the KIT receptor, which is a key factor to regulate hematopoietic progenitor cell and erythropoietic cell proliferation.

Alterations in miRNA expression patterns may be involved in hematological malignancies. For example, downregulation of the miR-15a/miR-16-1 cluster

due to deletion of 13q14 has been shown in the majority of chronic lymphocytic leukemia (CLL) cases, indicating that these miRNAs might serve as possible tumour suppressors.[11] The miR-17-92 cluster and miR-155 show overexpression in different types of lymphoma.[12;13] Furthermore, two case reports indicated that miRNAs might be involved in chromosomal translocations. First, in an acute lymphoblastic B-cell leukemia case, the pre-miRNA sequence of miR-125b-1 on 11q24 was inserted in the *IgH* gene locus. Second, a t(8;17) was observed in a patient with aggressive B-cell leukemia. The resulting fusion transcript contained part of the *MYC* gene, and the promoter, 5' portion and stem-loop sequences of miR-142 (originally termed *BCL-3*). Under the influence of the regulatory elements of miR-142, the translocated *MYC* gene was highly expressed.[14;15] Further work needs to be done to determine the contribution of both miRNAs in B-cell leukemia.

Leukemia is characterized by a differentiation block in hematopoietic progenitor cells that may be caused by a variety of mutations in factors that are required for normal hematopoiesis. Mutations that affect the retinoic acid receptor α (RARα) are characteristic for acute promyelocytic leukemia (APL). In 98% of the APL cases, a chromosomal translocation occurs involving chromosome 15 and 17, fusing the promyelocytic leukemia (*PML*) and the *RARA* genes. The resulting chimeric fusion protein, PML-RARα, has oncogenic potential and may interfere with the normal function of PML and RARα.[16] Pharmacological doses of the RARα ligand, all-*trans* retinoic acid (ATRA), overcomes the differentiation arrest of the leukemic blasts and activates granulocytic differentiation. To date, PML-RARα is known to be involved in the deregulation of several important RARα target genes in APL cells.[17-21] Recently, human miR-223 has been demonstrated to be specifically expressed during granulopoiesis, by studying retinoic acid induced myeloid differentiation in APL cells.[22] MiR-223 expression is positively regulated by binding of CCAAT/enhancer binding protein α (C/EBPα), and negatively regulated by binding of NFI-A to its promoter region. Binding competition between these two transcription factors determines the transcriptional activation of miR-223. C/EBPα directs myeloid precursor cells towards granulocytic maturation.[23;24] Fazi et al. demonstrated that miR-223 may control granulocytic differentiation by targeting the transcription factor NFI-A, thereby inducing sustained expression of miR-223.

In the present study, we investigated which miRNAs are expressed during APL cell differentiation and whether PML-RARα is directly involved in their transcriptional

regulation. We screened for the expression profile of 157 mature miRNAs during retinoic acid induced granulocytic maturation of APL cells, using a sensitive, quantitative PCR based method. We describe the direct involvement of PML-RARα in the transcription of several miRNAs.

Materials and Methods

Cell Culture

Leukemic cells were isolated from bone marrow of patients at diagnosis after informed consent. Mononuclear cells were isolated by means of a Ficoll-1077 (Amersham, Buckinghamshire, UK) gradient and cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 20% fetal calf serum (FCS). NB4, U937 and U937-PR9 (kindly provided by Prof. P.G. Pelicci) cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FCS. HEK 293 cells were cultured in DMEM (Life Technologies) supplemented with 10% FCS and 2 mM glutamine (Life Technologies). Cultures were incubated at 37°C in a 5% CO_2 completely humidified atmosphere. ATRA (Sigma, St Louis, MO, USA) was used at a concentration of 10^{-6} M, and where indicated 100 μM $ZnSO_4$ was added 16 hours prior to ATRA.

MiRNA reverse transcriptase reaction

RNA from cell lines and cultured primary APL patient cells were isolated using RNA-Bee (Tel-Test, Inc., Friendswood, TX, USA). Bone marrow mononuclear cells of AML patients were isolated by lysis of erythrocytes in 0.14 M NH_4Cl buffer for 30 min on ice, after which RNA was isolated using RNA-Bee (Tel-Test). Reverse transcriptase (RT) reactions contained 10 ng total RNA, 50 nM stem-loop RT primer (PN 4365381, Applied Biosystems, Foster City, CA, USA), 1x RT buffer, 1.0 mM dNTPs, 3.33 U/μl MultiScribe reverse transcriptase and 0.25 U/μl Rnase inhibitor (PN 4366597, Applied Biosystems). The 15 μl reactions were incubated in a Biozym PTC-100 Thermal Controller for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then kept at 4°C.

MiRNA PCR reaction

Quantitative PCR reactions contained 1.33 μl RT product, 1x TaqMan Universal PCR Master Mix (PN 4324018, Applied Biosystems), 0.2 μM TaqMan probe, 1.5 μM forward primer and 0.7 μM reverse primer (PN 4365381, Applied Biosystems). The 20 μl reactions were incubated in a 96-well plate for 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at

60°C. Quantitative PCR reactions were performed on the 7500 Real-Time PCR system (Applied Biosystems). Quantification of PCR product was based on the threshold cycle (Ct), which is defined as the fractional cycle number at which the amount of amplified target reached a fixed threshold. Let7a, a miRNA that is considered to be expressed in all kind of tissues, was used to correct for differences in the amount of total RNA added to a reaction and to compensate for different levels of inhibition during reverse transcription of RNA into cDNA and during PCR ($2^{Ct\ Let7a\text{-}Ct\ miR}$).

Chromatin immunoprecipitation (ChIP)

HEK 293 cells were transiently transfected with Flag-PML-RARα (kindly provided by A. Tomita[25] which was re-cloned into a pCMV expression vector) using the calcium phosphate precipitation method. ChIP assays were performed as described previously.[26] At 24 hours after transfection DNA-protein cross-linking was done for 30 min at room temperature by adding formaldehyde at a final concentration of 1% directly to the culture medium. Cross-linking was stopped by the addition of glycine to a final concentration of 125 mM for 5 min at room temperature. Cells were scraped and washed with ice-cold phosphate-buffered saline, buffer B (10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100, 20 mM HEPES [pH 7.6]), buffer C (1 mM EDTA, 0.5 mM EGTA, 0.15 M NaCl, 50 mM HEPES [pH 7.6]) and resuspended in incubation buffer (0.15% SDS, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6] and protease inhibitors) at $33x10^6$ cells/ml. Chromatin was sonicated using the Bioruptor (Cosmo Bio, Tokyo, Japan), at high intensity for 15 min with 0.5 min intervals. Insoluble material was removed by centrifugation at 4°C for 15 min. A total of 120 μl supernatant was incubated with 30 μl pre-coated protein A/G plus agarose beads 50% v/v (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 0.1% BSA, 36 μl 5x incubation buffer, protease inhibitors and 2-5 μg antibody (anti-FLAG (M2, Sigma) or non-specific IgG from human serum) and rocked at 4°C for 16 hours. Beads were harvested by centrifugation and washed twice with buffer 1 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES [pH 7.6]), once with buffer 2 (0.1% SDS, 0.1% NaDOC, 1% Triton X-100, 0.5 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES [pH 7.6]), once with buffer 3 (0.25 M LiCL, 0.5% NaDOC, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]), and twice with buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM HEPES [pH 7.6]). Chromatin antibody complexes were eluted by the addition of 1% SDS and 0.1 M NaHCO3 to the pellet and incubated for 20 min at room temperature. Cross-linking was reversed by the addition of NaCl (0.44 M final concentration) and incubation of the eluted samples for at least 4 hours at 65°C. DNA was recovered by phenol-chloroform-isoamylalcohol extraction followed by chloroform-isoamylalcohol extraction and precipitated

by the addition of 0.1 volume of 1 M sodium acetate (pH 5.2), and 2.5 volumes of ethanol. Precipitated DNA was dissolved in water, and input as well as immunoprecipitated DNA was analysed using quantitative PCR for genomic sequences of the indicated miRNA promoter regions. For calculation of the fold enrichment, quantitative PCR was performed for *ALBUMIN* genomic sequences before and after the immunoprecipitation. Quantitative PCR following ChIP was done using SYBR green PCR (Applied Biosystems). For genomic primer sequences used in the ChIP assay see Table 1. Quantitative PCR for the *ALBUMIN* genomic sequences was performed using probe ALB-VIC 5'-TGCTGAAACATTCACCTTCCATGCAGA-3' with the primers, sense 5'-TGAAACATACGTTCCCAAAGAGTTT-3', anti-sense 5'-CTCTCC-TTCTCAGAAAGTGTGCATAT-3'. Quantitative PCR reactions were performed on the ABI/PRISM 7700 Sequence Detection system (Applied Biosystems).

	Forward 5' → 3'	Reverse 5'→ 3'
miR-132 –0.9kb	CAGCACCTTCGCCTCACTTT	GAGGAGCATCGAGTGGAGGAC
miR-132 +0.3kb	CAGCCCGACCCCTTCTCT	CGTCCTGCGGAGTGCGTCT
miR-132 +1.1kb	CACCTGCCGAGGAGAAGAGAT	CCAACCTGGCATTATCAGCAA
miR-132 +1.6kb	TTCTTTCCCCTCTGACCACATT	GCTGTTGAGAGGTTCCTTGCTAA
miR-132 +5.0kb	AACTCCTGTTTAGCCCTCAGACATAG	TGCTGTGTCCCTGACCATAATTC
miR-135a (3) –0.3kb	GGTTTAGGAAAAGGGGAGCTTG	GATCATGCGAGGATCACCATAA
miR-135a (3) +0.15kb	GGAATAGAGGAACGGCTGTGTT	GGTTCTGACACTGAGCAA
miR-135a (3) +0.5kb	GCTTCTCCAGCCTCAGCTTG	GGGTGGGACAAACTTCAGCTCA
miR-135a (3) +2.2kb	CTGCATGACCAGGTGTTTTGC	CTCAGAGCCCATAAGGACAAACTG
miR-135a (3) +4.5kb	CACTCCTGATTTGAACCTAAACCTCTT	CCATTGACCTCCAGTCTTCTCTATTT
miR-135a (12) –2.7kb	GGGTTTGGTTAGCGTATAATCATTTT	GAGAGCCTTTCCGCCTAAATTG
miR-135a (12) –1.7kb	AAGCATGTGCCTCGACACTTT	AGCCTTGCATTTGCTCAGGTA
miR-135a (12) –0.3kb	GCTGAGTGTCTCTTGGTCTTGTTTC	TGACAGGAAGCACACTTGAATTG
miR-135a (12) –0.1kb	TGTGAAAAATCATCAACTAAGAAGG	CAGGCAGGTAGCAGAGCTATACAC
miR-223 –5.0kb	GACTACCCTACTTCCGCTCCAA	TCTACCAGAATAATTTATTGGCTCAGG
miR-223 –0.8kb	GAGACCACAAGAAATCAATGTCACA	TGCATGTAAAGCCTTTGGTTCA
miR-223 +1.0kb	TGGTCACACAAGGTTATACAGGTGTT	CTGTGGGGGAAAAGAAAATTCA

Table 1. Table showing genomic primer sequences used in the ChIP assay. The sequences of the forward and the reverse primers used for detection of specific genomic regions near the indicated miRNAs in the ChIP assay are presented in this table. For miR-135a, primers were designed for chromosome 3 (3) and 12 (12).

Results

MiRNA expression profile in NB4 cells

We measured the expression profile of 157 different mature miRNAs in the APL cell line NB4, before and after exposure to retinoic acid. For this, we used real-time quantification by stem-loop RT-PCR as described by Chen et al..[27] We found that more than 60% (101 out of 157) of the measured miRNAs were expressed in untreated NB4 cells, showing a broad range of expression levels (Supplemental S1). After stimulation of NB4 cells with ATRA, many different miRNA expression profiles were observed. We focussed on those miRNAs that showed a more than 10-fold up- or downregulation at 96 hours after ATRA stimulation. As shown in Figure 1, thirteen miRNAs were more than 10-fold upregulated at 96 hours after ATRA stimulation. No miRNAs were more then 10-fold downregulated. We confirmed upregulation of miR-223 (10-fold), which has already been reported, using Northern blot analysis, by Fazi et al..[22] Mir-132 and miR-135a were found to be most strongly induced by ATRA, 393-fold and 79-fold, respectively. These results show that ATRA is involved in the regulation of the expression of many miRNAs in NB4 cells, which suggests their involvement in granulocytic differentiation of leukemic blasts.

MiR-132 and miR-135a are upregulated by ATRA in APL patient cells

To confirm the expression patterns of miRNAs that showed a more than 10-fold induction in NB4 cells, we measured the miRNA expression level in primary APL patient cells. Bone marrow cells were isolated at diagnosis and cultured in the presence or absence of ATRA for several time intervals after which RNA was isolated. We confirmed the expression patterns of four miRNAs in primary APL patient cells. MiR-223 was slightly induced by ATRA in cells from patient 2, but no induction could be observed in cells from patient 1 (Figure 2A). MiR-326 showed elevated expression after culturing cells from patient 1 for 72 hours in the presence of ATRA (Figure 2B). Two miRNAs, miR-132 (Figure 2C) and miR-135a (Figure 2D), showed a clear and sustained ATRA-induced upregulation in both patient samples (see also Figure 2E).

Upregulation of miR-212 in NB4 cells

Interestingly, miR-132 is located 360 bp upstream of another miRNA, miR-212, suggesting that both miRNAs may be processed from the same pri-miRNA transcript.

As miR-212 was not included in the original panel of 157 Taqman miRNA assays, we tested whether miR-212 was also upregulated in NB4 cells following stimulation with ATRA. We found that miR-212 is induced to a similar extent and with similar kinetics as miR-132 (Figure 3A,B), suggesting that miR-212 and miR-132 are processed from a common pri-miRNA transcript.

When early time points were considered of miR-132, miR-135a, miR-212 and miR-223, we observed that the upregulation of miR-132, miR-212 and miR-223 was induced after more than 8 hours following ATRA stimulation (Figure 3A,B,C). This suggests that these miRNAs are not directly targeted by ATRA. For miR-223, this is supported by the report of Fazi et al., showing regulation of miR-223 by C/EBPα, a PML-RARα target gene. However, the upregulation of miR-135a was very fast with a more than 10-fold induction after 4 hours of ATRA stimulation, suggesting that miR-135a could be directly targeted by ATRA (Figure 3D).

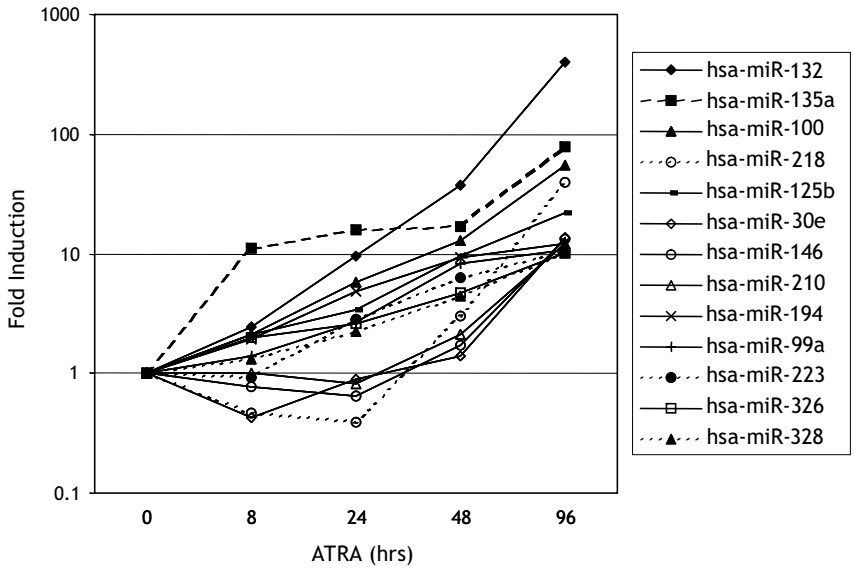
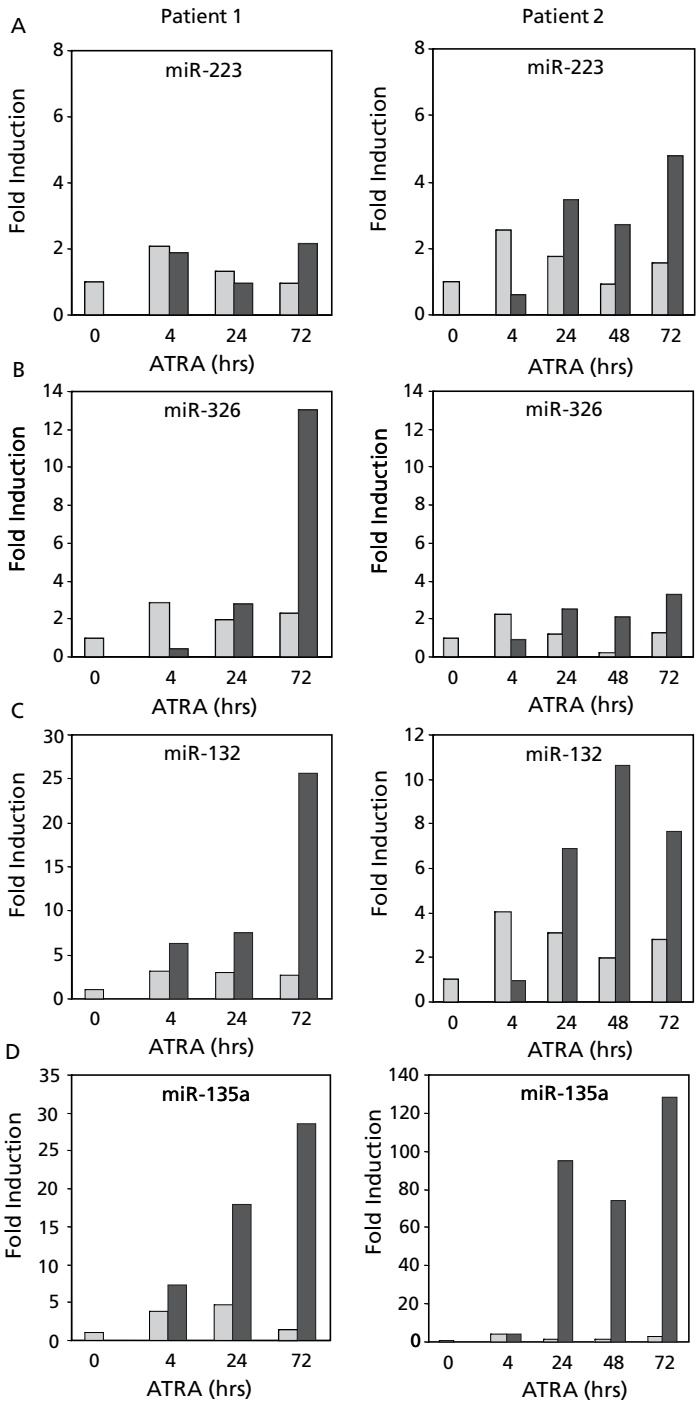


Figure 1. Top 13 ATRA-responsive miRNAs in NB4 cells. NB4 cells were cultured in the presence of 10^{-6} M ATRA for indicated time points after which RNA was isolated. These RNA samples were subjected to quantitative PCR for 157 different miRNAs. Presented are the more than 10-fold upregulated miRNAs, as measured by fold difference in expression level ($2^{\Delta Ct}$) between 0 and 96 hours of ATRA stimulation.


A
Patient 1
Patient 2
miR-223
Fold Induction
ATRA (hrs)
B
miR-326
C
miR-132
D
miR-135a

E

Patient 1	0	-ATRA (hrs) 72	+ ATRA (hrs) 72
miR-132	31,43	30,03	26,75
miR-135a	36,80	36,36	31,96
miR-223	21,36	21,42	20,24
miR-326	35,99	34,81	32,29

Patient 2	0	-ATRA (hrs) 72	+ ATRA (hrs) 72
miR-132	31,04	29,56	28,10
miR-135a	36,32	34,80	29,32
miR-223	22,43	21,79	20,17
miR-326	34,44	34,14	32,72

Figure 2. ATRA-induced miRNA expression in primary APL patient cells. Quantitative PCR for miR-223 (**A**), miR-326 (**B**), miR-132 (**C**) and miR-135a (**D**) was performed with total RNA from bone marrow derived mononuclear cells from two APL patients after *in vitro* exposure to 10^{-6} M ATRA for indicated time points (dark gray bars). As a control cells were cultured in the absence of ATRA (light gray bars). Plotted are the fold inductions compared to T=0 measured by $2^{\Delta Ct}$. (**E**) The Ct values, indicative for absolute expression, at T=0 and T=72 are presented in this table.

MiR-132, miR-135a, miR-212 and miR-223 are targets of PML-RARα

PML-RARα has been shown to act as a dominant oncogene, interfering with the normal function of the unrearranged RARα and PML proteins. We investigated whether miRNA expression may be (de)regulated by PML-RARα. To investigate this we used the parental U937 cell line and a U937 cell line stably transfected with a zinc-inducible PML-RARα expression cassette (U937-PR9). Both cell lines were stimulated with zinc, and the expression levels of miR-132, miR-135a, mir-212 and miR-223 were measured. Comparison of the expression levels in the parental U937 versus the U937-PR9 cell line after zinc-stimulation showed that expression of PML-RARα did not affect expression of miR-132 and miR-212 (Figure 4A,B). Expression of miR-223 was clearly downregulated upon expression of PML-RARα (Figure 4C). Compared to Let7a, miR-135a is hardly expressed in U937 cells, but in U937-PR9 cells we were able to detect low levels of miR-135a expression (Figure 4D).

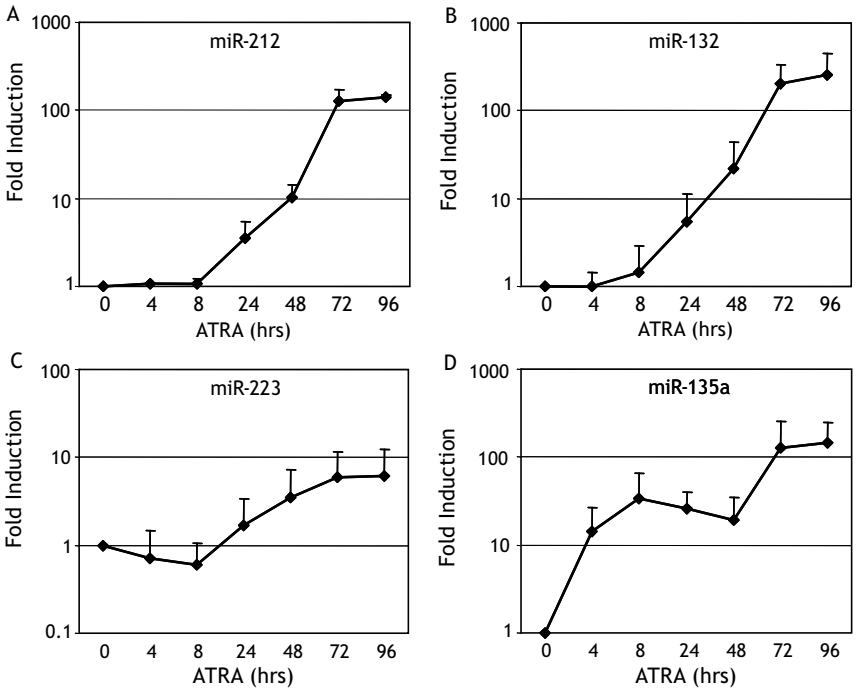


Figure 3. Kinetics of upregulation of miR-132, mir-135a, miR-212 and miR-223 in NB4 cells. Total RNA from non-stimulated and ATRA-stimulated NB4 cells (10^{-6} M ATRA was added for 0, 4, 8, 24, 48, 72, 96 hours) was used to perform quantitative PCR for miR-212 (**A**), miR-132 (**B**), miR-223 (**C**) and miR-135a (**D**) (n=2). The figure shows the mean fold inductions relative to non-stimulated NB4 cells.

To investigate more extensively whether expression of PML-RARα is directly associated with deregulation of miR-132, miR-135a, miR-212 and miR-223 expression, we measured the expression level of these miRNAs in different acute myeloid leukemia (AML) subtypes. APL accounts for 5-10% of all AMLs and is classified according to the French-American-British (FAB) classification system based on morphological criteria as AML-M3. Expression of the PML-RARα fusion protein is characteristic for APL. We analysed the expression level of miR-132, miR-135a, miR-212 and miR-223 in cells from patients diagnosed with AML subtypes M0 to M7, and we compared those with the level of expression in cells from patients diagnosed with AML-M3. No significant difference in expression was detected for miR-132, miR-135a and miR-212 (p-values

0.35, 0.23 and 0.18, respectively), indicating that unliganded PML-RARα does not affect the expression of these miRNAs (Figure 5A,B,C). These data suggest that leukemic transformation does not involve active downmodulation of these miRNAs by PML-RARα. However, a significant decrease in expression of miR-223 was observed in AML-M3 compared to AML M0 to M7 (p-value 0.001) (Figure 5D). These results are in line with the data from the U937 cells, indicating that PML-RARα may actively downmodulate the expression of miR-223.

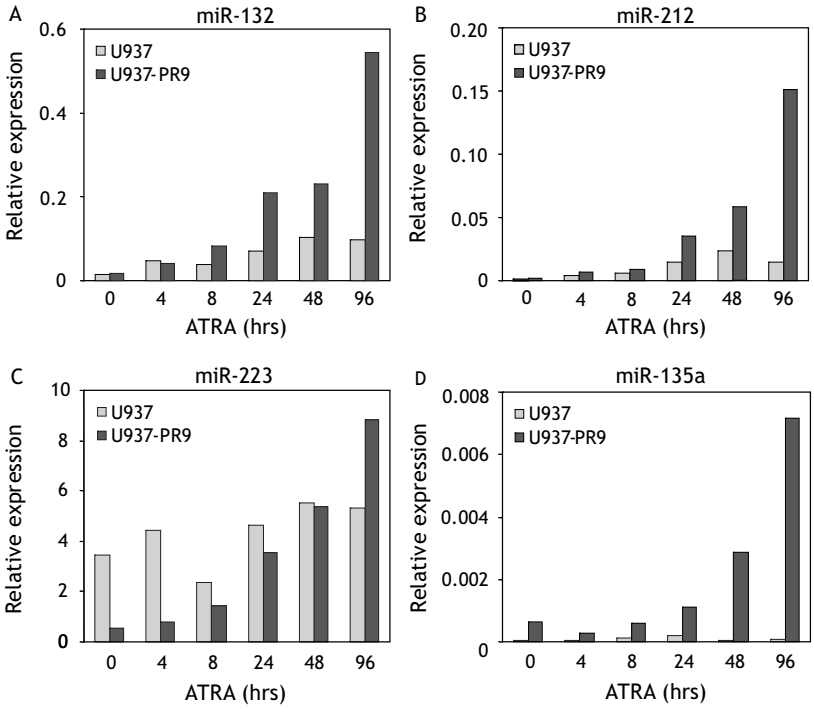


Figure 4. PML-RARα regulates expression of miR-132, miR-135a, miR-212 and miR-223 upon ATRA stimulation. Quantitative PCR for miR-132 (**A**), miR-212 (**B**), miR-223 (**C**) and miR-135a (**D**) was performed with total RNA extracted from U937 (light gray bars) and U937-PR9 (dark gray bars) cells. Cells were cultured in the presence of Zn^{2+} for 16 hours after which ATRA was added for indicated time points. Indicated is the relative expression compared to Let7a.

To study the effect of PML-RARα on miRNA expression during ATRA-induced differentiation, both the U937 and the U937-PR9 cell line were exposed to zinc followed by ATRA stimulation for several time intervals. Figure 4 shows that expression

of all four miRNAs was enhanced upon ATRA stimulation when PML-RARα was present, suggesting that PML-RARα is involved in the transcriptional regulation of miR-132, miR-135a, miR-212 and miR-223 during ATRA-induced differentiation.

PML-RARα binds to sequences near the miR-132 and miR-212 genes

To investigate whether PML-RARα binds in the vicinity of miRNA genes, we performed chromatin immunoprecipitation (ChIP) analysis. HEK 293 cells were transfected with FLAG-PML-RARα and immunoprecipitation was performed using chromatin from these cells and an antibody directed against FLAG or non-specific IgG as a control. The recovery of chromatin was measured by quantitative PCR using specific primer sets located at different positions near the miRNA genes (Figure 6A). In comparison, the recovery of a control region in an exon of the *ALBUMIN* gene was measured.

Two genes, located at chromosomes 3 and 12, code for an identical mature miR-135a. Since the quantitative PCR assay for detecting miRNA expression is based on the mature sequence it is impossible to discriminate between these two localisations. To study the presence of PML-RARα near the miR-135a genes we designed primer sets located in the vicinity of miR-135a sequences on both chromosomes. We were not able to detect binding of PML-RARα near mature miR-135a sequences located on chromosome 12 (Figure 6B). We do show some binding of PML-RARα in the vicinity of mature miR-135a sequences located on chromosome 3 (Figure 6C). However, we were not able to localize binding of PML-RARα to a specific region, as we detected binding of PML-RARα using primer sets spanning a region of ~5 kb. Whether or not PML-RARα directly binds to this gene remains unclear.

The upregulation of miR-223 has been described by Fazi et al..[22] They showed a direct involvement of the transcription factors C/EBPα and NFI-A in the transcriptional regulation of miR-223 and an indirect role for PML-RARα was proposed. The relatively late upregulation of miR-223 upon ATRA exposure suggests that this gene is not directly regulated by PML-RARα. In line with these observations, we could not show binding of PML-RARα to sequences near the miR-223 gene (Figure 6D). This suggests that the downregulation of miR-223 upon expression of PML-RARα both in the U937 cells (Figure 4C) and in primary AML patient cells (Figure 5D) is indirect.

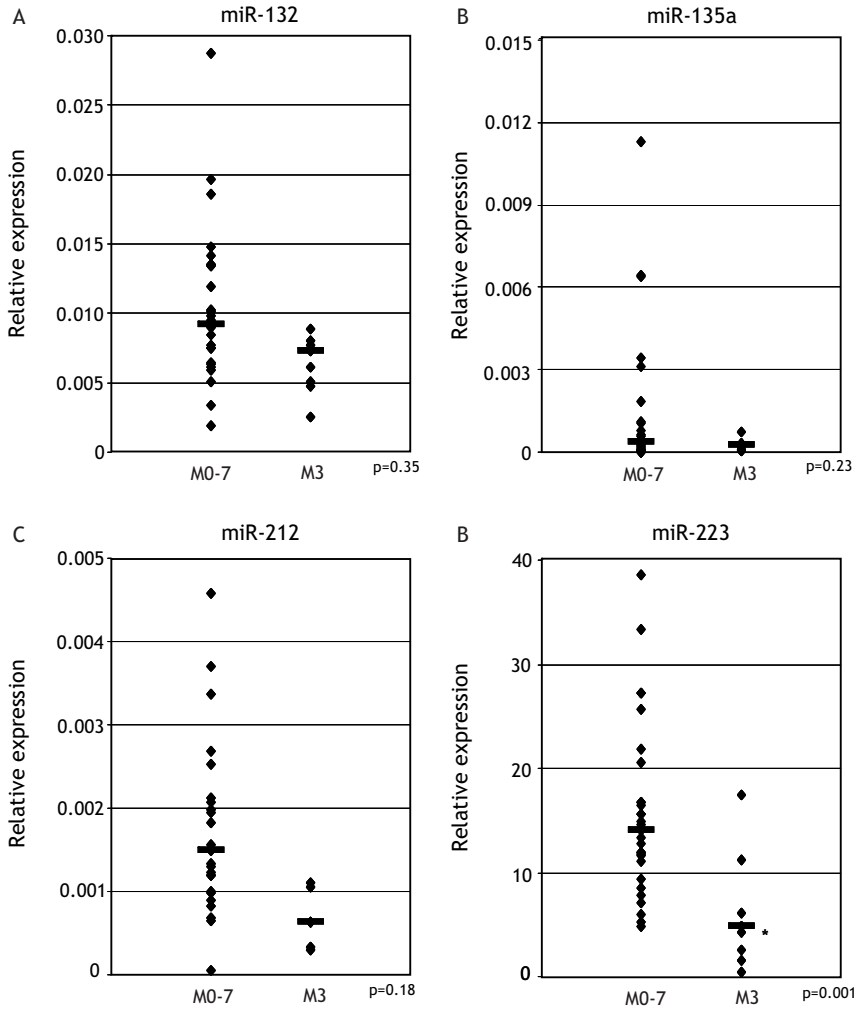


Figure 5. MiR-132, miR-135a, miR-212 and miR-223 expression in APL cells versus non-APL AML cells. RNA was isolated from bone marrow or peripheral blood mononuclear cells of newly diagnosed AML patients (M0 n=5 or 4, M1 n=6, M2 n=5 or 4, M3 n=9 or 7, M4 n=4 or 3, M5 n=4, M6 n=2 and M7 n=2). Quantitative PCR for miR-132 (**A**), miR-135a (**B**), miR-212 (**C**) and miR-223 (**D**) was performed and values were normalised for Let7a expression. Compared are the expression levels of miR-132, miR-135a, miR-212 and miR-223 in cells from AML-M3 patients (M3) versus cells from AML-M0 to M7 patients (M0-7). The black horizontal bar indicates the median. Statistically significant differences between the groups M0-7 and M3 (using Student's *t*-test, $P<0.05$) are indicated with an asterix.

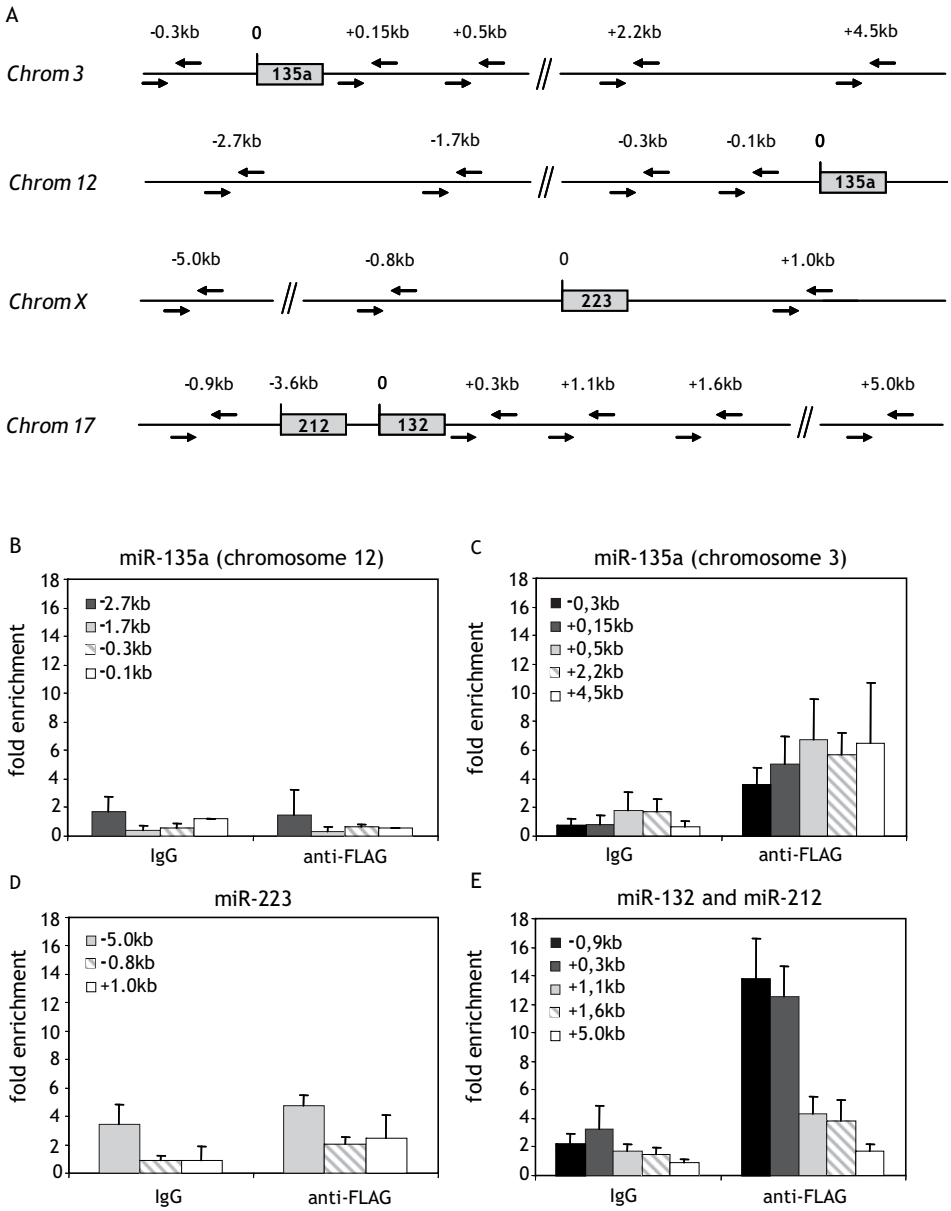


Figure 6. PML-RARαα binds to DNA sequences near the miR-132 and miR-212 genes. (**A**) Schematic representation of the location of the quantitative PCR primer sets (for sequences see Table 1) as used in the ChIP assay. Indicated at the left are the chromosomes on which the miRNAs are located. (**B** to **E**) Sonicated chromatin of HEK 293 cells overexpressing FLAG-PML-

RARα was used for ChIP analysis using anti-FLAG antibody or non-specific IgG as a control (n=3). Quantitative PCR was used to measure the amount of immunoprecipitated genomic region near the miRNA indicated, which was compared to the control genomic sequence of *ALBUMIN*. The legend in each figure indicates the primer sets used (see also Table 1 and Figure 6A). The percentage of recovery of the indicated genomic region near the miRNA was plotted as the fold enrichment over the percentage of recovery of *ALBUMIN*.

In contrast, ChIP analysis revealed binding of PML-RARα to sequences near the miR-132 and miR-212 genes (Figure 6E). We observed binding of PML-RARα when using primer sets located 0.9 kb upstream and 0.3 kb downstream of mature miR-132 sequences. The ChIP was not positive when using primer sets located 1.1 kb, 1.6 kb and 5.0 kb downstream of mature miR-132 sequences. These data suggest that binding of PML-RARα localizes to a region upstream of the mature miR-132 sequence or at a distance less than ~1 kb downstream of the mature miR-132 sequence. The data strongly suggest that ATRA-induced upregulation of miR-132 and miR-212 is mediated by association of PML-RARα to the promoter region of these miRNAs.

Discussion

In this study we investigated the miRNA expression profile during ATRA-induced myeloid differentiation of APL cells. We found that many miRNAs are expressed in APL cells and that several miRNAs are strongly upregulated upon ATRA induction. This suggests that they contribute to the post-transcriptional regulation of gene expression in these cells. As each miRNA is believed to regulate multiple genes, multiple target genes may be affected by the upregulation of these miRNAs. Recently, Garzon et al. reported the expression profile of miRNAs during ATRA treatment of APL cells by using a miRNA microarray platform.[28] When comparing their and our data set, some differences can be observed which might be due to the different (hybridization) methods used.

MiRNAs can be located both in intergenic- as well as in intronic regions. When located in introns, miRNAs share their regulatory elements with their host genes. When located in intergenic regions, it is postulated that miRNAs have their own promoter regions. MiR-132, miR-135a, miR-212 and miR-223 are located in intergenic regions and have their own promoter region. Therefore, we studied whether these

miRNAs are directly regulated by PML-RARα. Using ChIP analysis we showed that PML-RARα binds to sequences near the miR-132 and miR-212 genes, suggesting that PML-RARα is directly involved in the transcriptional regulation of these miRNAs. The rapid upregulation of miR-135a in NB4 cells suggests that miR-135a is directly regulated by PML-RARα. However, we were not able to localize binding of PML-RARα to regions near the miR-135a genes. It remains to be clarified whether or not PML-RARα directly binds to this gene. Fazi et al. demonstrated that miR-223 is a direct target of the transcription factors C/EBPα and NFI-A, which in turn are regulated by PML-RARα. In line with these results, we could not show binding of PML-RARα to sequences near the miR-223 gene.

PML-RARα may silence normal retinoic acid receptor target genes at physiological concentrations of retinoic acid. It has been postulated that these target genes mediate PML-RARα-induced transformation. At high concentrations of ligand, PML-RARα (re)activates the transcription of genes, forcing the leukemic cells into terminal differentiation. We studied whether unliganded PML-RARα is able to deregulate expression of miR-132, miR-135a, miR-212 and miR-223 by investigating the miRNA expression levels in U937 cells with or without expression of PML-RARα and in various AML subtypes. Expression of PML-RARα in U937 cells downregulates miR-223 expression. In addition, a significantly lower expression level of miR-223 was observed when comparing cells from APL patients with cells from non-APL AML patients. In the absence of ligand, expression of PML-RARα does not modulate the expression of miR-132, miR-135a and miR-212, both in U937 cells as well as in primary AML patient cells. However, a clear PML-RARα dependent regulation was demonstrated following ATRA-induced differentiation. This suggests a role for miR-132, miR-135a and miR-212 in differentiation rather than in PML-RARα-mediated transformation. The reduced expression of miR-223 in the presence of PML-RARα may suggest that miR-223 is involved during transformation.

In summary, we identified miRNAs that are differentially regulated upon ATRA stimulation of APL cells. These observations suggest a potential role for these miRNAs in APL cell differentiation. Functional studies should reveal the biological relevance of the induction of miR-132, miR-135a and miR-212 expression during ATRA-induced granulocytic differentiation. Our study demonstrates for the first time a direct interference of an oncoprotein with miRNA expression.

Acknowledgements

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	ATRA (hrs)				
	0	8	24	48	96
let-7a	24,61	24,62	24,77	23,93	23,65
miR-16	21,59	21,86	21,34	20,81	20,50
miR-9	28,68	28,30	28,19	27,27	26,90
miR-9*	31,19	31,53	31,09	31,16	30,09
miR-10a	ND	ND	ND	ND	37,09
miR-15a	24,61	24,64	24,76	24,58	23,07
miR-15b	24,06	24,44	23,97	23,60	23,48
miR-17-3p	28,01	28,78	29,95	28,53	28,62
miR-17-5p	22,39	22,78	22,68	23,00	24,09
miR-19a	22,25	22,28	22,69	22,00	22,89
miR-20	23,27	23,19	23,53	23,92	24,17
miR-21	25,05	25,79	25,63	25,94	23,38
miR-23a	25,73	25,51	25,54	25,23	24,42
miR-23b	28,50	28,76	28,16	27,85	27,81
miR-25	23,22	23,59	23,62	23,32	23,21
miR-26a	26,09	26,04	25,90	24,34	23,52
miR-26b	24,96	25,23	25,21	25,08	24,51
miR-27a	27,43	26,99	27,53	27,95	27,20
miR-27b	28,68	28,23	28,79	28,66	28,13
miR-28	30,66	29,84	29,77	29,70	28,24
miR-29a	24,62	25,25	24,51	24,41	23,14
miR-29b	25,58	26,22	26,09	26,23	24,49
miR-29c	24,40	24,57	23,83	24,01	23,04
miR-30a-3p	30,49	30,95	30,44	30,50	29,67
miR-30b	24,85	25,02	24,96	24,77	23,68
miR-30c	24,43	24,48	24,49	24,05	23,32
miR-30d	27,77	27,88	27,68	26,94	26,23
miR-30e	28,91	30,12	29,06	28,41	25,13
miR-31	ND	ND	ND	ND	ND
miR-34a	36,50	34,62	35,23	35,38	36,71
miR-34b	ND	ND	ND	ND	ND
miR-34c	ND	ND	ND	ND	ND
miR-92	20,65	21,56	20,72	21,31	22,19
miR-95	ND	ND	ND	37,54	34,69
miR-96	30,31	32,02	32,02	32,65	33,27
miR-98	29,68	29,92	29,56	28,82	27,63
miR-99a	31,51	31,02	30,10	28,47	28,09
miR-100	33,20	32,14	30,68	29,51	27,41
miR-103	23,92	23,92	23,41	23,46	21,89
miR-104	37,48	38,82	ND	39,10	38,61

Supplemental S1. MiRNA expression profile in NB4 cells. NB4 cells were cultured in the presence of 10^{-6} M ATRA for indicated time points after which RNA was isolated. These RNA samples were subjected to quantitative PCR for 157 different miRNAs. The level of miRNA expression is shown as Ct value, which is indicative for absolute expression. ND = not detectable.

	ATRA (hrs)				
	0	8	24	48	96
miR-105	36,08	39,78	ND	37,99	38,84
miR-106a	23,59	24,07	23,23	23,83	24,08
miR-107	29,54	31,80	28,68	29,83	27,12
miR-122a	ND	ND	ND	ND	ND
miR-124a	28,58	29,06	27,97	27,01	25,64
miR-124b	31,73	31,12	30,35	30,33	28,67
miR-125a	30,72	30,19	30,01	30,26	28,30
miR-125b	32,90	31,84	31,13	29,65	28,45
miR-126	ND	ND	ND	36,83	36,58
miR-127	38,35	37,72	38,74	39,62	38,69
miR-128a	30,97	31,25	30,86	31,86	30,53
miR-128b	32,57	32,80	32,65	33,37	32,29
miR-129	30,70	31,07	30,56	30,20	29,50
miR-130a	33,49	33,43	33,23	34,27	34,42
miR-130b	25,37	25,69	25,78	25,79	25,53
miR-132	32,53	31,23	29,26	27,30	23,91
miR-133a	36,24	36,06	34,52	34,74	36,42
miR-133b	37,55	33,50	34,84	36,16	35,38
miR-134	35,07	36,15	36,18	36,20	38,94
miR-135a	36,54	33,05	32,55	32,47	30,24
miR-135b	ND	ND	ND	ND	ND
miR-137	37,67	ND	ND	ND	38,08
miR-138	ND	ND	38,37	39,21	37,70
miR-139	35,17	35,31	34,05	35,13	34,52
miR-140	26,07	26,35	25,48	25,57	24,62
miR-141	34,02	33,55	32,59	32,35	31,19
miR-142-3p	21,11	21,10	22,25	21,25	21,07
miR-142-5p	25,50	25,89	25,01	26,92	25,08
miR-144	37,57	ND	ND	37,27	38,73
miR-145	29,56	28,09	27,97	27,26	27,19
miR-146	26,81	27,17	27,43	26,03	23,09
miR-147	35,73	38,11	37,53	37,54	39,32
miR-148a	27,01	31,12	27,01	29,14	26,53
miR-149	ND	ND	ND	ND	ND
miR-150	33,38	32,60	31,93	33,28	33,73
miR-151	ND	ND	ND	ND	ND
miR-152	34,18	34,20	32,85	32,53	31,46
miR-154	ND	ND	ND	ND	ND
miR-154*	ND	ND	ND	ND	ND
miR-155	27,37	27,72	28,19	29,39	28,80

	ATRA (hrs)				
	0	8	24	48	96
miR-181a	25,06	24,38	24,43	24,17	23,50
miR-181b	24,39	25,43	25,01	24,29	24,48
miR-181c	29,50	30,48	30,21	30,30	29,36
miR-182	28,51	28,45	29,11	29,93	30,16
miR-182*	35,51	36,10	35,12	36,11	36,69
miR-183	32,13	33,44	34,02	33,40	33,19
miR-184	37,66	ND	ND	35,94	ND
miR-185	31,85	30,87	31,34	31,12	29,67
miR-186	27,75	26,67	27,14	26,19	26,58
miR-187	38,78	36,20	34,45	36,71	33,03
miR-189	35,88	ND	38,38	35,98	36,13
miR-190	36,65	35,68	36,84	35,43	35,01
miR-191	23,13	23,19	22,60	22,49	21,97
miR-193	29,18	30,37	30,37	30,89	30,23
miR-194	31,36	30,41	29,08	28,14	27,76
miR-195	26,84	27,35	27,50	27,28	26,24
miR-197	27,98	27,39	27,39	26,69	26,26
miR-198	ND	ND	ND	ND	ND
miR-199a	35,65	35,48	35,72	36,35	36,39
miR-199a*	26,02	26,26	26,09	25,81	25,84
miR-199b	26,41	26,99	27,18	27,05	27,15
miR-199-s	32,90	33,44	32,17	32,39	32,35
miR-200a	ND	36,90	37,00	35,25	35,88
miR-200b	ND	ND	39,00	35,84	35,16
miR-200c	32,13	31,66	31,23	31,04	30,61
miR-203	ND	ND	37,34	ND	36,09
miR-204	26,75	26,80	27,39	27,23	27,59
miR-205	ND	ND	ND	ND	ND
miR-210	29,54	29,53	29,83	28,45	25,92
miR-211	ND	ND	ND	ND	ND
miR-213	28,65	28,86	28,79	28,55	28,23
miR-214	32,49	32,67	33,03	33,23	32,19
miR-215	32,40	33,00	32,64	31,43	29,92
miR-216	38,09	39,43	ND	ND	38,04
miR-218	37,08	38,16	38,44	35,47	31,78
miR-219	36,72	36,40	ND	36,53	35,33
miR-220	ND	ND	ND	ND	ND
miR-221	24,26	25,19	23,97	23,29	23,36
miR-222	24,39	24,58	24,23	23,44	23,35

	ATRA (hrs)				
	0	8	24	48	96
miR-223	22,42	22,54	20,90	19,78	19,02
miR-224	ND	ND	ND	ND	ND
miR-296	34,96		35,43	33,81	33,94
miR-299	ND	ND	ND	ND	ND
miR-301	26,61	26,34	25,63	24,97	24,24
miR-302a	ND	ND	ND	ND	ND
miR-302b	ND	ND	ND	ND	ND
miR-302b*	ND	ND	ND	ND	ND
miR-302c	ND	ND	ND	ND	ND
miR-302c*	ND	ND	ND	ND	ND
miR-302d	ND	ND	ND	ND	ND
miR-320	25,18	25,52	25,37	24,51	24,36
miR-323	ND	ND	ND	ND	ND
miR-324-5p	27,77	27,42	27,12	26,84	26,01
miR-325	37,18	38,13	39,04	38,02	37,01
miR-326	33,01	32,00	31,64	30,76	29,66
miR-328	30,72	30,34	29,55	28,57	27,40
miR-330	32,12	32,66	32,32	32,37	31,44
miR-331	27,58	27,27	26,61	26,78	25,83
miR-335	28,22	28,49	28,29	28,11	28,19
miR-337	ND	ND	ND	ND	ND
miR-338	37,37	38,07	37,39	35,24	34,39
miR-339	25,37	25,68	26,03	24,92	25,24
miR-340	32,07	32,49	32,94	33,08	31,13
miR-342	26,08	26,06	25,22	24,75	23,16
miR-367	ND	ND	ND	ND	ND
miR-368	ND	ND	ND	ND	ND
miR-370	ND	ND	ND	ND	ND
miR-371	ND	ND	ND	ND	ND
miR-372	ND	ND	ND	ND	ND
miR-373	36,64	37,52	38,22	39,84	35,94
miR-373*	ND	ND	ND	ND	ND
miR-374	27,78	27,37	27,74	27,56	25,94
let-7b	28,19	27,99	28,61	27,90	27,67
let-7d	25,14	25,03	25,30	25,13	24,20
let-7e	34,54	34,97	34,29	35,01	32,75
let-7g	25,86	26,22	25,62	25,39	24,28
let-7i	29,54	30,07	29,16	28,11	26,63

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Chapter 7

Summary
&
General discussion

Summary

Leukemia is a hematological malignancy that is characterized by the clonal expansion of immature hematopoietic cells, which have escaped from the tightly coordinated cell cycle regulation, differentiation and apoptosis controls. In general, leukemia is characterized by a variety of mutations in pathways that are required for normal hematopoiesis. This thesis describes target genes of the mutated transcription factor PML-RARα, which is expressed in acute promyelocytic leukemia (APL) cells. APL, which accounts for 5-10% of all acute myeloid leukemias (AMLs), represents the best prognostic group amongst the different forms of leukemia having the highest curability. APL requires a unique form of treatment, which constitutes the first successful form of leukemia therapy that is based on induction of differentiation of the malignant cells, using all-*trans* retinoic acid (ATRA). The addition of pharmacological doses of ATRA to standard chemotherapy has led to an improvement of cure rates from 40% with chemotherapy alone to up to 90% when combined with ATRA.[1-3] The identification of the chromosomal translocation t(15;17), which causes the expression of the chimeric oncoprotein PML-RARα and which is the genetic hallmark of APL, is important to establish the diagnosis. ATRA has been proven to directly target the product of this molecular defect and the APL model provides an example of targeted therapy based on differentiation induction.[4] In this thesis we describe the identification of three ATRA-responsive genes in APL cells that are transcriptionally regulated by PML-RARα via a novel mechanism. Their biological function and their mechanism of action were studied in more detail in APL cells.

In **chapter 2** we describe the identification of inhibitor of DNA binding 1 (*ID1*) and *ID2* as two direct ATRA-responsive genes in APL cells. Both genes are rapidly induced by ATRA in the APL cell line NB4 as well as in primary patient cells. To test the biological relevance of the induction of ID1 and ID2 in APL cells, both proteins were overexpressed in NB4 cells using retroviral transduction. We show that overexpression of ID1 and ID2 induced inhibition of APL cell proliferation due to an increased accumulation of leukemic cells in the G0/G1 phase of the cell cycle. We conclude that ID1 and ID2 play a role in cell cycle arrest during APL cell differentiation. In **chapter 3** we studied the molecular mechanisms by which *ID1* and *ID2* are regulated in APL cells. We found that the oncogenic fusion protein PML-RARα is able to associate with the promoters of *ID1*

and *ID2* and that PML-RARα is able to transactivate both promoters in the presence of ATRA, in contrast to the wild type RARα/RXR complex. We show that direct binding of PML-RARα to the DNA is not required for transcriptional activation of *ID1* and *ID2* and that the transcription factors Sp1 and NF-Y are involved in PML-RARα-induced gene transactivation. We define a novel, Sp1 and NF-Y-dependent mechanism by which PML-RARα interferes with gene transcription, which implicates that PML-RARα (de) regulates an as yet unrecognized class of genes that are normally not regulated by retinoic acid receptors. In **chapter 4**, we identified *HES1* as another direct ATRA-responsive gene that is transcriptionally regulated by PML-RARα in APL cells through a similar mechanism as has been described for *ID1* and *ID2* in chapter 3. Further studies are necessary to elucidate the biological function of HES1 in APL cells.

To understand the function of ID2 during myelopoiesis, we searched for ID2-interacting proteins using a mammalian yeast two-hybrid assay and in **chapter 5** the results of this study are shown. We found that the transcriptional repressor methyl-CpG binding protein 1 (MBD1) is able to interact with ID2 in yeast cells and we confirmed this interaction in mammalian cells. We hypothesize that the binding of ID2 to MBD1 may alter the function of MBD1, which contributes to the (re)expression of genes that were silenced by MBD1. Further studies should help to clarify the relevance of the interaction.

The discovery of microRNAs (miRNAs) has led to new insights into the regulation of gene expression. In **chapter 6** we investigated whether miRNA expression is regulated during ATRA-induced differentiation of APL cells. We demonstrate that several miRNAs are differentially regulated upon ATRA stimulation and we identified miRNA-132, miRNA-212 and miRNA-135a as being most profoundly induced by ATRA in APL cells. Our data show that PML-RARα transcriptionally regulates their expression in the presence of ATRA. We found that PML-RARα is able to bind to sequences near the miRNA-132 and miRNA-212 genes indicating a direct involvement of the oncoprotein in the transcriptional regulation of these miRNAs.

General discussion

Identification of PML-RARα target genes

In APL cells, the mutated transcription factor PML-RARα has been demonstrated to be involved both in leukemic transformation as well as in ATRA-induced differentiation. The identification of PML-RARα target genes will broaden our understanding of the molecular mechanisms involved in the development of APL and ATRA-induced differentiation of APL cells. In this thesis we describe the identification of three PML-RARα target genes, *ID1*, *ID2* and *HES1*, which are transcriptionally regulated by the oncogenic fusion protein in a ligand-dependent manner. We demonstrate that *ID1*, *ID2* and *HES1* are not regulated by unrearranged RARα following stimulation with ATRA. This indicates that PML-RARα not only interferes with regular RARα target genes but that an additional class of genes that are normally not regulated by RARα is (de)regulated by PML-RARα. We demonstrate that transcriptional regulation of these three genes does not require the DNA-binding domain of the fusion protein and we propose a model in which PML-RARα binds to a DNA-bound Sp1-NF-Y complex. Therefore, the range of genes that are dysregulated by PML-RARα in APL may be much wider than initially assumed. The ChIP-on-chip assay, which is a powerful tool to study chromatin-protein interactions across the entire genome, can be used to identify PML-RARα target genes. In addition, using this technique, it may be interesting to study the requirement of the DNA-binding domain of PML-RARα to associate with promoters by using a mutant form of PML-RARα that is no longer able to directly bind to DNA. The results of these studies may reveal additional genes, coding for regular proteins and miRNAs, that are (de)regulated by the oncoprotein and may provide insight into the molecular mechanisms of transcriptional regulation by PML-RARα.

To confirm the involvement of newly identified PML-RARα target genes in APL pathogenesis and during ATRA-induced differentiation of APL cells, these target genes should be examined for their expression and their biological function should be studied by ectopic expression or by inhibition of their expression. In addition, it would be interesting to study whether the DNA-binding mutant of PML-RARα is able to induce leukemic transformation and/or ATRA-stimulated differentiation in transgenic mice, as this may help to identify important PML-RARα target genes that are involved in these processes.

ID1 and ID2 and their role in granulocytic differentiation

The differential regulation of ID1 and ID2 following ATRA stimulation of APL cells suggests a different role for these proteins during neutrophilic differentiation of APL cells. However, when overexpressing ID1 or ID2 in APL cells both proteins are associated with an inhibition of proliferation due to an accumulation of cells in the G0/G1 phase of the cell cycle. In line with these data, inhibition of ID2 expression in normal $CD34^+$ cells induced proliferation during both eosinophil and neutrophil development.[5] Overexpression of ID1 or ID2 in normal $CD34^+$ cells also demonstrated similar roles for ID1 and ID2 during neutrophil development, both leading to enhanced neutrophil development.[5] Furthermore, in normal hematopoiesis it has been demonstrated that ID2 is required for maturation of neutrophils, since inhibition of ID2 expression in $CD34^+$ cells blocked terminal maturation of neutrophils.[5]

It has been demonstrated that the various ID proteins may have similar as well as different binding partners and that ID1 and ID2 are able to interact with each other. If ID1 and ID2 interact with the same proteins in APL cells, ID2 may take over the function of ID1 during APL cell differentiation. Redundancy of murine Id proteins was illustrated by the observation that breeding of various single *Id* gene knockout mice did not produce viable offspring carrying null mutations in two different Id proteins.[6] ID1 and ID2 may exert similar biological functions through different molecular mechanisms. Different binding partners may reflect a differential molecular mechanism for ID1 and ID2 during APL cell differentiation. Therefore, the identification of ID specific interacting proteins may help to elucidate the function of both proteins in APL cell differentiation. Not only bHLH proteins but also non-bHLH proteins have been found to interact with ID proteins. The relevance of these various interactions in mediating biological functions of ID proteins in a truly physiological context *in vivo* has now to be established.

The relevance of the interaction between ID2 and MBD1

Upregulation of ID2 during terminal granulocytic differentiation might implicate that ID2 abrogates the function of proteins that are involved in the transcriptional repression of granulocyte-specific genes. In this context, MBD1 has become an interesting newly identified partner of ID2. A large and growing number of genes show hypermethylation in cancer, including APL. DNA methylation, which is mediated by specific DNA methyltransferases and MBD-proteins, is associated with

transcriptional repression.[7] In APL cells, silencing of RARβ expression is mediated by binding of PML-RARα and the recruitment of a corepressor complex containing histone deacetylase activity. In addition, it has been found that DNA methylation contributes to the repressive effect of PML-RARα on RARβ transcription. ATRA has been demonstrated to induce demethylation and re-expression of RARβ in APL cells,[8] while the expression of ID2 is induced. It is tempting to speculate that ID2 might be involved in the abrogation of the function of MBD1, and in the re-expression of genes silenced by hypermethylation. Overexpression of ID2 in the APL cell line NB4 may reveal whether ID2 expression results in re-expression of genes that were silenced by hypermethylation by comparing the methylation profile between normal NB4 cells and ID2-overexpressing NB4 cells.

In conclusion, with the identification of *ID1*, *ID2* and *HES1* as ATRA-responsive genes in APL cells, this thesis is a step towards the unravelling of the involvement of these helix-loop-helix proteins in myeloid hematopoiesis. The elucidation of the molecular pathways implicated in the process of APL cell differentiation may have a much broader implication than APL per se.

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Chapter 8

Nederlandse samenvatting

De bloedcelvorming of hematopoïese is een gecontroleerd en continu proces dat voornamelijk plaats vindt in het beenmerg. Vanuit pluripotente hematopoïetische stamcellen die het vermogen hebben zich te vermenigvuldigen (prolifereren), ontstaan alle soorten rijpe functionele bloedcellen via een proces van uitrijping (differentiatie). Deze bloedcellen verlaten het beenmerg en komen in de bloedcirculatie terecht. De meeste soorten rijpe bloedcellen hebben maar een korte levensduur en verlaten de bloedcirculatie vrij snel via natuurlijke celdood (apoptose). Tijdens normale hematopoïese bestaat een balans tussen proliferatie, differentiatie en apoptose van bloedcellen. Verstoring van deze balans leidt tot het ontstaan van bloedziekten. Leukemie is een verzamelnaam voor verschillende vormen van bloedkanker en de aandoening wordt gekarakteriseerd door ongecontroleerde proliferatie van onrijpe, meestal witte bloedcellen. De niet-functionele leukemische bloedcellen hopen zich op in het beenmerg en verdringen het normale beenmerg. Dit leidt tot een tekort aan normale bloedcellen, wat samengaat met symptomen zoals bloedarmoede, malaise, spontane bloedingen, terugkerende infecties en koorts.

Per jaar krijgen wereldwijd ongeveer 300.000 mensen leukemie en 222.000 mensen overlijden jaarlijks aan leukemie. Over het algemeen is leukemie gekarakteriseerd door een verscheidenheid aan mutaties in genen die van belang zijn tijdens de normale hematopoïese. Deze verscheidene typen mutaties hebben verschillende invloeden op de overleving van een patiënt. Het diagnosticeren van de onderliggende genetische verandering kan betekenisvolle informatie verschaffen over de prognose. De behandeling van leukemie richt zich voornamelijk op het vernietigen van de sneldelende cellen. De manier van behandelen, bijvoorbeeld de intensiteit van de chemotherapie of de keuze om een beenmergtransplantatie uit te voeren, is niet alleen afhankelijk van de leeftijd van een patiënt of de beschikbaarheid van een donor, maar wordt tevens afgestemd op de prognose die gerelateerd is aan de onderliggende genetische verandering. Een nieuwe trend is de ontwikkeling van doelgerichte therapie waarbij gebruik wordt gemaakt van de genetische eigenschappen van de leukemische cellen. De behandelingsstrategie van leukemie patiënten wordt steeds individueler door middel van medicijnen die zich specifiek richten op de onderliggende genetische verandering.

Acute myeloïde leukemie (AML) is de meest frequente vorm van acute leukemie in volwassenen en komt voor in 90% van alle gevallen van acute leukemie. Acute promyelocyten leukemie (APL) is een subtype van AML en komt voor in 5-10% van alle gevallen van AML. Bij dit type leukemie zijn onrijpe granulocytaire voorlopercellen (promyelocyten) geblokkeerd in hun vermogen om te differentiëren tot rijpe granulocyten. Van alle verschillende vormen van leukemie heeft APL de beste prognose met het hoogste genezingspercentage door middel van een unieke behandelingsmethode. Onderzoek heeft uitgewezen dat het toedienen van een hoge dosis vitamine A (all-*trans* retinoic acid (ATRA)) het differentiatie vermogen van de leukemische cellen herstelt en in combinatie met standaard chemotherapie leidt dit tot een genezingspercentage van bijna 90%.

APL wordt gekenmerkt door een chromosomale translocatie t(15;17) die resulteert in de expressie van het PML-RARα fusie-eiwit. De identificatie van deze chromosomale afwijking is belangrijk voor het vaststellen van de diagnose. PML-RARα heeft oncogene eigenschappen en het is gebleken dat PML-RARα kan interfereren met de normale functies van het PML-eiwit en het RARα-eiwit. RARα is een ligand-afhankelijke transcriptiefactor die in een complex met het RXR-eiwit de expressie van genen kan reguleren. In afwezigheid van ATRA, het ligand van deze transcriptiefactoren, werkt het complex als een transcriptionele repressor door de rekrutering van een co-repressor complex. In aanwezigheid van ATRA wordt het co-repressor complex vervangen door een co-activator complex waardoor de transcriptie van target genen wordt geactiveerd. Het PML-RARα fusie-eiwit heeft een sterkere binding met het co-repressor complex dan RARα. Bij een fysiologische concentratie ATRA wordt het co-repressor complex niet vervangen door het co-activator complex en de target genen van PML-RARα worden niet tot expressie gebracht. Echter, bij een zeer hoge dosis ATRA wordt het co-repressor complex alsnog vervangen door het co-activator complex waardoor de transcriptie van PML-RARα target genen wordt geactiveerd. Het APL model verschaft een uitstekend voorbeeld van doelgerichte therapie waarbij gebruik wordt gemaakt van de genetische verandering van de leukemische cellen. De unieke vorm van behandeling van APL vormt de eerste succesvolle therapie voor leukemie welke gebaseerd is op de inductie van differentiatie van leukemische cellen.

We hebben de genen *ID1* en *ID2* geïdentificeerd als twee directe ATRA respons genen in APL. ATRA stimuleert hogere expressie van beide genen zowel in NB4 cellen

(een APL cellijn) als in primaire APL patiënten-cellen. Om de biologische relevantie van de expressie van ID1 en ID2 in APL te bestuderen hebben we beide eiwitten door middel van retrovirale transductie tot overexpressie gebracht in NB4 cellen. In **hoofdstuk 2** laten we zien dat overexpressie van ID1 en ID2 de groei van APL cellen remt door een ophoping van leukemische cellen in het G0/G1-stadium van de celcyclus. We concluderen dat ID1 en ID2 een rol spelen in de celcyclus-stop tijdens ATRA geïnduceerde differentiatie van APL cellen. In **hoofdstuk 3** hebben we de moleculaire mechanismen bestudeerd waardoor *ID1* en *ID2* worden gereguleerd in APL cellen. We hebben ontdekt dat het PML-RARα fusie-eiwit kan binden aan de promoter regio's van de twee *ID* genen en dat PML-RARα beide genen kan transactiveren in de aanwezigheid van ATRA. Dit fenomeen treedt niet op bij het wild type RARα/RXR eiwit complex. We tonen aan dat de transcriptiefactoren Sp1 en NF-Y betrokken zijn bij deze PML-RARα afhankelijke regulatie van *ID1* en *ID2*. We definiëren een nieuwe functie voor PML-RARα en laten zien dat het fusie-eiwit betrokken is bij de transcriptionele activatie van een tot nu toe onbekende klasse van genen die normaal niet door ATRA wordt gereguleerd. In **hoofdstuk 4** identificeren we *HES1* als een nieuw ATRA respons gen welke transcriptioneel gereguleerd wordt door PML-RARα in APL cellen. We tonen aan dat deze transcriptionele regulatie van *HES1* net als die van *ID1* en *ID2* afhankelijk is van de transcriptiefactor NF-Y. Verdere studie zal moeten ophelderen wat de biologische functie van het HES1 eiwit in APL is.

Om de biologische functie van ID2 tijdens granulocytaire differentiatie beter te begrijpen hebben we het gist twee-hybride systeem gebruikt om eiwitten te identificeren die een interactie aangaan met ID2. In hoofdstuk 5 laten we de resultaten van deze studie zien. We hebben ontdekt dat het methyl-CpG-bindende eiwit 1 (MBD1) een interactie aangaat met ID2 en we hebben deze interactie bevestigd door middel van co-immunoprecipitatie studies. Verdere studies zullen duidelijk moeten maken wat de relevantie is van de interactie tussen MBD1 en ID2.

De ontdekking van het bestaan van microRNAs (miRNAs) in de begin jaren negentig heeft geleid tot nieuwe inzichten op het gebied van de regulatie van gen expressie. In **hoofdstuk 6** hebben we bestudeerd of miRNAs transcriptioneel gereguleerd worden tijdens ATRA geïnduceerde differentiatie van APL cellen. We tonen aan dat miRNAs differentieel tot expressie komen na ATRA stimulatie en we laten zien dat miRNA-132, miRNA-212 en miRNA-135a het sterkste worden opgereguleerd door ATRA in APL cellen. We hebben ontdekt dat PML-RARα betrokken is bij de ATRA geïnduceerde

transcriptionele regulatie van deze miRNAs en tonen aan dat het PML-RARα fusie-eiwit aan sequenties kan binden die in de buurt van de miRNA-132 en miRNA-212 genen liggen. Deze bevinding suggereert dat PML-RARα direct betrokken is bij de transcriptionele regulatie van deze miRNAs.

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Curriculum Vitae

Jeannet Nigten, de auteur van dit proefschrift, werd geboren op 14 februari 1979 te Grave. Na de basisschool de Raamdonk te Grave volgde zij de VWO opleiding aan het Kruisheren Kollege te Uden. In 1997 behaalde zij haar VWO-gymnasium diploma waarna zij de studie Medische Biologie begon aan de Universiteit van Amsterdam. Gedurende haar studie volgde zij een wetenschappelijke stage op de afdeling Anatomie en Embryologie aan de Universiteit van Amsterdam, onder leiding van Prof. dr. Antoon Moorman. Vervolgens vertrok zij naar het Verenigd Koninkrijk voor een tweede wetenschappelijke stage aan de University of Reading, onder leiding van Dr. Gavin Brooks. In september 2001 behaalde zij haar doctoraal diploma. In mei 2002 is zij begonnen als Junior Onderzoeker bij het Centraal Hematologisch Laboratorium aan de Radboud Universiteit te Nijmegen. Het onderzoek dat zij daar heeft uitgevoerd onder begeleiding van Dr. Joop Jansen en Dr. Bert van der Reijden, met Prof. dr. Theo de Witte als promoter, is beschreven in dit proefschrift. In september 2007 is zij begonnen aan de opleiding tot Klinisch Chemicus bij Medial medisch diagnostische laboratoria te Haarlem en Hoofddorp.

List of publications

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