Aus dem Universitätsklinikum Münster
Klinik und Poliklinik für Innere Medizin A
- Direktor: Univ.-Prof. Dr. med. Wolfgang E. Berdel -

Id1 and Id2 are relevant target genes of C/EBPα in acute myeloid leukemia

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1. Berichterstatter: Priv.-Doz. Dr. med. Utz Krug
2. Berichterstatter: Univ.-Prof. Dr. med. Steffen Koschmieder

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Zusammenfassung

Id1 und Id2 sind relevante Zielgene von C/EBPα in akuter myeloischer Leukämie

Svenja Erichsen

Die Induktion der Differenzierung von Leukämiezellen ist eine vielversprechende Möglichkeit, die akute myeloische Leukämie (AML) zu behandeln. Während all-trans-Retinolsäure (ATRA), welches die granulozytäre Differenzierung induziert, schon sehr erfolgreich in der Therapie der akuten Promyelozytenleukämie (APL) eingesetzt wird, gibt es für Patienten mit anderen AML-Subtypen weit weniger gute klinische Ergebnisse. Das Triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oidsäure (CDDO) induziert die granulo-monozytäre Differenzierung zumindest teilweise durch Aktivierung der Translation des Transkriptionsfaktors CCAAT/Enhancer Binding Protein alpha (C/EBPα) (Koschmieder et al, Blood 2007). Die Identifikation von C/EBPα-Zielgenen ist daher ein Schlüssel zum Verständnis der Funktionsweise der Substanzen ATRA und CDDO und verspricht zugleich neue Ansätze für Differenzierungstherapien. Im Rahmen der vorliegenden Arbeit wurde gezeigt, dass die Behandlung von HL60-Zellen mit ATRA zu einer Induktion der Id1-und Id2-Transkription und die Behandlung mit CDDO zu einer Steigerung der Id2-Transkription führt. Mittels Chromatin-Immunopräzipitation und Luziferase-Assays in HL60-, 32D- und 293T-Zellen wurden Id1 und Id2 als direkte Zielgene von C/EBPα identifiziert. C/EBPα-Bindung an den Enhancer „3PRE“ induzierte zudem stark Id1. Auch mit C/EBPβ und C/EBPδ konnte eine Id1-Induktion erreicht werden. Diese Ergebnisse weisen stark darauf hin, dass Id1 und Id2 für die Differenzierungsinduktion durch ATRA und CDDO relevant sind und als Zielgene für neue Therapien in Frage kommen.

Tag der mündlichen Prüfung: 4. Juli 2012

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Id1 and Id2 are relevant target genes of C/EBPα in acute myeloid leukemia

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First Commentator: Priv.-Doz. Dr. med. Utz Krug
Second Commentator: Univ.-Prof. Dr. med. Steffen Koschmieder

Summary

Id1 and Id2 are relevant target genes of C/EBPα in acute myeloid leukemia

Svenja Erichsen

The differentiation induction in leukemic cells is a promising means to treat human acute myeloid leukemia (AML). However, while all-trans retinoic acid (ATRA), which induces granulocytic differentiation, has been applied successfully in the therapy of acute promyelocytic leukemia, the results for patients suffering from other AML subgroups have been much less promising. The triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) induces granulomonocytic differentiation. This was at least in part mediated by translational activation of the transcription factor CCAAT Enhancer Binding Protein alpha (C/EBPα) (Koschmieder et al, Blood 2007). Therefore, the identification of C/EBPα target genes is a key to understand the way of action of the compounds ATRA and CDDO and at the same time a promising way to find new approaches for differentiation therapies. In the present thesis it has been shown that treatment of HL60 cells with ATRA leads to an induction of Id1 and Id2 transcription and treatment with CDDO to an increase of Id2 transcription. Via chromatin immunoprecipitation and luciferase assays in HL60, 32D and 293T cells, Id1 and Id2 were identified as direct target genes of C/EBPα. Also binding of C/EBPα to the enhancer "3PRE" strongly induced Id1. Additionally, also with C/EBPβ and C/EBPδ expression an Id1 and Id2 induction was achieved. These results strongly indicate that Id1 and Id2 are relevant for differentiation induction by ATRA and CDDO and that they should be considered as target genes for differentiation therapies.

Date of oral examination: 4th of July 2012

Meiner Familie gewidmet, die mich immer unterstützt hat.

List of abbreviations

A	Adenosin
Ab	Antibody
Ad	To
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
ATO	Arsenic trioxide
ATRA	All-trans retinoic acid
BCA assay	Bicinchoninic acid protein assay
bHLH protein	Basic Helix-loop-helix protein
BSA	Bovine serum albumin
B1	Enhancer of the gene Id1
B1s	Mutated enhancer of the gene Id1
C	Cytosine
CD	Cluster of differentiation
CDDO	2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
ChIP	Chromatin immunoprecipitation
CHOP	C/EBP homologous protein
CMV	Cytomegaly virus
CO_2	Carbon dioxide
Ct	Cycle threshold
Cu	Copper
dd	Double distilled
DEPC	Diethylpyrocarbonate
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E.	Escherichia
EDTA	Ethylenediaminetetraacetic acid
et al.	Et alii (and others)
FAB	French-American-British-Cooperative Group
FACS	Fluorescent-activated cell sorting
FCS	Fetal calf serum
FISH	Fluorescent in situ hybridization
FLT3	Fms-related tyrosine kinase 3
G	Guanine
g	Gram
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
G-CSFR	Granulocyte-colony stimulating factor receptor
gDNA	Genomic deoxyribonucleic acid
GM-CSF	Granulocyte-monocyte colony stimulating factor
G0	Non-dividing, quiescent phase of the cell cycle (Gap-zero-phase)
G1	Postmitotic phase of the cell cycle (Gap-one-phase)
h	Hour

HCl	Hydrochloric acid
H_2O	Water
HL60	Human promyelocytic leukemia cells
HSC	Hematopoietic stem cell
Id	Inhibitor of differentiation / Inhibitor of DNA-binding
Ig	Immunoglobulin
Ig-EBP	Immunoglobulin Enhancer binding protein alpha
IL	Interleukin
kb	Kilobases
K562 cells	Human erythroid leukemia cells
l	Liter
LB	Lysogeny broth medium
LiCl	Lithium chloride
µ	Micro (10^{-6})
m	Meter
M	Molar
MDS	Myelodysplastic Syndrom
miRNA	Micro deoxyribonucleic acid
MPD	Myeloproliferative Disease
mRNA	Messenger ribonucleic acid
M-CSF	Monocyte-colony stimulating factor
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
n	Nano (10^{-9})
NB4 cells	Human promyelocytic leukemia cells
NaCl	Sodium chloride
NaF	Sodium flouride
$NaHCO_3$	Sodium hydrogen carbonate
Na_3VO_4	Sodium orthovanadate
NPM1	Nucleophosmin 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Negative decadal logarithm of molar concentration of hydrogen ions
p-value	Probability value
RARα	Retinoic receptor alpha
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
RPMI-Medium	Roswell Park Memorial Institute-Medium
SDS	Sodium dodechyl sulfate
siRNA	Silencing ribonucleic acid
T	Thymine
TAE	Tris-Acetate-EDTA buffer
taq	Thermus aquaticus polymerase
TE	Tris-EDTA buffer
Tris	Tris-(hydroxyethyl)aminomethane
TSA	Trichostatin A
U	Unit
UV	Ultraviolet
V	Volt
WBC	White blood cell count
WHO	World Health Organization
3PRE	3 prime regulatory element

32D cells	Mouse myeloid progenitor cells
293T cells	Human embryonic kidney cells
%	Percent
°C	Degrees Celsius

Table of contents

1. Introduction

1.1 Hematopoiesis and leukemia

1.1.1 The course of hematopoiesis

Hematopoiesis is the development of cellular components of the blood. In a healthy adult person 10^{11} to 10^{12} new blood cells are produced every day (56). Although the produced cells perform completely different tasks like the transport of oxygen, hemostasis and infection defense, they all derive from hematopoietic stem cells (HSCs) which reside in the bone marrow. Besides proliferation, the HSCs are also self-renewing, as one blood cell and one HSC arise from every cell division (2).

For the differentiation of a blast cell into a mature cell of one lineage, changes of gene expression are essential. During the process of maturation of a blast cell which derives from a pluripotent stem cell, a variety of transcription factors take influence on the development of the cell to bring it to its final cell type (32).

Primarily the cell is directed into the myeloid or the lymphoid cell line. A lymphoid progenitor cell develops into a B or T lymphoid cell, whereas the myeloid cell line contains the other types of blood cells which are the different kinds of granulocytes and the monocytes, erythrocytes and megakaryocytes, the producer cells of the thrombocytes (2).

Several proteins that control granulopoiesis, the differentiation from myeloblasts to mature granulocytes, have been identified and analyzed, for instance the cytokines granulocyte-colony stimulating factor (G-CSF) (73) and interleukin-3 (IL-3) (40) which activate signaling pathways by binding to their corresponding receptor tyrosin kinases (75), or the transcription factors C/EBPα (104), PU.1, GATA-1 and RUNX1 which regulate gene transcription and are themselves influenced by protein-protein interactions, competition for DNA binding and transcription regulation (60). These

proteins induce the myeloid lineage and thereby generate immune reactions. The relevance of transcription factors has been proved by the development of knockout mice which do not express the according transcription factors. These mice present severe defects in granulopoiesis (60).

Figure 1.1 provides an overview over the different steps in hematopoiesis and the influence of certain important transcription factors during differentiation.

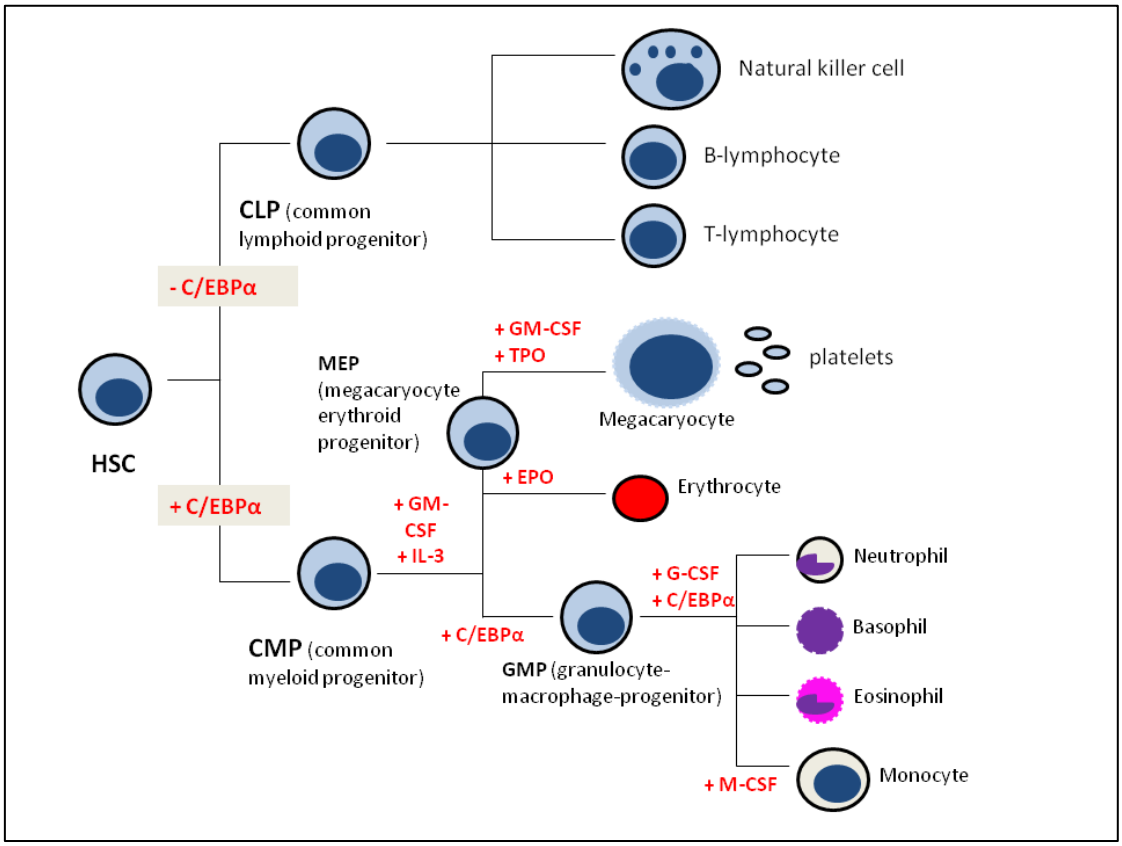

Figure 1.1: The course of hematopoiesis and the influence of certain transcription factors. EPO = erythropoietin; TPO = thrombopoeitin. Source: Modified from Akashi et al., Nature 404, 193-197(9 March 2000)

Among white blood cells, the granulocytes count for about 60%, although the amount of granulocytes can increase rapidly when inflammations occur in the body. The high proliferation and degradation rate of granulocytes also implicates the risk of degeneration, which leads to uncontrolled, malign cell division and deductively to myeloid leukemia.

1.1.2 Acute myeloid leukemia (AML)

Acute myeloid leukemia is characterized by both a block of differentiation at an immature stage of hematopoiesis and an uncontrolled proliferation and inhibition of apoptosis. The autonomous proliferation of immature myeloid cells leads to a displacement of other hematopoietic cell lines in the bone marrow. It is hypothesized that leukemia develops from the alteration of two genes, of which one gives the cells a proliferative advantage and apoptosis inhibition (class I mutation) and another one causes a block of differentiation (class II mutation) (87). Examples for class I mutations are activating mutations of tyrosine kinases, i.e. of the fms-like tyrosine kinase 3 (FLT3), whereas class II mutations constitute of aberrations of transcriptional modulators, i.e. a reciprocal translocation with the involvement of a transcription factor of the core binding factor (CBF) complex.

The incidence of AML is 2.5 / 100.000 and it increases with age, the average age of diagnosis ranging at 63 years. Rarely it can also occur in children (76). Although AML is a relatively rare disease, its incidence is expected to be increasing in the industrial countries due to the rising age of the population.

Classification of AML

According to the French-American-British-Cooperative Group (FAB) classification, eight subtypes of AML have been defined with regard to the morphological differences between the cells concerning the different maturation states.

However, in recent years increasing emphasis lies on the different genetic changes connected to leukemia, underlining the important role they play in leukemia therapy and as prognostic factors. This development led to the introduction of the new AML classification by the World Health Organization (WHO), which divides AML into four groups distinguished by the etiology of the disease (129). The WHO classification is shown in table 1.1.

Table 1.1: The WHO classification of AML. Source: Vardiman et al. (2009): The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood, 114, pp. 937-951.

Name	Description
1. AML with genetic abnormalities	Includes t(8;21), t(15;17), inv(16) and other genetic aberrations; provisional entity: AML with NPM1 mutations and C/EBPα mutations
2. AML with myelodysplasia-related changes	Prior myelodysplastic syndrome (MDS) or prior myeloproliferative disease (MPD)
3. Therapy related myeloid neoplasms	Prior chemotherapy and/or radiation
4. AML, not otherwise specified	Other subtypes of AML

The first group applies to AML with the typical genetic aberrations, of which AML1/ETO and PML/RARα are frequently identified. The translocation t(8;21) is one of the most frequent chromosome abnormalities in AML (81). It combines the genes AML1 and ETO (67), leads to the repression of numerous tumor suppressor genes like PU.1 and C/EBPα (93, 128) and interferes with many differentiation regulating pathways, finally ending up in uncontrolled proliferation of myeloblasts.

The other fusion protein which is frequently found is PML-RARα. It derives from the chromosomal translocation t(15;17) (q22;q12) and occurs in almost all patients with the AML subtype called acute promyelocytic leukemia (APL) (35). It joins the promyelocytic leukemia gene (PML) with the retinoic acid receptor α (RARα) (24, 55). This subtype of AML plays a particularly important role in this work and for this reason it is outlined more detailed in section 1.1.3.

Risk factors and prognostic factors

Risk factors for AML are previously received radiation or chemical substances like cytostatic drugs, especially topoisomerase II inhibitors and alcylating agents, as well as benzol and genetic dispositions like Down syndrome and Klinefelter syndrome (76).

The AML cases arising from earlier cancer therapies like radiation and cytostatic drugs are often connected to more complicated cytogenetic abnormalities. On this account they have a worse prognosis than the so-called de-novo AML cases (76).

AML treatment is divided into two phases: induction and consolidation therapy. Induction therapy for AML usually consists of one to two courses of a cytarabine and anthracycline (or anthracedenione)-based combination therapy (107). For AML patients younger than 60 years, complete remission rates of 70-80% are achieved with this approach (13). A risk-stratified postremission therapy reduces the risk of relapse in patients in complete remission. Potential postremission therapy approaches are an intensive, short-term cytarabine-based consolidation therapy, prolonged maintenance therapy, an autologous or allogeneic hematopoietic stem cell transplantation or combinations thereof. (107). Consolidation therapy reduces the overall relapse rate to approximately 50% (13), even though the effect of a postremission therapy in elderly AML patients over the age of 60 years remains unclear.

Prognostic factors are a fundamental instrument to be able to give a relatively precise evaluation of the patient's prognosis and to aid in selection of the appropriate therapy. A very important factor for the prognosis of AML is the age of the patient, as AML patients older than 60 years have a substantially lower CR rate and a shorter overall survival (6). Secondary AML evolving from a myelodysplastic or myeloproliferative syndrome or occurring after cytotoxic radio- or chemotherapy represent approximately 20% of all AML cases and are also associated with lower complete remission rates and shorter overall survival (63). Additionally, the white blood cell count (WBC) at diagnosis is a useful indicator for the prognosis, as a comparably low WBC implicates a better outcome (14).

The karyotype at diagnosis is one of the strongest prognostic factors for AML (36, 37). In 60% of AML cases an abnormal karyotype can be found (117). Very frequent cytogenetical aberrations are the translocations t(15;17) and t(18;21), which are described above. These translocations, together with the inversion (16), have a relatively good prognosis, with complete remission rates reaching 90% and a 5-year-survival rate of approximately 65% (15, 37, 115). In addition, mutations in certain genes are important prognostic factors for AML, for example internal tandem duplications (ITD)

of the FLT3 gene (43, 85), NPM1 (26, 33, 106) and the tumor suppressor gene C/EBPα (27, 69, 92, 103, 132).

Pathology and diagnosis

The symptoms of AML are rather unspecific, which makes it difficult to diagnose AML clinically. In general, symptoms are directly attributed to the bone marrow infiltration and the resulting cytopenia: Due to the lack of mature granulocytes, patients are vulnerable to bacterial infections and mycoses. Sometimes pyrexia without the finding of an infectious focus occurs. The deficit in erythrocytes leads to anemia with typical symptoms like pallor, dyspnea and fatigue. Thrombocytopenia results in frequent bleedings, especially in the frequent incidence of hematomas, petechiae, epistaxis and upper intestinal bleedings. Also lymph node turgor and splenomegaly, resulting from the leukemic infiltration, can be symptoms of AML (76).

The diagnosis of AML is based on two criteria according to WHO guidelines (129):

1st: A portion of immature blasts in the bone marrow of at least 20%, determined by cytological examination of a bone marrow smear (or histology in case of a dry tab) (see figure 1.2).

2nd: The identification of blasts as part of the myeloid cell line by cytochemical examinations, immunohistological staining or immunophenotyping.

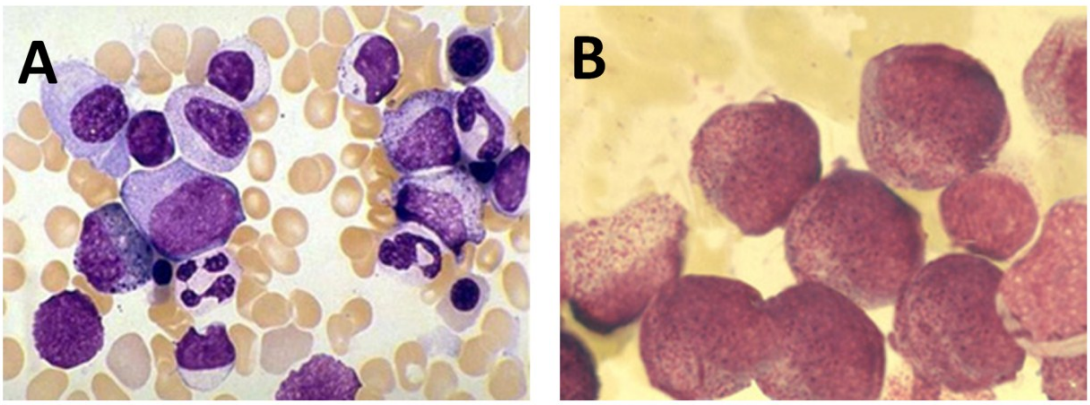

Figure 1.2: Normal bone marrow smear (A) and bone marrow smear from an APL patient (B).
Source: Leukemia information website, Department of Medicine A, University Hospital of Münster, http://meda-muenster.de/Leukaemietherapie.37.0.html

In addition of establishing the diagnosis 'AML' by cytology, the following methods are employed for the determination of the subtype according to the FAB and WHO classifications and for prognostication:

- Immunophenotyping with fluorescence activated cell sorting (FACS) helps to ensure the diagnosis by analyzing specific surface proteins of the cell.

- Cytogenetical analysis, (classical karyotyping via G-banding, accompanied by screening for frequent recurrent abnormalities by fluorescent in situ hybridization (FISH)), provides information about structural or numerical chromosomal aberrations.

- Mutational analysis by classical Sanger sequencing, or recently by next generation sequencing, allow the identification of typical AML-associated mutations with prognostics impact, for example biallelic C/EBPα mutations, mutations of the nucleophosmin gene (NPM) or FLT3 internal tandem duplication mutations.

1.1.3 Acute promyelocytic leukemia (APL)

The FAB subtype AML-M3, also called acute promyelocytic leukemia, counts for 10-15% of all AML cases. APL almost always contains the translocation t(15;17)(q22;q12) (35). This translocation leads to the fusion of the gene PML with the retinoic acid receptor α (RARα) (24, 55). In normal differentiating cells, RARα forms multiprotein complexes with corepressors like NCoR, SIN3A, SMRT and histone deacetylases (HDAC) and regulates the transcription of a number of genes associated with myeloid differentiation (80). The fusion protein PML/RARα is an aberrant retinoid receptor with altered DNA binding activity (25, 95) and tighter corepressor binding (38, 70) which causes a block of myeloid differentiation in the promyelocytic stage. Further genetic changes lead to uncontrolled proliferation of the promyelocytes (8). Figure 1.3 demonstrates the mode of action of the PML/RARα fusion protein. Treatment with retinoid acid (RA) in therapeutical concentrations leads to the dissociation of

corepressors from the PML/RARα transcriptional repressor complex and recruitment of transcriptional coactivators and ultimately to the transcription of differentiation-associated genes, starting the differentiation process.

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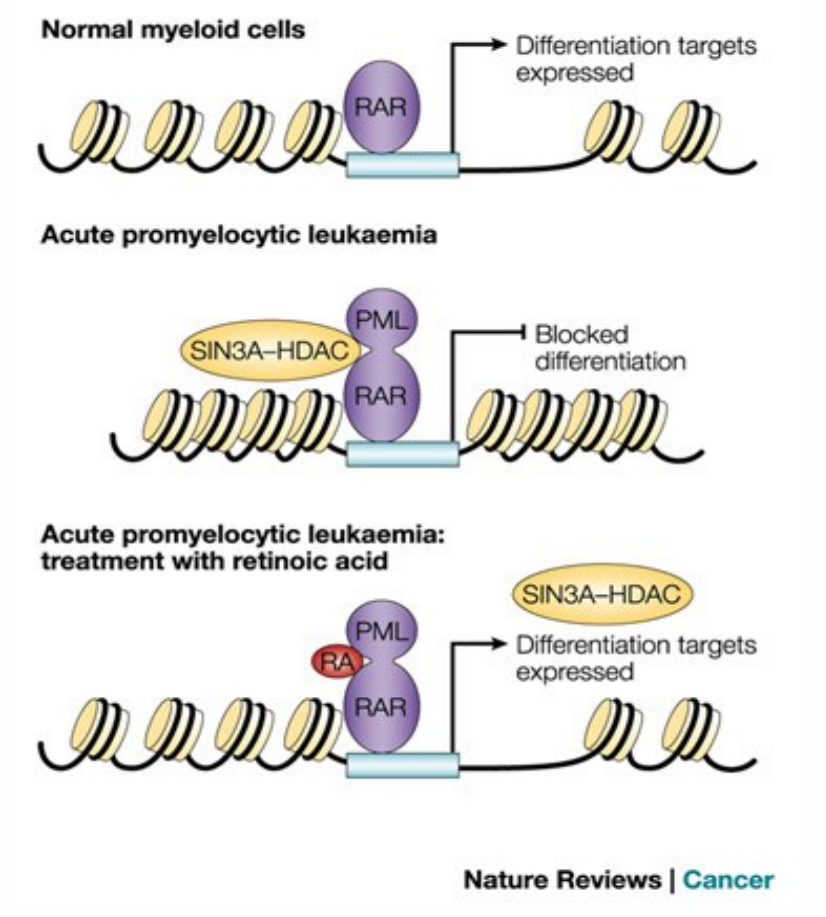


Figure 1.3: RARα normally regulates transcription of genes associated with myeloid differentiation. The fusion gene PML-RARα with altered binding activity causes a transcriptional repression of genes associated with myeloid differentiation in APL by recruitment of corepressor complexes, ATRA (RA) reverses this condition and activates transcription of these repressed genes. Roberts C and Orkins S (2004): The SWI/SNF complex - chromatin and cancer. Nature Reviews Cancer 4, pp. 133-142.

In addition to the typical symptoms of AML, APL is often characterized by a high incidence of disseminated intravascular coagulation (DIC), a serious syndrome which arises from an activation of coagulation mechanisms in the vessels, leading on the one hand to a disruption of the normal blood flow in the organs, especially in the kidney, and on the other hand to a massive consumption of platelets and coagulation factors

and, therefore, to spontaneous bleedings (41, 105). Without adequate therapy, APL is fatal in 100% of the cases, which is mainly due to these catastrophic bleeding disorders (123).

However, since the targeted therapy with all trans retinoic acid (ATRA) plus anthracycline based chemotherapy and subsequently arsenic trioxide (ATO) was introduced, most APL patients, even relapsed cases, can be cured (124). The most threatening aspect of the disease is still the initial phase, in which consequent blood product support and an early start of ATRA therapy are the most important actions to reduce the early mortality rate, which could be demonstrated to be in the range of 30% in a recent population-based exploration (66). Furthermore, 20% of the treated APL patients relapse. On that account, the identification of prognostic factors which detect patients who have a higher risk for relapse and individualization of the therapy have become a major focus of research. For example, a high white blood cell count at diagnosis, old age and certain genetic mutations like FLT3-internal tandem duplication mutations are prognostic factors predicting a probable relapse and an early death (17, 78, 111).

1.2 The therapy of acute promyelocytic leukemia

1.2.1 Therapy of APL

The final goal of APL therapy is a complete, stable remission. In contrast to other subtypes of AML, APL is unique in its reaction to differentiation therapy with the compound ATRA.

In the initial phase, aggressive support of blood products and coagulation factors for the prevention and treatment of bleeding complications and an early start of ATRA treatment are pivotal for the patient's survival. The following induction therapy aims at a complete remission, defined as elimination of all symptoms, normalization of the blood count and clearance of the immature blasts in the bone marrow (blast count of less than 5% without evidence for residual leukemic blasts, i.e. Auer rods). Induction

therapy of APL consists of ATRA in combination with anthracyclines, for example daunorubicine or idarubicin, and it results in a complete remission in approximately 90% of the treated patients. For maintenance of the complete remission, treatment with ATRA and low dose chemotherapy are continued for one to two years to prevent relapses (123).

When patients are unable to receive anthracyclines, complete remission can be achieved with ATRA combined with arsenic trioxide (ATO). For patients with relapsed APL, ATO is a considerable alternative to stem cell or bone marrow transplantation achieving high cure rates (123).

ATRA is a representative of a novel therapeutic strategy that has a different way of action than chemotherapy with its disadvantages: While chemotherapy is based on the destruction of all fast-dividing cells in the body and causes a severe DNA damage with the consequential risk of secondary cancer diseases, differentiation therapy is able to force the malign cells to overcome their block of differentiation and behave like normal body cells. Thus, ATRA does not cause the typical side effects of cytotoxic therapies.

ATRA therapy for APL patients is still an unequaled example for this therapeutic strategy. Treatment of PML-RARα positive cells with ATRA causes them to differentiate into mature neutrophils (11), an effect thought to be mediated by binding to RARα and thereby normalizing the RARα transcription factor activity. ATRA also leads to concentration changes of transcription factors like c-myc (10), PU.1 (84) and C/EBP proteins (29, 96, 104). However, the precise mechanisms by which ATRA reverses the block in neutrophil differentiation are still unclear and the understanding is complicated by the paradox fact that the fusion protein PML-RARα, which causes APL, is at the same time essentially involved in the effects of ATRA (82, 96) and that ATRA resistance is mainly associated with a PML-RARα defect (28, 46).

1.2.2 Differentiation therapy with CDDO

The anti-cancerous effects of the triterpenoid 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) are still a relatively new detection. Its differentiating and anti-apoptotic effects for a variety of solid tumor cell lines and leukemic cell lines in vitro and in vivo have first been described in 1999 (98, 119). CDDO is currently evaluated in clinical trials. Various different pathways have been identified to play a role for CDDO induced apoptosis and differentiation (45, 48, 57, 120). CDDO also strongly induces apoptosis and differentiation of leukemic cells (52, 58, 98, 119), encouraging the hope that the analysis of its mechanisms of action and intracellular pathways evolve in new differentiation therapy approaches for APL and other types of leukemia or even other types of cancer.

1.3 The C/EBP transcription factors and their role in granulocytic differentiation

Both ATRA and CDDO have an effect on C/EBPα protein synthesis. CDDO induces granulocytic differentiation by translational up-regulation of the active form p42 of the transcription factor C/EBPα, which means that it acts on the translational level (59), whereas during ATRA induced neutrophil differentiation, C/EBPα mRNA is increased on the transcriptional level (104). C/EBPα is one of the critical transcription factors which orchestrate granulocytic differentiation and is crucial for granulocytic differentiation, as C/EBPα knockout mice show a selective block in differentiation of neutrophils (137). Several of C/EBPα's target genes which are associated with myelopoiesis have been identified, for instance the growth factors M-CSF, G-CSF and GM-CSF (42, 116, 136) and the transcription factors E2F (102) and c-myc (53). In nearly 10% of AML patients, mutations in the gene of the transcription factor C/EBPα which impair the protein function occur (94). Also down-regulation of C/EBPα mRNA and protein expression is often observed in AML cells (77, 93).

These findings indicate that C/EBPα plays a key role in the mechanisms of action of ATRA and CDDO and let the idea arise that restoration of normal C/EBPα function or C/EBPα overexpression could be a trigger for differentiation induction in vivo. This is supported by the finding that overexpression of C/EBPα in leukemia mouse models can abrogate leukemia and increase survival (65, 127).

The C/EBP protein family includes the six different subtypes C/EBPα, β, γ, δ, ε and ζ (3, 5, 21, 62, 97, 108). They all contain two conserved regions, namely a dimerization leucine zipper region and a DNA binding domain, and therefore belong to the basic loop-helix-loop (bHLH) transcription factor family. Both regions and dimerization with other members of the bHLH family are requirements for DNA binding (131). But only C/EBPα, β, δ and ε exhibit transactivation domains and thereby work as direct transcription factors. In contrast C/EBPγ, also called IG/EBP, and C/EBPζ, also called CHOP, which stands for C/EBP homologous protein, lack the transcriptional activation domains and work as inhibitors of other C/EBP proteins (7, 22). C/EBPγ is expressed ubiquitously, which indicates that it could be a common buffer for C/EBP activity (22). C/EBPα is transcribed in two different isoforms, the longer isoform p42 and the shorter isoform p30. While p42 is the active form of C/EBPα, p30 lacks the transcriptional activation domains and probably inhibits the activity of p42 (83, 94).

Besides C/EBPα, also C/EBPβ and C/EBPε have been shown to be involved in myeloid development. While C/EBPα is particularly essential for the initiation of granulocytic differentiation (104, 137), C/EBPε seems to be important for terminal differentiation (133). C/EBPβ knockout mice do not show any defects of myeloid differentiation, but it has been shown that expression of C/EBPβ from the C/EBPα locus is sufficient for normal hematopoiesis in vivo (54) although C/EBPα is crucial for granulopoiesis (137). This indicates that C/EBPβ has a similar function as C/EBPα concerning granulopoiesis.

As C/EBPα induces granulocytic differentiation, it is likely that the favorable effect of ATRA and CDDO on leukemic cells is a consequence of C/EBPα up-regulation. However, it has been shown that the synergistic effect of forced C/EBPα or C/EBPε expression and ATRA treatment is stronger than either of the arrangements alone, suggesting that ATRA therapy includes more mechanisms than simply increasing C/EBP activity (65). Understanding the pathways of C/EBPα induced differentiation is

an important goal in order to comprehend the precise mechanisms of differentiation therapy as a promising new strategy against cancerous diseases.

1.4 The genes Id1 and Id2

"Inhibitors of differentiation", also called "inhibitors of DNA binding" are a group of dominant inhibitors of basic helix-loop-helix transcription factors (9). They lack a basic region adjacent to the HLH domain, which means that they are unable to bind DNA themselves, but they can associate specifically with HLH proteins and impair their ability to bind DNA. This function of the Id proteins is outlined in figure 1.4.

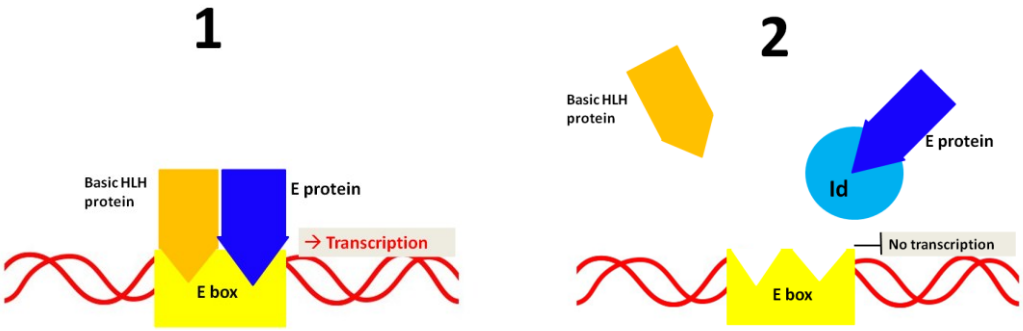


Figure 1.4: Id proteins are dominant negative regulators of basic helix-loop-helix proteins. Source: Modified by Perk et al. (2005): ID Family of Helix-Loop-Helix Proteins in Cancer. Nat Rev Cancer, pp 603-14

It has been shown in previous publications (12, 23) that Id1 expression is up-regulated at the start of granulocytic differentiation and decreased during final maturation. Ectopic, constitutive expression of Id1 inhibits eosinophil development, whereas in contrast neutrophil differentiation is enhanced. At the same time, Id2 is expressed strongly during final differentiation of neutrophils, and inhibition of Id2 expression blocks differentiation (12). Additionally, Id1 and Id2 overexpression results in the inhibition of proliferation and induces a G0/G1 accumulation of promyelocytic leukemia cells (86). These findings indicate that Id proteins play a role in the differentiation of granulocytes. In fact it has been shown that Id1 is essential for

C/EBPα induced myeloid differentiation and that C/EBPα strongly induces Id1 in CD34+ cells and K562 cells (130).

The connection between Id proteins and granulocytic differentiation induction is remarkable because Id proteins have been shown to promote excessive proliferation by inhibiting the transcription of the tumor suppressor gene p16/Ink4a (4, 89) and by binding and inhibiting the product of the retinoblastoma tumor suppressor gene pRB (44, 64). Id proteins also have been shown to protect cells against drug-induced apoptosis (68, 71, 72). Additionally, overexpression of Id1 has been found in many types of human cancer and is correlated with advanced tumor stages for example in breast cancer (31, 68, 113), prostate cancer (90), pancreatic cancer (79) and cervical cancer (112). Even in murine hematopoietic cells Id1 induces proliferation, and when it is expressed unregulated, it leads to immortalization of hematopoietic progenitors in vitro and to a myeloproliferative disease in vivo, which is contradictory to the observed inhibition of proliferation in promyelocytic leukemia cells (79). In human hematopoietic cells, a knockdown of Id1 inhibits leukemic cell growth. These data suggest that there is a causal relationship between Id1 overexpression and hematopoietic malignancy (118).

To sum up, Id proteins are usually known as proliferation inducing proteins and associated with the development of different types of human cancer, but interestingly, they are up-regulated during granulocytic differentiation. Their dysregulation in human cancers and their involvement in hematopoiesis makes it desireable to evaluate the involvement of Id1 and Id2 in leukemogenesis and hematopoiesis.

1.5 The ChIP-on-Chip technology as a method to identify C/EBPα target genes

The ChIP-on-Chip technology should be briefly explained here as the results from this technology, namely the identification of Id2 as a C/EBPα target gene (Steffen Koschmieder and Felix Sprenger, unpublished data), were the starting point for the presented experiments.

The ChIP-on-Chip technology as it has been introduced by Odom et al. (88) is a method which combines chromatin immunoprecipitation (ChIP) with microarray technology (chip). It allows the genome wide identification of all binding sites of a transcription factor in vivo.

In the first step, chromatin immunoprecipitation is performed as explained in 3.1.1. The resulting sample containing the DNA to which the transcription factor has bound is amplified via PCR and then denaturated. The single strands are tagged with a fluorescent marker and poured over the surface of a microarray, which carries single strand fragments from the genome region of interest. Whenever a fragment finds its complementary fragment, they hybridize. The array is illuminated with fluorescent light and the DNA fragments which are hybridized emit a signal light (50).

In the ChIP-on-Chip experiment that forms the basis for this work, the above described tumor suppressor transcription factor C/EPBα has been found to bind to the promoter of the gene Id2. Most of the problems with ChIP-on-chip experiments arise from the last work phase, which includes the correct chip read-out and the statistical analysis of the data. For this reason, the verification of Id2 as a target gene of C/EBPα, as it has been done in this thesis by conventional chromatin immunoprecipitation, is an important step to confirm the accuracy of the ChIP-on-chip results.

1.6 Hypothesis and aims

In this dissertation, the induction of Id1 and Id2 by C/EBP proteins and the effects of ATRA and CDDO on this condition have been investigated, especially emphasizing the localization of C/EBP binding sites in the regulatory elements of Id1 and Id2. The purpose of this examination is to obtain a better understanding of differentiation therapy in order to find new therapy approaches. The success of ATRA therapy for APL arises the hope to find other drugs that induce differentiation, like CDDO, which is currently clinically tested, for other types of leukemia or even for other types of human cancer. Genes and proteins which are involved in the effects of ATRA and CDDO are candidates for target genes for differentiation therapy. This makes it so important to

identify these genes and characterize their effects on cell development. Figure 1.5 gives an overview over the topic of this work concerning Id1.

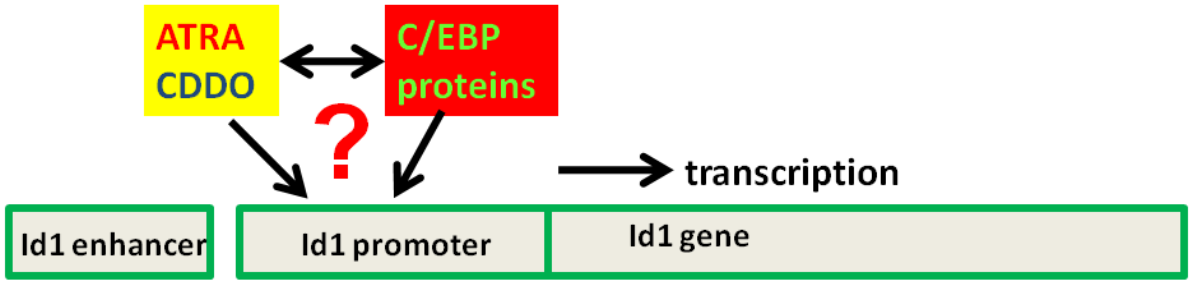


Figure 1.5: Research question – How do ATRA, CDDO and the C/EBP proteins regulate the Id1 transcription activity?

2. Materials

2.1 Chemicals

Agarose for gel electrophoresis	Biozym, Vienna, Austria
Ampicillin sodium salt	Sigma-Aldrich, Saint Louis, MO, USA
All trans retinoic acid (ATRA)	Sigma-Aldrich, Saint Louis, MO, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, Saint Louis, MO, USA
CDDO	Gift from Dr. Michael Sporn, Dartmouth, NH, USA
Complete-Protease inhibitor	Roche, Basel, Switzerland
ddH_2O (Aqua ad iniectabilia)	B. Braun, Melsungen, Germany
Deoxycholic acid (DOC)	Sigma-Aldrich, Saint Louis, MO, USA
Deoxy-ribonucleotides (dNTPs)	eBioscience Inc, Frankfurt, Germany
Dimethyl sulfoxide (DMSO)	Serva Electrophoresis, Heidelberg, Germany
EDTA	Serva Electrophoresis, Heidelberg, Germany
Ethanol	AppliChem, Darmstadt, Germany
Ethidium bromide	Carl Roth, Karlsruhe, Germany
Formaldehyde 37 %	Mallinckrodt Baker Inc., Deventer, Netherlands
Glutamine	Gibco, Carlsbad, CA, USA
Glycine	Carl Roth, Karlsruhe, Germany
Hydrochloric acid (HCl)	Carl Roth, Karlsruhe, Germany
Isopropanol	SAV Liquid Production, Flintsbach am Inn, Germany
Lithium chloride (LiCl)	Merck, Darmstadt, Germany
Loading dye (Qiagen gel pilot™)	Qiagen, Hilden, Germany
Marker (GeneRuler DNA ladder mix™)	Fermentas, Ontario, Canada
Methanol	AppliChem, Darmstadt, Germany

NP 40	Sigma-Aldrich, Saint Louis, MO, USA
Protein A/G Plus Agarose Beads	Santa Cruz Biotechnologies, Santa Cruz, CA, USA
RNAse inhibitor (RNAse out)	Invitrogen, Carlsbad, CA, USA
Sodium chloride	AppliChem, Darmstadt, Germany
Sodium fluoride (NaF)	Sigma-Aldrich, Saint Louis, MO, USA
Sodium hydrogen carbonate ($NaHCO_{3)}$	Merck, Darmstadt, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, Saint Louis, MO, USA
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Saint Louis, MO, USA
Trichostatin A (TSA)	Sigma-Aldrich, Saint Louis, MO, USA
Triton-X 100	Carl Roth, Karlsruhe, Germany
2- Mercaptoethanol	Sigma-Aldrich, Saint Louis, MO, USA

2.2 Enzymes

KpnI, HindIII, BamHI, BspHI, NcoI, XbaI, SmaI and according buffers	New England Biolabs, Ipswich, MA, USA
M-MLV reverse transcriptase and according buffer	Promega, Madison, WI, USA
Nanofectin™	PAA, Pasching, Austria
Proteinase K	Carl Roth, Karlsruhe, Germany
SYBRgreen™ qPCR MasterMix	Thermo Fisher Scientific, Waltham, MA, USA
Biotherm™ Taq Polymerase and according buffer	GeneCraft, Cologne, Germany
Ribonuclease A (RNAse A)	Sigma-Aldrich, Saint Louis, MO, USA
Trypsin	Gibco, Carlsbad, CA, USA

2.3 Primers

cDNA human Id1	**Sequence**
Forward Primer	5’ GCGGCGTCCCTTCCA 3’
Reverse Primer	5’ GCATGCCGCCTGTGAAA 3’

cDNA human Id2	**Sequence**
Forward Primer	5’ CTG TCC TTG CAG GCT TCT G 3’
Reverse Primer	5’ AAC ACC GCT TAT TCA GCC A 3’

cDNA human GAPDH	**Sequence**
Forward Primer	5’ GAA GGT GAA GGT CGG AGT 3’
Reverse Primer	5’ GAA GAT GGT GAT GGG ATT TC 3’

gDNA human Id1 enhancer “3PRE” (130)	**Sequence**
Forward Primer	5’ GCC CTT ATC TCC CTG GAC CT 3’
Reverse Primer	5’ CCA CTG TCC TCC CTT TAA CCC 3’

gDNA human Id1 promoter sequence “5PCE” (130)	**Sequence**
Forward Primer	5’ TTG TCG TCT CCA TGG CGA C 3’
Reverse Primer	5’ CTG CGG AGC TAC AGT CTC CC 3’

gDNA human Id1 promoter, C/EBP motif 1	Sequence
Forward Primer	5’ AGG GAG ACC CTG CTC TGA GGT CT 3’
Reverse Primer	5’ GTG AGG CTG CAT TCG ATT CCA CCT 3’

gDNA human Id1 promoter, C/EBP motif 2	Sequence
Forward Primer	5’ CCA CCG ACC CAC CCT TGC TG 3’
Reverse Primer	5’ ACC CGC GCT CCT AGG TCC AG 3’

gDNA human Id2 promoter, C/EBP motif 1	Sequence
Forward Primer	5’ GGG TAA GGG CTC CCT CTT GCC A 3’
Reverse Primer	5‘ CCG AAT GTT CCC GGT CCT GTT ATC T 3‘

gDNA human Id2 promoter, C/EBP motif 2-4	Sequence
Forward Primer	5’ CCA GGA TCA AAG CCA TTC GGC GAG 3’
Reverse Primer	5’ AGC CGG GAG AGG GAG AAG AAA GAA 3’

2.4 Antibodies

c-terminal C/EBPα Antibody	Epitomics, Burlingame, CA, USA
Polyclonal rabbit IgG	New England Biolabs, Ipswich, MA, USA

2.5 Plasmids

Name	Properties	Origin
pGL3-Id1h	pGL3 vector (Promega, Madison, WI, USA) with human Id1 promoter and luciferase gene	Kindly provided by Pierre-Yves Desprez, Geraldine Brush Cancer Research Institute, California Pacific Medical Centre, San Francisco, CA, USA
pGL3-Id2h	pGL3 vector with human Id2 promoter and luciferase gene	Kindly provided by Takashi Tokino, Department of Molecular Biology, Cancer Research Institute, Sapporo Medical Universitiy, Sapporo, Japan
pfLuc-B1	pBluescript vector (Stratagene, Santa Clara, CA, USA) with human Id1 enhancer and luciferase gene	Kindly provided by Xiao-Hong Sun, Department of Cell Biology, New York University Medical Centre, New York, NY, USA; now Oklahoma Medical Research Foundation, OK, USA
pfLuc-B1s	pBluescript vector with human Id1 enhancer containing a substitution mutation and luciferase gene	Kindly provided by Xiao-Hong Sun, Department of Cell Biology, New York University Medical Centre, New York, NY, USA; now Oklahoma Medical Research Foundation, OK, USA
pcDNA3-0 **pcDNA3-C/EBPα** **pcDNA3-C/EBPβ** **pcDNA3-C/EBPδ**	pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA) containing the gene C/EBPα, β or δ or empty vector	From own laboratory stock
pRL-CMV	pRL vector containing CMV promoter and Renilla gene	Promega, Madison, WI, USA

2.6 Laboratory equipment

Agarose Gel Sub-Cell GT electrophoresis cell	Bio- Rad, Hercules, CA, USA
Cell culture flasks CellStar; 50, 250 and 550 ml	Greiner bio-one, Solingen, Germany
Electronic Balance Mettler PM2000™	Mettler Toledo, Switzerland
Electroporation Pulse Generator EPI 2500™	Dr. L. Fischer, Heidelberg, Germany
Eppendorf table centrifuge 5415D™	Eppendorf, Hamburg, Germany
Gel Documentation System	Intas, Göttingen, Germany
Incubator innova 4230™	New Brunswick Scientific, Edison, NJ, USA
Luminometer TD-20/20 DLReady™	Turner Designs, Sunnyvale,CA, USA
Micro Amp™ 96-well reaction plates with barcode Optical adhesive films for Real-Time PCR	Applied Biosystems, Foster City, CA, USA Applied Biosystems, Foster City, CA, USA
Petri dishes	Sarstaedt, Nümbrecht, Germany
NanoDrop Spectrophotometer ND-1000™	Peqlab Biotechnologie, Erlangen, Germany
Sigma 4K15™ Refrigerated Centrifuge	Sigma, Osterode am Harz, Germany
Sigma 5417R™ Refrigerated Centrifuge	Sigma, Osterode am Harz, Germany
Branson Sonifier 250 D	Branson, Danbury, CT, USA
Thermocycler (mastercycler personal™)	Eppendorf, Hamburg, Germany
Thermomixer comfort™	Eppendorf, Hamburg, Germany
0,2 ml PCR tubes	Biozym, Vienna, Austria
0,6 / 1,5 / 2 ml reaction tubes	Biozym, Vienna, Austria
0,6 ml low-binding reaction tubes	Biozym, Vienna, Austria
14 ml PP Round-Bottom Tubes	BD Falcon, Franklin Lakes, NJ, USA
20-ml Tubes	Corning, Lowell, MA, USA
50-ml Tubes	Corning, Lowell, MA, USA

6-, 12-, 24-well-plates for cell culture	Greiner bio-one, Solingen, Germany
7500 Fast Real-Time PCR System	Applied Biosystems, Foster City, CA, USA

2.7 Buffers and solutions

ChIP Elution buffer	1% SDS (10 %); 0,1 M $NaHCO_3$
High Salt buffer	0,1% SDS; 1% Triton-X 100; 2mM EDTA; 20 mM Tris-HCl pH 8,1; 5 M NaCl
LiCl buffer	0,25 M LiCl; 1% NP40; 1% DOC; 1 mM EDTA; 10 mM Tris-HCl pH 8,1
Low Salt buffer	0,1% SDS; 1% Triton-X 100; 2mM EDTA; 20 mM Tris-HCl pH 8,1; 1,5 M NaCl
Dulbecco PBS buffer (sterile)	Sigma-Aldrich, Saint Louis, MO, USA
Dulbecco PBS buffer	AppliChem, Darmstadt, Germany
RIPA buffer	15 ml NaCl (5 M); 5 ml NP40 (igepal); 25 ml DOC (10%); 5 ml SDS (10%); 25 ml Tris HCl pH 8 (1 M); filled up to 500 ml with ddH_2O
RIPA buffer containing ChIP Inhibitors	RIPA buffer prepared as above, containing 50 ml RIPA; 2,5 ml NaF (1 M); 50 µl Na_3VO_4; 83,5 µl TSA.
TAE buffer (50x)	
TE buffer for ChIP	2 M Tris Base; 100 mM EDTA; pH 8,0
	10 mM Tris-HCl pH 8,0; 1 nM EDTA

2.8 Culture media

DMEM	Gibco, Carlsbad, CA, USA
FCS	PAA, Pasching, Austria
LB medium	25 g Luria Broth Base (Invitrogen, Carlsbad, CA, USA) ad 1l ddH_2O, autoclaved; with 0,1 mg/ml Ampicillin added after cooling
LB-Amp agar plates	12,5 g Luria Broth Base and 7,5 g Agar ad 1l ddH_2O, autoclaved; with 0,1 mg/ml Ampicillin added after cooling
RPMI	Gibco, Carlsbad, CA, USA

2.9 Computer software

NCBI Primer Blast

PASW Statistics 18.0 (former SPSS)

Primer Express 3.0

TF SEARCH Programme, version 1.3 (Searching Transcription Factor Binding Sites) (39)

2.10 Miscellaneous

Dual Luciferase Reporter Assay System	Promega, Madison, WI, USA
JetStar Plasmid Purification Maxi Kit 2.0	Genomed, Löhne, Germany
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, MA, USA
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany
QIAgen RNeasy Mini Kit	Qiagen, Hilden, Germany

3. Methods

3.1 Molecular biological methods

3.1.1 Chromatin immunoprecipitation (ChIP)

ChIP is a method to analyze DNA protein interactions in chromatin in vivo by the localization of transcription factors linked to the genome. Precipitation of the DNA sequences is reached by the use of antibodies specific for the transcription factor of interest. Probable promoter binding sites were found with the online program "TF Search – Searching Transcription Factor Binding Sites", version 1.3 (39).

"Crosslinking" and cell lysis

2 x 10^7 HL60 cells per immunoprecipitation were centrifuged and resuspended in 10 ml PBS buffer. A permanent linkage between DNA and proteins was achieved by addition of 270 µl of 37% formaldehyde to a final concentration of 1% and incubation at room temperature for 10 minutes. Formaldehyde binds predominantly to amino groups of proteins as well as side groups of DNA bases. In that way it covalently fixes all proteins bound to DNA. After incubation, 650 µl 1M Glycine were added. Glycine buffers the residual formaldehyde to prevent toxic impact on the cells. In the following, the HL60 cells were washed two times with 25 ml ice cold PBS for 5 minutes at 4000 rpm. The cells were lysed by solving the pellets in 400 µl fresh-prepared RIPA buffer containing all the proteinase inhibitors described in section 2.7 and 10 minute incubation on ice. Thus, the crosslinked chromatin was released by the lysed cells.

Fragmentation of chromatin via sonication

To fragment the chromatin into pieces of 100 to 500 base pairs, the lysates were exposed to ultrasound. The sonication was carried out in 1.5 ml tubes filled each with 400 µl lysate and which were cooled on ice during the whole procedure. Conditions for the sonication were tested out and settled as follows:

- 30 sonication cycles
- 3 seconds on-time
- 5 seconds off-time
- 15% amplitude.

The correct fragment size was verified by agarose gel electrophoresis (see also section 3.1.8). After successful sonication, the lysates were centrifuged at 4°C for 10 minutes at 13000 rpm to separate the chromatin from the remaining cell detritus. The supernatant which contains the chromatin was transferred to a 2 ml tube.

Immunoprecipitation

To eliminate other molecules than the chromatin in the sample, 100 µl A/G plus agarose beads were added and the tubes were rotated at 4°C for one hour. The beads, which bind to unspecific molecules, were separated from the dilution by a 2 minute centrifugation at 4°C and 4000 rpm. The supernatant was transferred to a new tube. A BCA-assay (see section 3.2.1) was accomplished to determine the protein concentration in each lysate.

For each immunoprecipitation, 0.5 mg of protein were used and the volumes were transferred to a siliconized 0.6 ml tube, respectively. The siliconized tubes were employed because of their low-binding surface to avoid binding of antibodies and beads to the tube barriers. To compare the result of the experiment to a control sample, 100 µg of protein were used as input control, which was not immunoprecipitated. Then each tube was filled up to 200 µl with RIPA buffer containing proteinase inhibitors. After that, 3 µg of the according antibody was added to the lysates. The monoclonal c-terminal C/EBPα antibody (Epitomics) was used to precipitate C/EBPα binding sites, and, as a control antibody, a polyclonal rabbit IgG antibody was applied in one sample. No antibody was added to the input control. The tubes were rotated at 4°C over night to let the antibodies attach to the proteins.

Wash steps and elution

After immunoprecipitation over night 40 µl of A/G plus agarose beads were added to the samples. During a one hour rotation at 4°C the beads bind to the antibody. After the rotation the samples contain a linkage between agarose beads, antibodies, proteins and

according DNA-fragments. Via centrifugation for 2 minutes at 4°C at 4000 rpm, these linked chains were separated from the rest of the sample and the supernatant was discarded. Then the beads were washed with the IP wash buffers in the according order presented in the grey box below. From each buffer, 400 µl were added to the bead pellets and the tubes were shaken 2 minutes with each wash step, respectively, and centrifuged at 4°C at 4000 rpm for 2 minutes. After every wash step, the supernatants were discarded. The wash buffers were used as follows:

1x Low salt buffer
2x High salt buffer
1x Lithium chloride buffer
2x TE buffer.

After the last wash step with TE buffer, the beads were shaken for 15 minutes with 100 µl elution buffer, respectively, which breaks up the linkage between beads and antibodies. The tubes were centrifuged for 2 minutes at 4°C and 4000 rpm and the supernatants were collected in new tubes. To increase the outcome of the immunoprecipitation, the elution step was carried out two times, resulting in a 200 µl volume after combining the two eluates. During wash steps and elution, the input control was kept on ice.

"Reverse Crosslinking" and removal of proteins

By reverse crosslinking, the linkage between the transcription factor proteins and the DNA breaks up. This was accomplished by addition of 8 µl 5 M sodium chloride and 2 µl ribonuclease A of a concentration of 32.5 mg/ml. The samples were incubated at 65°C over night. From this step on, the input control was included again. After the incubation, 4 µl 0.5 M EDTA, 4 µl 1 M Tris HCl (pH 6.5) and 4 µl proteinase K were added and the tubes were incubated at 42°C. Thereby the transcription factors bound to the DNA as well as the antibodies are decomposed.

DNA purification

For the purification of the remaining DNA from the ChIP, the "Qiagen PCR purification kit" was employed. 200 µl of ChIP product were mixed with 1 ml PB buffer and applied to Qiagen columns in two steps. The columns were centrifuged at 13000 rpm for 1 minute. After that the DNA on the membranes was washed with 750 µl PE buffer and the column was once centrifuged without addition of buffer. To eluate the purified DNA, the columns were incubated at room temperature for 15 minutes after addition of 30 µl ddH2O and then centrifuged for 1 minute at 13000 rpm. After this step, the sample contains only the purified, unbound DNA fragments of interest. Figure 3.1 represents the procedure of the ChIP with C/EBPα as an example for a transcription factor. The results of the ChIP were analyzed with quantitative realtime PCR, as described in section 3.1.1.

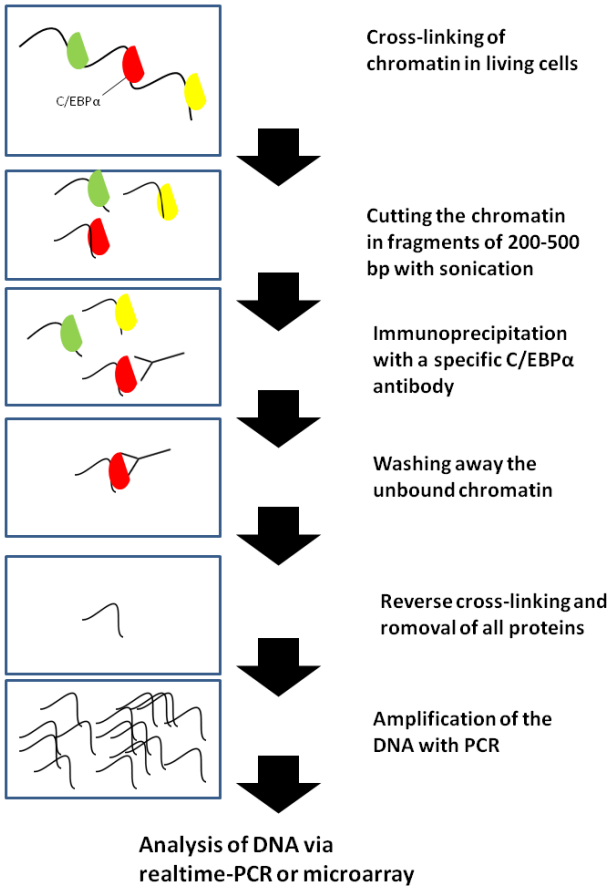


Figure 3.1: Finding transcription factor binding sites of C/EBPα with ChIP.
Source: Modified by Orlando, V. (2000) Mapping chromosomal proteins in vivo by formaldehyde-immunoprecipitation. Science, 0004(March), 99-104.

3.1.2 Amplification of DNA fragments via polymerase chain reaction (PCR)

PCR is employed for amplification of defined DNA fragments. This is achieved by quick changes of the reaction temperature and an enzyme called polymerase. To provide a double strand start site for the polymerase enzyme, primers, which are approximately 20 bases long oligonucleotides, are used. These primers are complementary to the start and the ending of the fragment which should be amplified. At first, the double strand template DNA is denaturated to single strands. Next follows the annealing phase, in which the primers hybridize with the complementary DNA sequence. During the third phase, the elongation, the polymerase attaches desoxyribonucleotides to the single strand, starting from the primer sites. After that a new denaturation follows, in which the sample contains twice as many single strands as before. By repetition of this procedure for 30 to 50 cycles, the amount of complementary single strands increases exponentially. A terminal elongation step serves to complete the fragments. PCR was performed following the program stated below:

Step 1: denaturation: 2 minutes, 93°C
Step 2: denaturation: 3 minutes, 93°C
Step 3: annealing: 30 seconds, 60°C
Step 4: elongation: 1.5 minutes, 72°C
} 35 cycles
Step 5: elongation: 40 minutes, 72°C

The ideal annealing temperature depends on the base composition of the primers used. For a higher GC content in the primers, a higher annealing temperature should be chosen.

The reaction mixture for one PCR contained:

0.5 µl Biotherm taq polymerase
2.5 µl Biotherm buffer
1 µl dNTPs
0.5 µl forward primer (dilution 1:100)
0.5 µl reverse primer (dilution 1: 100)
2 µl DNA template
13 µl ddH2O.

The primers employed were constructed with the programs „Primer Express 3.0“ and “NCBI Primer Blast”. The sequences are listed in section 2.3.

3.1.3 Isolation and purification of mRNA from HL60 cells

Incubation of HL60 cells (see 3.4.2) was stopped after 24 hours by centrifuging the cells at 1400 rpm for 5 minutes, washing with PBS buffer and resuspending the pellet in 350 µl 1x RLT buffer. The isolation of mRNA was carried out with the Qiagen RNeasy Kit. First, the lysates were applied to the “Qia Shredder Columns”, respectively, and centrifuged at 13000 rpm for 2 minutes. The columns were discarded and the flow-through in the collection tubes was mixed with 350 µl 70% RNAse-free ethanol. After that, the content was applied to the “easy-mini columns” and again centrifuged for 2 minutes at 13000 rpm. The flow-through was discarded and the column was washed by centrifuging it with 350 µl RW1 buffer for 2 minutes at 13000 rpm. To remove the DNA from the column, 70µl RDD buffer was mixed with 10 µl DNAse I and the solution was dripped onto the membrane of each column. The columns were left for 15 minutes at room temperature. In the following, the column was washed again with 350 µl RW1 buffer and two times with RPM buffer. After each centrifugation, the flow-through was discarded and the column was centrifuged again without buffer addition.

Finally, the eluation of the RNA was performed with 30 µl RNAse-free H_2O into RNAse-free tubes. While measuring the RNA concentration (see 3.1.5) the eluted RNA was put on ice immediately and afterwards stored at -80°C.

3.1.4 Reverse transcription

Reverse transcription is a method to obtain complementary DNA (cDNA) from the isolated RNA using the enzyme "reverse transcriptase" (RT). This enzyme synthesizes one RNA-DNA hybrid strand from each RNA single strand, it separates the RNA strand from the DNA strand and produces a DNA double strand from the DNA single strand. For each reverse transcription, 1 µg isolated RNA was exerted and diluted to 15.75 µl with DEPC H_2O. 1 µl RNAse out and 1 µl random primers were added and the solution was kept 5 minutes at 70°C, 5 minutes on ice and then 5 minutes at room temperature. This procedure inhibits the RNAse enzymes in the solution. Then the reverse transcription was started by adding 5 µl 5x M-MLV buffer, 1.25 µl desoxy-ribonucleotides (dNTPs) and 1 µl M-MLV reverse transcriptase (RT). The samples were incubated for 1 hour at 42°C. Then the reaction was stopped by incubation at 70°C for 15 minutes. The produced cDNA was filled up to 200 µl with ddH_2O and stored at -20°C.

3.1.5 Photometric DNA and RNA concentration measurement

To determine DNA and RNA concentrations in samples, a NanoDrop spectrophotometer was used. 2 µl of the sample were applied to the pedestal of the device and photometric measurement at a wavelength of 260 nm was started. To exclude protein contamination, absorption at a wavelength of 280 nm was also measured.

3.1.6 Quantitative realtime PCR (SYBRgreen)

Quantitative realtime PCR, a method based on conventional PCR (see section 3.1.2), employs fluorescence measurement to quantify DNA amounts during the DNA amplification. The marker "SYBRgreen" starts to fluoresce when it intercalates with DNA. The fluorescence correlates with the amplification of DNA and is measured during the entire PCR reaction. Realtime PCR was used for the quantification of mRNA transcripts in HL60 cells after reverse transcription, as well as for the quantification of DNA resulting from chromatin immunoprecipitations. Due to the different amounts of template DNA in the samples, 40 PCR cycles were carried out when mRNA quantification was needed, but 50 cycles for chromatin immunoprecipitation DNA amplification.

The reaction mixtures for the realtime PCR were prepared in 96-well plates under sterile conditions to avoid contamination with DNA from the environment. Each mixture contained:

6.25 µl SYBRgreen Mastermix
3.15 µl ddH20
0.3 µl forward primer (dilution 1:100)
0.3 µl reverse primer (dilution 1:100)
2.5 µl template DNA.

The primers were constructed using the programs "Primer Express 3.0" and NCBI Primer Blast. Each primer sequence is specified in section 2.3. For mRNA quantification, exon-spanning primers, which bind specifically to the cDNA of the according mRNA transcript crossing a linkage between two exons, were constructed with Primer Express 3.0. For chromatin immunoprecipitation analysis primer pairs that bind to the gDNA sequence were constructed for locations in the Id1 and Id2 promoters, which have been identified with the program TFsearch (39). To evaluate the Id1 enhancer, two primer pairs were constructed, one of them in the assumed enhancer region of the gene, which is in the 3' region 4998 basepairs downstream from Id1 ("3PRE", see 2.3), and the other one 928 bp basepairs upstream from Id1 ("5PCE").

Binding of the transcription factor of interest to the enhancer was verified by the detection of an enrichment of the DNA fragment for “3PRE” in comparison to “5PCE”. The primers for the locations “3PRE” and “5PCE” were first constructed by Wagner et al. (130).

To control the purity of the reaction mixtures, every realtime PCR had a control mixture without addition of template DNA. This control did not show any DNA amplification. After completion of the realtime PCR, the sample temperature was increased slowly and continuously, and the denaturation point of the DNA, the moment in which the SYBRgreen marker is released and fluorescence decreases rapidly, was measured. This additional step was used to exclude contaminations of the samples with unspecific PCR products by determination of the DNA melting point.

The results were interpreted on the basis of Ct (cycle threshold) values: The cycles are counted until the DNA amount increases exponentially, which leads to a sudden increase of the fluorescence which exceeds the background fluorescence. The Ct value is the number of cycles performed until a certain threshold of fluorescence is reached.

3.1.7 Restriction digest of DNA plasmids

Restriction digests were performed to check the plasmid identities before transfection. In a restriction digest the DNA is fragmented with bacterial enzymes called restriction endonucleases. In this case, type II endonucleases, which are enzymes that cut DNA at a specific, usually a palindromic sequence, were employed, for example BamHI, HindIII, KpnI, BspHI and NcoI. One restriction digest contained:

- 2 U restriction enzyme
- 2 µl buffer recommended by the enzyme manufacturer
- 1 µg DNA
- ddH2O to a total volume of 20 µl.

The tubes were incubated for 2 hours at the temperature recommended by the enzyme manufacturer which was in most cases 37°C. The success of the digest was checked by agarose gel electrophoresis (see section 3.1.8).

3.1.8 DNA fragment separation by agarose gel electrophoresis

Agarose gel electrophoresis is a method to separate DNA fragments according to their length. By applying an electric field to the gel, the nucleic acid molecules move through the gel matrix to the positive pole because they are electronegative. The shorter fragments migrate faster than the longer ones because they can move through the gel more easily.

Agarose gel electrophoresis was performed to check the success of PCR amplifications of DNA, to evaluate chromatin immunoprecipitation results, to analyse restriction digests and to verify the correct fragment size of sonicated DNA for chromatin immunoprecipitation. Depending on the size of the fragments which should be separated, the gel was prepared with 0.5 to 2.5% agarose. The agarose was diluted in TAE buffer, boiled and poured into a gel chamber to cool down and become solid. The DNA was mixed with 5x concentrated loading dye and 20 µl of each DNA solution were placed in each slot, respectively. A length marker was added into one slot of each gel as a scale. The voltage applied to the gels was 120 V for 45 to 75 minutes, depending on the agarose concentration and the size of the fragments. To visualize the DNA bands after agarose gel electrophoresis, the gels were shaken slowly in an ethidium bromide bath for 30 minutes. Ethidium bromid intercalates with DNA and fluoresces in ultraviolet light. With the help of ultraviolet illumination the bands were visualized and the gel was photographed for documenation.

3.2 Protein biochemical methods

3.2.1 Protein concentration measurement by BCA assay

In a BCA assay the protein concentration of solutions can be determined by colorimetric measurements of a colour change from green to purple. The BCA solution contains cupric sulfate in a highly alkaline solution. The peptide bonds of proteins reduce the Cu^{1+} ions from cupric sulfate as soon as the sample is kept in a warm temperature, ideal is 37°C. The amount of Cu^{1+} ions in the solution depends on the protein concentration of the solution. Two molecules of bicinchoninic acid (BCA) form chelates with each of the Cu^{1+} ions and a purple-coloured product results which absorbs light at a wavelength of 562 nm.

The BCA-assays were performed with a Pierce BCA protein assay kit. The reaction mixtures were incubated for 30 minutes at 37°C and the measurement at 562 nm was performed with the NanoDrop photospectrometer. Using a dilution series of bovine serum albumin (BSA), the samples of interest were compared to the calibration line and the protein concentration was determined.

3.3 Microbiological methods

3.3.1 Transformation of competent E. coli cells

Transformation is the uptake and expression of DNA which is integrated into the cell through the cell wall. Transformation of E. coli bacteria was performed to achieve a quick, effective amplification of plasmids which were needed for further experiments. For each plasmid transformation, 50 µl of DH5α competent E. coli bacteria were thawed slowly on ice and transferred into 15 ml tubes. Depending on the concentration of the DNA, 1 to 5 µl of DNA were added to the bacteria and the tubes were incubated on ice for 30 minutes. To cause pores in the cell walls, the cells were heat shocked in a 42°C water bath for 50 seconds. After that a two minute incubation on ice followed.

Then 800 µl of LB medium was added and the cells were shaken in a 37°C incubator for 1 hour. To select the positive clones which express the plasmid DNA, 100 µl of the culture were streaked on LB-Amp plates. All the transformed plasmids include an ampicillin resistance gene, which means that only cells that express the plasmid can grow on the ampicillin containing plates during the incubation at 37°C over night.

After 14 hours, one of the grown colonies was picked up with a pipette tip and dropped in a 1000 ml Erlenmeyer flask with 250 ml LB medium containing 1 µl/ml ampicillin. The inoculated cultures were shaken over night in a 37°C incubator. Before the plasmid was isolated from the bacteria, a bacteria stock was prepared out of 900 µl bacteria and 70 µl DMSO, quickly cooled on ice and stored at -80°C. A new culture was inoculated with approximately 1 µl of this bacteria stock when needed.

3.3.2 Plasmid isolation from bacteria (Maxi prep)

To isolate the produced plasmids from the bacteria cultures, plasmid preparations were performed using the "JetStar 2.0 Plasmid Purification Maxi Kit". At first the bacteria were centrifuged for 30 minutes at room temperature and 4300 rpm, resuspended in 10 ml cell suspension buffer and transferred to 50 ml tubes. The cells were lysed by adding 10 ml alkaline cell lysis buffer and incubating the suspension for 5 minutes. To neutralize the lysate, 10 ml neutralization buffer was added. To precipitate proteins and genomic DNA the tubes were directly inverted 5 times and centrifuged at 4300 rpm for 20 minutes. The maxi prep columns were equilibrated with 30 ml equilibration buffer. The clear supernatant from the centrifuged lysate was added to the column. In this step the plasmid DNA binds to the column. By adding 60 ml washing buffer to the column in two steps, the DNA was washed and finally, the plasmids were eluted with 15 ml elution buffer into a fresh tube.

To precipitate the plasmid DNA, the elution was mixed with 10.5 ml isopropanol and centrifuged for 45 minutes at 4°C at 5000 rpm. After that, the DNA was washed by dissolving the pellet in 5 ml 70 % ethanol and centrifuging for 15 minutes under the same conditions as above. The DNA pellet was dried and dissolved in 300 µl ddH2O.

The DNA concentration was determined using the NanoDrop photospectrometer (see section 3.1.5). In some cases restriction digests (see section 3.1.7) were performed to verify the plasmid identity. The plasmid DNA solution was stored at -20°C.

3.4 Cell biological methods

3.4.1 Cell culture

All cell lines were kept in a cell incubator at 37°C, 5% CO_2 and 90% relative air humidity. The cells were passaged every 2 to 3 days, never exceeding 75% of the maximum density according to the provider.

HL60 and 32D cells are suspension cells and were cultured in RPMI medium containing 10% fetal bovine serum (FCS), 1% glutamine and 1% penicillin/streptomycin. As 32D cells are IL-3 dependent, the medium for 32D cells additionally contained 10% IL-3.

293T cells, which are adherent cells, were kept in DMEM medium with 10% FCS and 1% penicillin/streptomycin. For passaging the medium was removed, the cells were washed with 10 ml PBS on a surface of 75 cm^3 and detached from the surface with 1 ml 1x trypsin. After detaching the cells, they were split using the same procedure as for suspension cells, which means to discard part of the suspension and to fill the rest up with fresh medium.
For longer storage the cells were dissolved in FCS containing 10% DMSO in a cryotube and frozen at -80°C, protected from a quick temperature decrease by a paper envelope. After some days the tubes were transferred to a liquid nitrogen tank.

3.4.2 Incubation of HL60 cells

For mRNA analysis and chromatin immunoprecipitation, HL60 cells were incubated with differentiation-inducing agents (see below). For each condition, $2x10^7$ cells were

cultured in 50 ml RPMI medium containing 10% fetal bovine serum, 1% glutamine and 1% penicillin/streptomycin. Cells were incubated with

- 0.01 % DMSO
- 0.25 mM CDDO
- 0.5 µM CDDO
- 1 µM ATRA.

The duration of incubation (measured between addition of the compound to the medium and washout) was between 2 and 24 hours for mRNA analysis and constantly 24 hours for chromatin immunoprecipitation.

3.4.3 Transfection of 293T cells

Transfection is the process of bringing genetic material into eukaryotic cells. It was used to transfer luciferase and renilla vectors as well as other expression vectors into cells to determine promoter activities under different conditions. 293T cells can be transfected using the lipofectamine method. 24 hours before transfection, 24 well plates were filled with $5x10^4$ cells per well in 1 ml medium, respectively. For each transfection a tube containing the DNA to be transfected, dissolved in 50 µl NaCl (150 mM) and another tube containing 3.2 µl Nanofectin in 50 µl NaCl were prepared. Both solutions were mixed well, put together, mixed again and incubated for 15 to 30 minutes at room temperature. During the incubation time, DNA and Nanofectin form a complex which can pass the cell membrane.

Applied DNA amount for $5x10^4$ cells:

Luciferase construct: 1 µg
pcDNA3 construct with or without CEBP (α / β / δ): 40 ng
pRL-CMV (Renilla): 40 ng.

Before transfection, 500 µl medium were removed out of each well from the surface. Then 100 µl of the DNA Nanofectin mix was added to the cells drop by drop, in order not to detach the adherent cells. After 4 hours, the wells were filled up with 500 µl of fresh medium. 24 hours later, luciferase assays were performed to measure the promoter activities.

3.4.4 Transfection of 32D cells

32D cells were transfected by electroporation. This method uses the fact that cell membranes become permeable from the impact of the electric voltage and the cells incorporate the DNA. In sterile cuvettes, the DNA was added to $3x10^6$ cells and mixed to resuspend all cells.
Applied DNA amounts for $3x10^6$ cells:

- Luciferase construct: 15 µg
- pcDNA3 vector with or without CEBP (α / β / δ): 400 ng
- pRL-CMV (Renilla): 400 ng

The conditions for electroporation were settled at 320 V and 8 ms. After electroporation, the content of each cuvette was partitioned into 3 wells with 4 ml medium, so every well contained approximately $1x10^6$ cells. The luciferase assays were performed 24 hours after transfection.

3.4.5 Luciferase reporter gene assays

For a luciferase reporter gene assay, a plasmid is needed which contains the promoter that should be analyzed in front of the luciferase reporter gene, so that upon activation of the promoter, the expression of the reporter gene is induced. Luciferase is able to transform the substrate D-luciferine to oxyluciferine and light. By measuring the light intensity in relative light units during this reaction, conclusions can be drawn about the

amount of luciferase enzyme expressed in the cells and, consequentially, the promoter activation. Figure 3.2 explains the idea of luciferase reporter gene assays graphically.

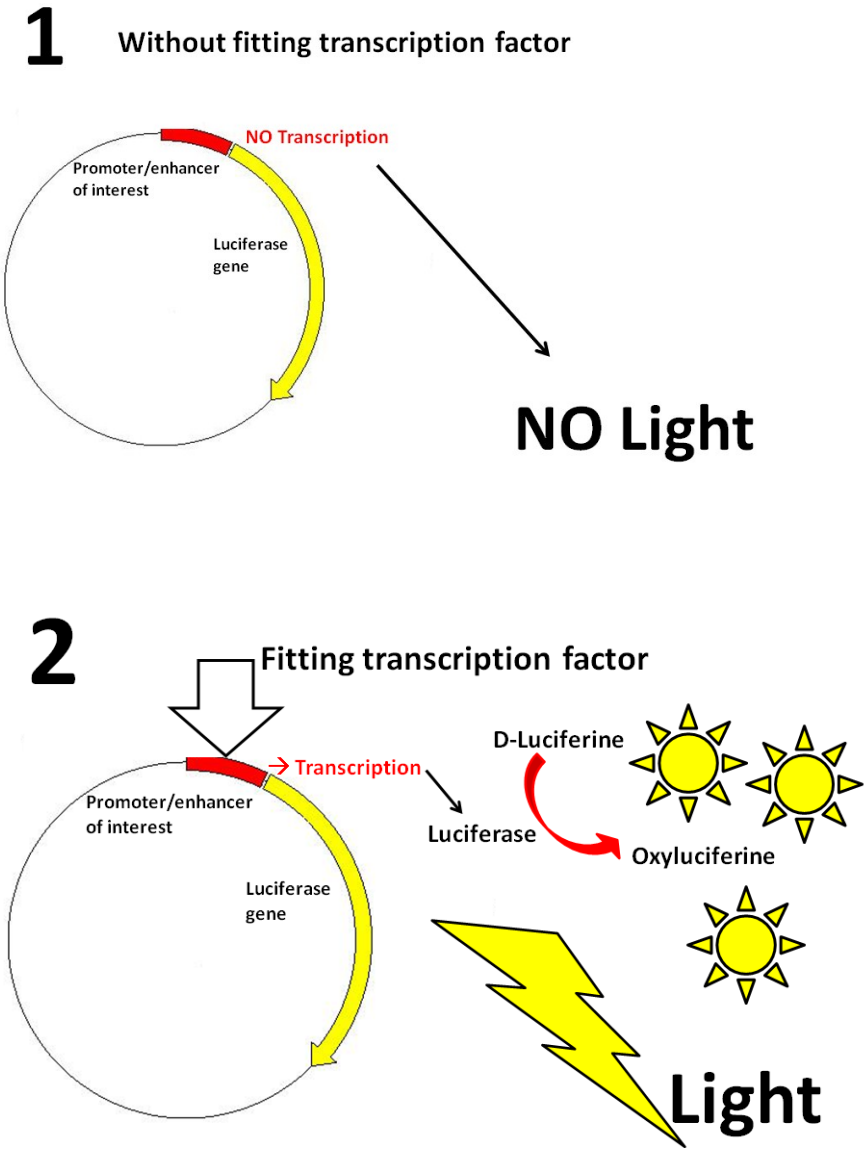


Figure 3.2: Analysis of the activity of genetic regulatory elements via luciferase assays.

In both transfection methods, the lipofectamin method as well as electroporation, besides the luciferase construct, another construct was transfected, which contained the reporter gene "renilla" with the strong promoter of CMV. Renilla was employed to reflect the transfection efficiency, which eliminates mistakes in the experiment

evaluation due to different transfection outcomes in the wells. The ratio of luciferase and renilla value expresses the real induction of the protein.

For determination of the promoter activation of the genes Id1 and Id2, pGL3 vectors by Promega were used, which contained the promoters of each gene, respectively, and the luciferase gene. Additionally, pBluescript vectors were used, which contained a full Id1 promoter (pfLuc-B1) and a mutated promoter (pfLuc-B1S). A detailed description of the different vectors can be found in section 2.5. Luciferase assays were performed with the Dual Luciferase Reporter Assay System by Promega. 24 hours after transfection, the cells were centrifuged, washed with PBS and resuspended in 100 µl 1x passive lysis buffer from the kit. A 15 minute incubation at room temperature followed to complete cell lysis. For lysis of the adherent 293T cells, the medium was removed from the surface, cells were washed with PBS and lysed with 100 µl 1x PLB. After the incubation, the lysates were kept on ice.

Before starting the photometric measurement, the sensitivity was adjusted to the light intensity, which depends on the cell line used for the assay. For 293T cells, in which the transfection efficiency was very high and most of the cells survived the transfection treatment, the sensitivity had to be decreased to 39.9%. In some cases the values were still above the maximum level of the measuring device. In that case the lysates were diluted in 300 to 500 µl 1x passive lysis buffer. For 32D cells, which were electroporated for transfection, a high sensitivity value of 60.1% was chosen, because transfection efficiency was not that high and approximately 50% of the cells did not survive electroporation.

100 µl LAR II was provided in a luciferase measurement cuvette, 20 µl lysate was added and mixed with the buffer, then the tube was brought into the luminometer and the measurement was started. When the luciferase activity had been measured, 100 µl Stop&Glo buffer was added to the lysate, which stops the luciferase reaction and starts the renilla reaction. To get a representative and comparable value, the ratio of luciferase and renilla value was used to express the real activation of the promoter independent from the transfection efficiency.

3.5 Statistical evaluation of data

The results from realtime PCRs and luciferase assays were evaluated with the statistics program “PASW Statistics 18.0” (former SPSS). For all values, Student’s t-tests for paired samples were performed. For all statistical analyzes, the significance level was set to $\alpha = 0.01$. Unless mentioned otherwise, all diagrams of this work present the mean value and the standard deviation of triplicate measurements.

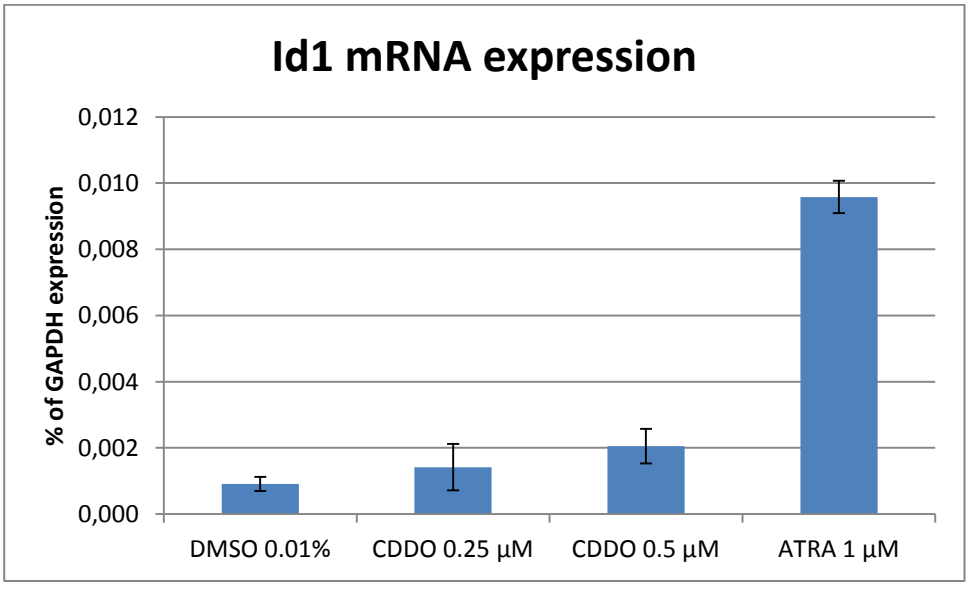
4. Results

4.1 The mRNA expression of Id1 and Id2 is increased by ATRA, of Id2 also by CDDO in HL60 cells

In realtime PCRs with cDNA from HL60 cells treated with DMSO, CDDO or ATRA, Id1 mRNA was 10.5 times increased after 24 hours of ATRA treatment compared to treatment with the vehicle DMSO ($p = 0.0000014$). There was no significant effect after 24 hour CDDO treatment (compare figure 4.1 a). Id1 mRNA expression in HL60 cells was ranging at 0.0009% of GAPDH expression after 24 hours of treatment with DMSO. After 24 hours of ATRA treatment it was increased 10.5 times to a value of 0.0096% of GAPDH expression.

For Id2, the total mRNA content in HL60 cells treated only with the vehicle DMSO for 24 hours was 0.58% of the GAPDH mRNA amount. ATRA treatment increased Id2 mRNA 3.8 times ($p = 0.0004$) to a value of 2.2% of the GAPDH mRNA. With 0.25 µM CDDO treatment, mRNA expression was 0.87%, which is 1.49 times as high as with DMSO ($p = 0.001$). With 0.5 µM CDDO it was 1.52%, which is 2.63 times as high as with DMSO ($p = 0.002$) (compare figure 4.1 b).

a.



b.

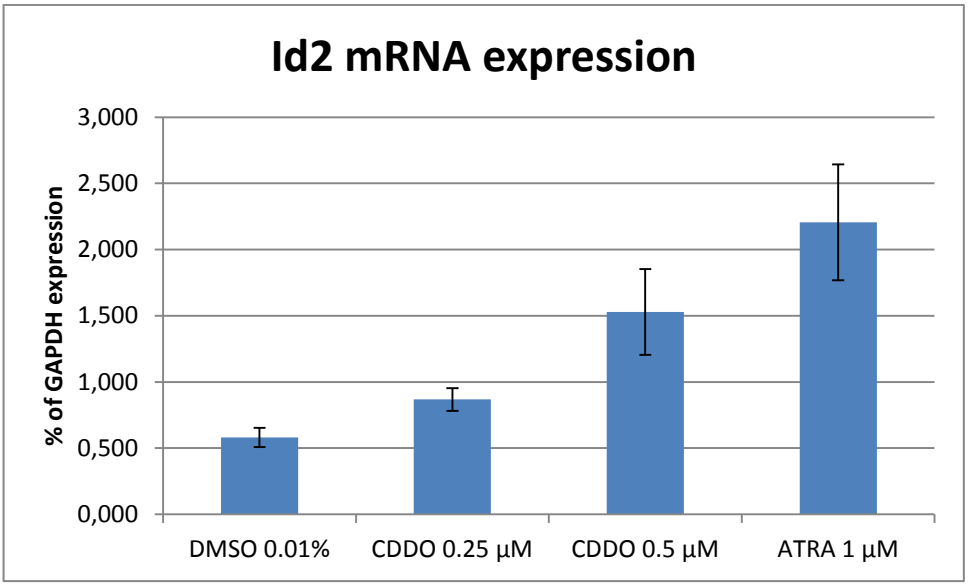


Figure 4.1 a-b: Id1 mRNA is increased by ATRA, Id2 mRNA is increased by CDDO and ATRA. Realtime-PCR with cDNA from HL60 cells. The cells were cultured with 0.01% DMSO (vehicle), 0.25 µM and 0.5 µM CDDO and 1 µM ATRA for 24 hours. The mRNA was isolated and reverse transcription was performed. SYBRgreen realtime-PCR was performed for 35 cycles.

a. Id1

b. Id2

4.2 C/EBPα binds to the Id1 enhancer and to certain binding sites in the Id1 and Id2 promoters in HL60 cells

Binding sites of C/EBPα in the Id1 enhancer and in the Id1 and Id2 promoters were identified using chromatin immunoprecipitation. For immunoprecipitation, binding of a C/EBPα antibody was compared to a rabbit IgG antibody. Another sample, called input, was not immunoprecipitated or washed. For the Id1 enhancer "3PRE", which stands for "3' prime regulatory element", a strong enrichment in the C/EBPα sample was detected compared to the IgG sample. The promoter sequence "5PCE" was also tested and no enrichment could be detected in the C/EBPα sample compared to the IgG sample.

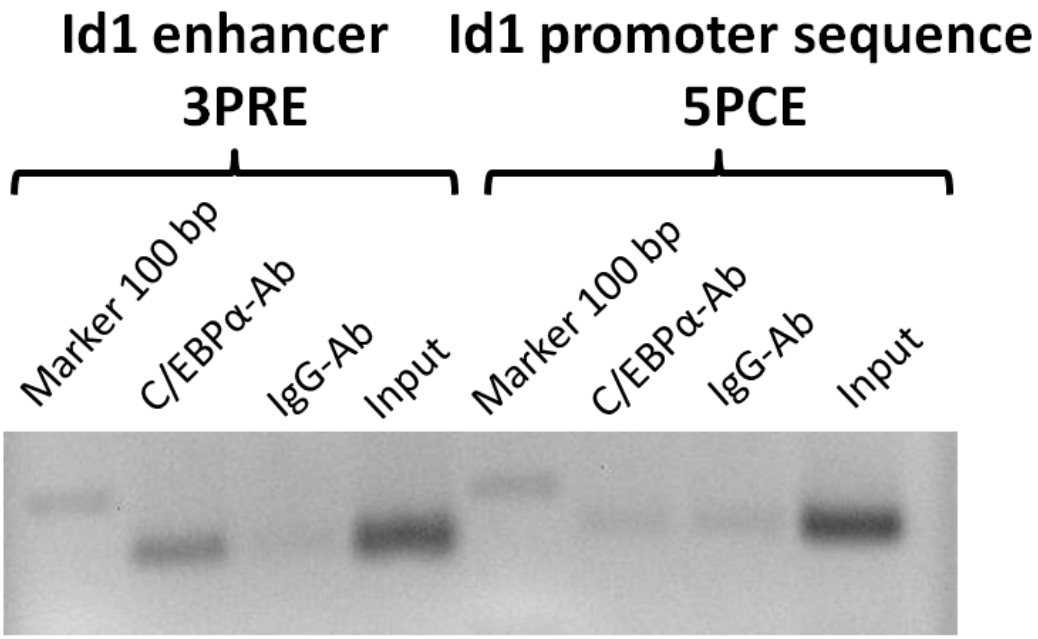


Figure 4.3: C/EBPα binds to the Id1 enhancer "3PRE". Agarose gel electrophoresis of chromatin immunoprecipitation material that was amplified with conventional PCR. Chromatin immunoprecipitation was performed with DNA of HL60 cells using a c-terminal C/EBPα antibody and an IgG antibody. The Input contains the DNA which was not immunoprecipitated and washed. PCR was performed with 34 cycles with primers for the Id1 enhancer sequence "3PRE" and the promoter fragment "5PCE".

The same method was also applied to the assumed C/EBPα binding sites in the Id1 and Id2 promoters which were identified with the program TFsearch (39). Enrichment could be found in the following C/EBPα binding sites (see also figure 4.4):

Id1 promoter:

Id1 motif 2 5' CTTTCCACAC 3' (TFsearch score for C/EBPα: 89.2)

Id2 promoter:

Id2 motif 2-4 5' TGCTTTGGCAACCT 3' (TFsearch score for C/EBPα: 91.5)

5' CCTTTTGCAAAAG 3' (TFsearch score for C/EBPα: 86.5)

5' GTTGCAAAAACA 3' (TFsearch score for C/EBPα: 90.7)

The binding sites in the Id2 promoter "Id2 motif 2-4" are located very close to each other so that only one primer pair could be used for a sequence that includes all three

sequences. This means that the enrichment indicates that at least one of the sequences is a binding site of C/EBPα. No enrichment could be detected for the assumed binding sites Id1 motif 1 (5' CATTAAAAAA 3') (TFsearch score for C/EBPα: 85.3) in the Id1 promoter and Id2 motif 1 (5' CTTATGTAATC 3') (TFsearch score for C/EBPα: 85.7) in the Id2 promoter, as well as in the "5PCE" promoter sequence presented together with the enhancer sequence above.

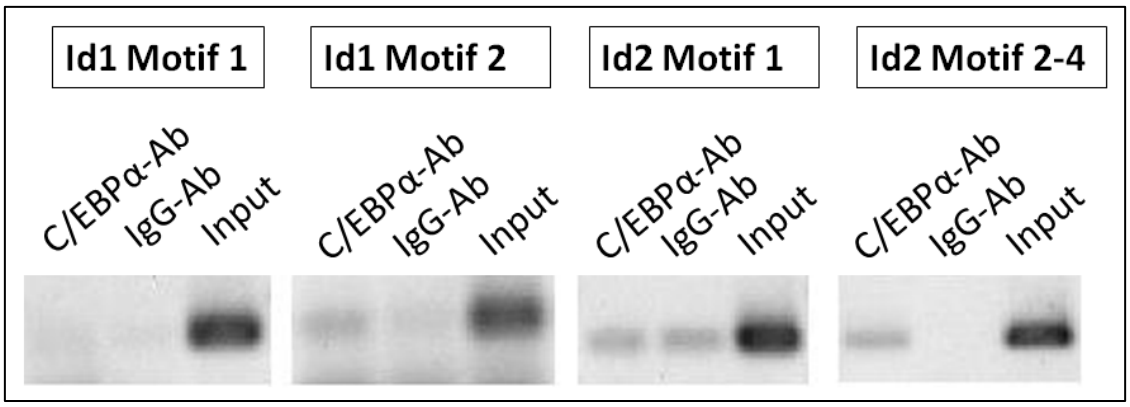


Figure 4.4: C/EBPα binds to the binding sites "Id1 motif 2" and "Id2 motif 2-4". Agarose gel electrophoresis of chromatin immunoprecipitation material that was amplified with conventional PCR. Chromatin immunoprecipitation was performed with DNA of HL60 cells using a c-terminal C/EBPα antibody and an IgG antibody. The input contains the DNA which was not immunoprecipitated and washed. PCR was performed with 34 cycles with primers for the different assumed C/EBPα binding sites in the Id1 and the Id2 promoter.

To sum up, C/EBPα binding in HL60 cells could be confirmed for the Id1 enhancer "3PRE" and for one binding site in the Id1 promoter as well as at least one of the binding sites 2-4 in the Id2 promoter.

4.3 C/EBPα binding to the Id1 enhancer is increased by CDDO and ATRA in HL60 cells

The chromatin immunprecipitation described above was repeated with HL60 cells treated with DMSO, CDDO and ATRA. PCR and agarose gel electrophoresis were performed with primers for the Id1 enhancer "3PRE". For the samples from cells treated with DMSO, the C/EBPα and IgG antibody band both had the same intensity, which

could mean that there is not enough C/EBPα binding to demonstrate it with the performed washing steps. After the same washing steps were performed with samples from CDDO and ATRA treated HL60 cells, an enrichment of the C/EBPα antibody band compared to the IgG band was visible in the agarose gel. The result of the agarose gel is presented in figure 4.5.

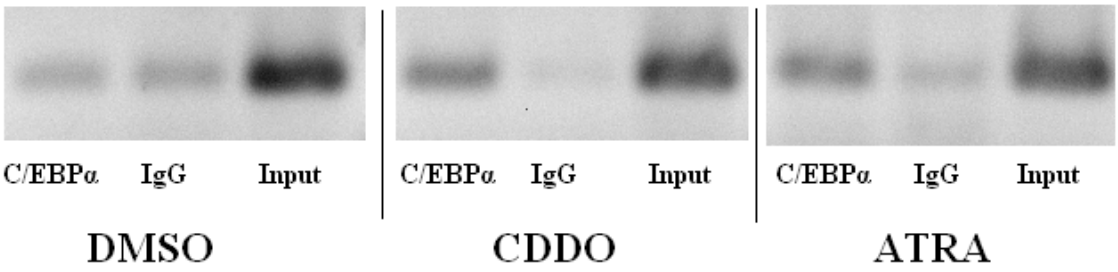
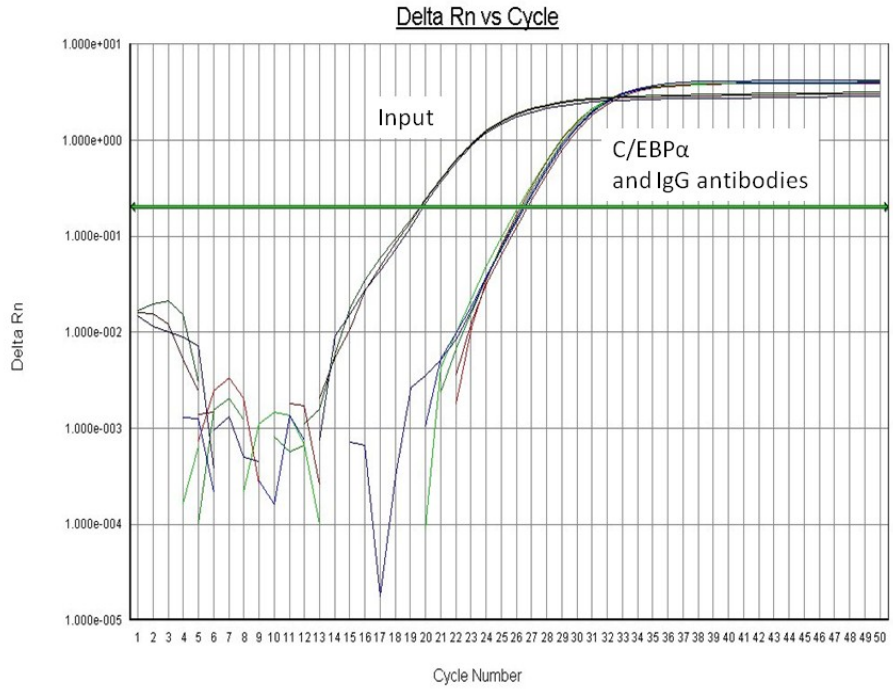
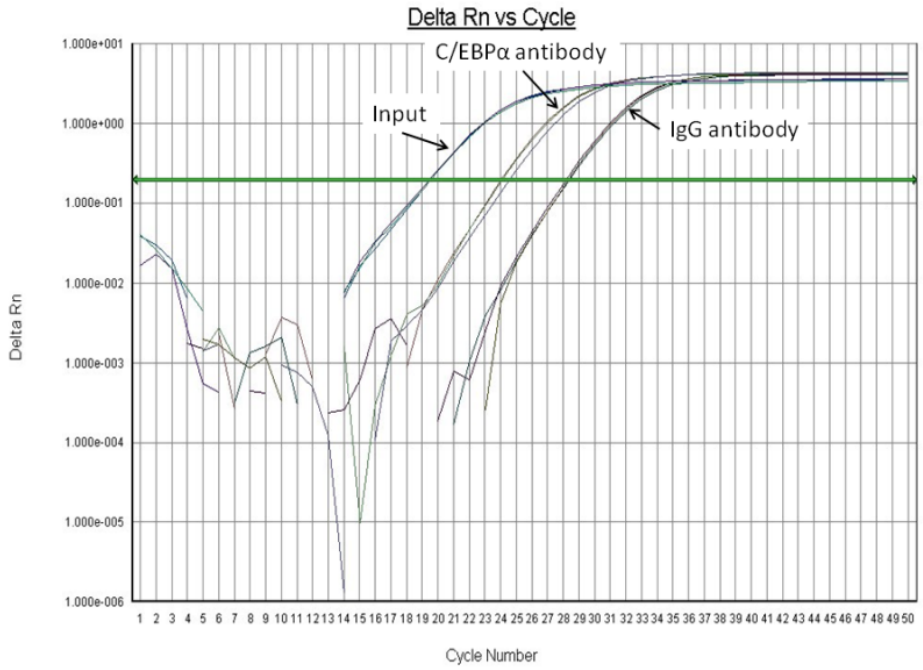


Figure 4.5: C/EBPα binding to the Id1 enhancer is increased by CDDO and ATRA treatment in HL60 cells. Realtime-PCR in triplicates after chromatin immunoprecipitation in HL60 cells. The cells were cultured with 0.01% DMSO, 0.5 µM CDDO and 1 µM ATRA for 24 hours. DNA was isolated and chromatin immunoprecipitation was performed using a c-terminal C/EBPα antibody and an IgG antibody. The input contained DNA which was not immunoprecipitated and washed. Conventional PCR with 35 cycles was performed, the resulting DNA was applied to an agarose gel.

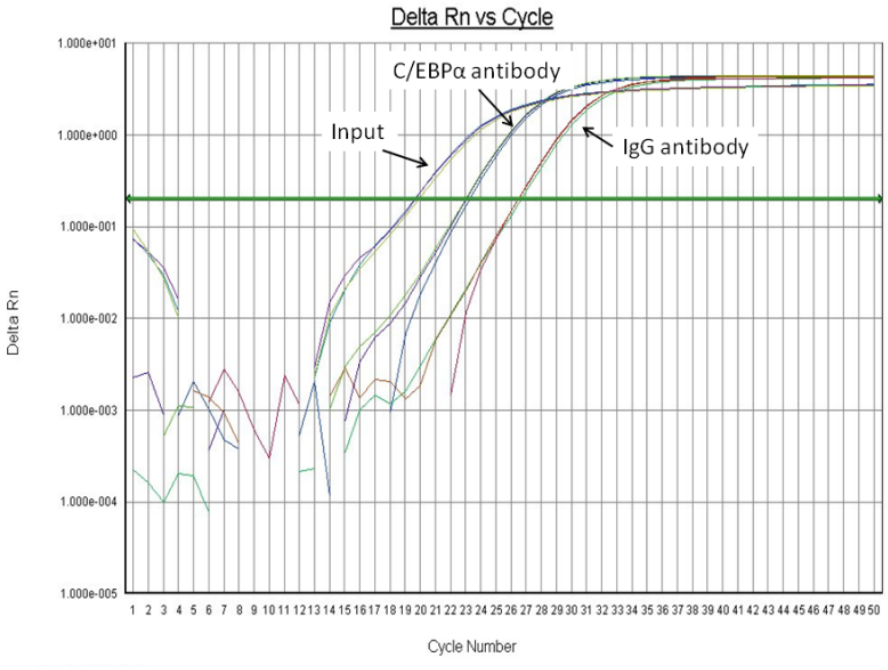
For an exact quantification of the difference between C/EBPα binding in DMSO, CDDO and ATRA treated cells, realtime PCR was carried out. As observed in the agarose gel, for DMSO the C/EBPα antibody and the IgG treated sample contained the same amount of DNA in the realtime PCR. This can be seen in figure 4.56 a, where the graphs for the C/EBPα sample and the IgG sample have the same course and the same Ct value. In samples from CDDO and ATRA treated cells, the C/EBPα sample contained more Id1 enhancer DNA than the IgG sample. Thus it appears that the graphs of the C/EBPα sample rise earlier, meaning after less cycles, than the ones of the IgG sample. In numbers, the enrichment was 4 Ct values for the CDDO treated cells and 3.5 Ct values for the ATRA treated cells. The difference between the Ct values, which is where the graphs cross the green cycle threshold line of the coordinate system, can be observed in figure 4.6 a-c.



a. Cells treated with 0.01% DMSO



b. Cells treated with 0.5 μM CDDO



c. Cells treated with 1 µM ATRA

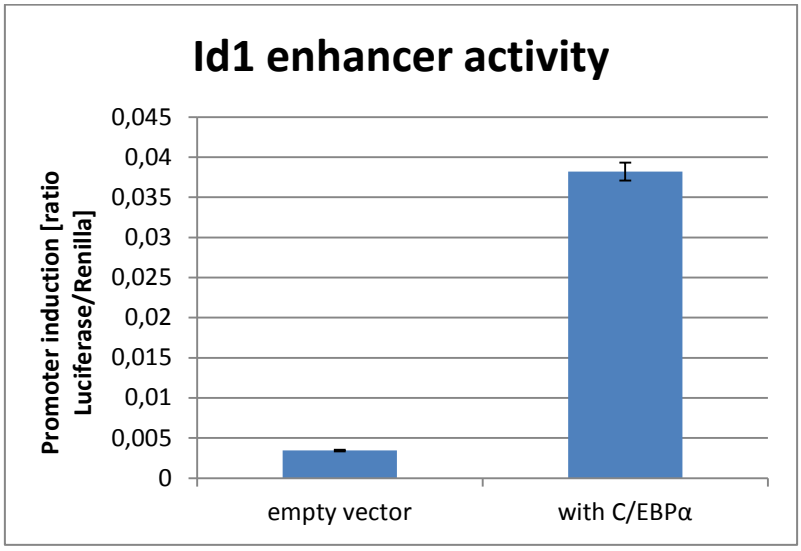
Figure 4.6 a-c: C/EBPα binding to the Id1 enhancer is increased by CDDO and ATRA treatment in HL60 cells. Realtime-PCR in triplicates after chromatin immunoprecipitation in HL60 cells. The cells were cultured with 0.01% DMSO, 0.5 µM CDDO and 1 µM ATRA for 24 hours. DNA was isolated and chromatin immunoprecipitation was performed using a c-terminal C/EBPα antibody and an IgG antibody. The input contained DNA which was not immunoprecipitated and washed. SYBRgreen realtime PCR with 50 cycles was performed in triplicate measurements using the primer for the Id1 enhancer.

4.4 C/EBPα induces the Id1 enhancer tenfold in 293T cells

The effect of C/EBPα on the Id1 enhancer was analyzed with luciferase assays using the luciferase expression regulated by the Id1 enhancer as a marker for the enhancer induction and the renilla expression regulated by a common promoter as an indicator for the expression activity of the cells of each sample. Together with the luciferase and the renilla vector, the cells were transfected either with an expression vector containing C/EBPα or with an empty expression vector as a control. A tenfold higher activity of the Id1 enhancer was measured when the 293T cells were transfected with a C/EBPα

expression vector compared to cells transfected with an empty expression vector. In numbers, the luciferase renilla ratio was increased by a factor of 10, from 0.0034 to 0.038 ($p = 0.0002$). The same experiment was performed with an Id1 enhancer containing a substitution mutation. For the mutated Id1 enhancer the induction was only fourfold, from a ratio of 0.005 to a ratio of 0.022.

a.



b.

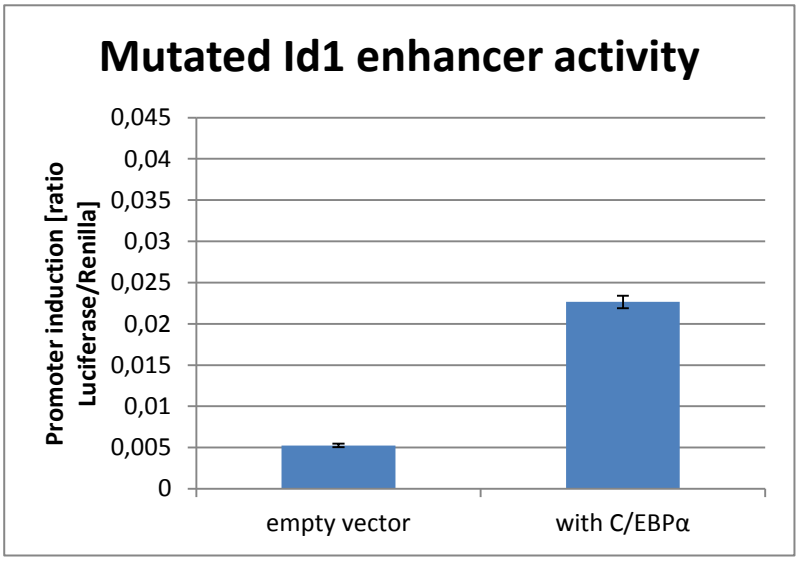


Figure 4.7 a-b: C/EBPα induces the Id1 enhancer tenfold and the mutated Id1 enhancer fourfold in 293T cells. Luciferase assay with Id1 enhancer in 293T cells. 293T cells were transfected either with an expression vector containing the C/EBPα gene or with an empty expression vector. Additionally, the cells were transfected with a luciferase vector containing the Id1 enhancer or the mutated Id1 enhancer and the luciferase gene and with a renilla expression vector. After 24 hours of incubation, the cells were lysed and luciferase assays were performed. The diagrams show the ratio of luciferase and renilla values.

a. Id1 enhancer
b. Mutated Id1 enhancer

4.5 C/EBPβ and C/EBPδ also induce the Id1 enhancer; C/EBPδ has a fourfold stronger effect than C/EBPα, C/EBPβ induction is half as strong as C/EBPα induction on the Id1 enhancer in 293T cells

The luciferase assays with the Id1 enhancer vector were also performed in 293T cells with C/EBPβ and C/EBPδ expression vectors instead of the C/EBPα expression vector. In this experiment, C/EBPδ had a fourfold stronger effect on the Id1 enhancer than C/EBPα ($p = 0.005$) which means that the enhancer is 40 times more active in cells transfected with a C/EBPδ expression vector compared to cells with an empty expression vector ($p = 0.001$).

With the C/EBPβ expression vector, the activity of the Id1 enhancer was only half as strong as with C/EBPα ($p = 0.011$). This means that the Id1 enhancer is 5 times as active as in cells with an empty expression vector ($p = 0.013$).

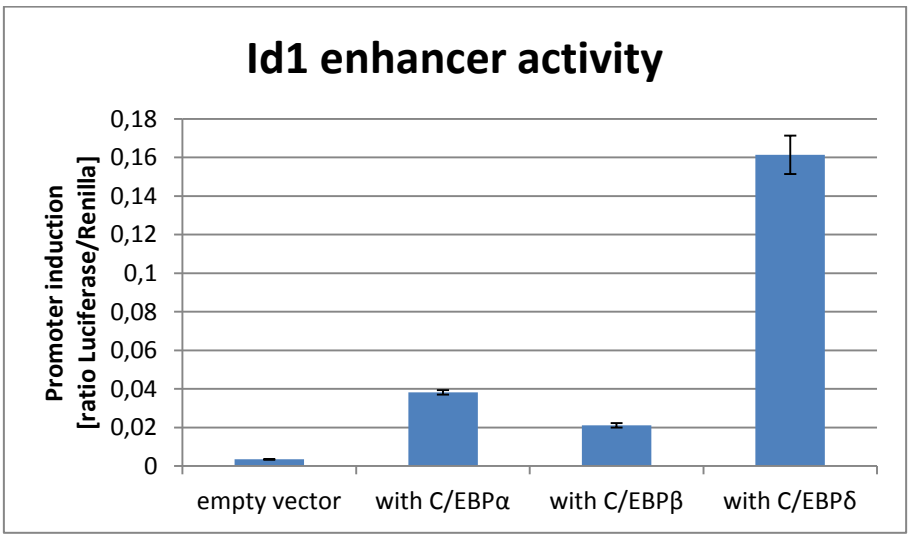


Figure 4.8: C/EBPδ has a fourfold stronger effect on the Id1 enhancer than C/EBPα; C/EBPβ is only half as strong as C/EBPα. Luciferase assay with the Id1 enhancer in 293T cells. 293T cells were transfected either with an expression vector containing the C/EBPα, β or δ gene or with an empty expression vector. Additionally, the cells were transfected with a luciferase vector containing the Id1 enhancer and the luciferase gene and with a renilla expression vector. After 24 hours of incubation, the cells were lysed and luciferase assays were performed. The diagrams show the ratio of luciferase and renilla values.

4.6 C/EBP proteins activate the Id1 promoter in 32D cells, but have no effect on the Id1 promoter in 293T cells

Luciferase assays with a luciferase vector containing the Id1 promoter or the Id2 promoter were performed in 293T and in 32D cells. Additionally, the cells were transfected with an empty expression vector or an expression vector containing C/EPBα, β or δ and with a renilla vector as described above.

A 2.5-fold induction of the Id1 promoter from 0.036 to 0.091 was observed in 32D cells with a C/EBPα expression vector ($p = 0.001$) and a 3.8-fold induction from 0.036 to 0.136 in 32D cells with a C/EBPδ vector ($p = 0.008$) compared to cells with an empty vector. C/EBPβ had no significant effect on the Id1 promoter in 32D cells (compare figure 4.8). The C/EBPα and C/EBPδ induction is less strong than on the Id1 enhancer (shown in item 4.5).

In contrast to the results from 32D cells, there was no significant effect observed in 293T cells for any of the tested C/EBP proteins on the Id1 and Id2 promoters (compare figure 4.9).

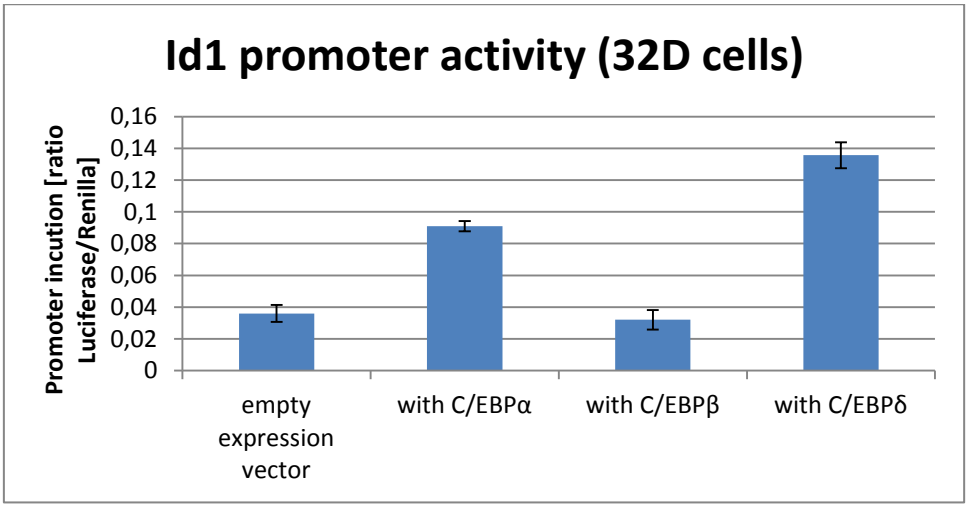


Figure 4.9: The effect of the C/EBP proteins on the Id1 promoter in 32D cells is similar as on the enhancer in 293T cells. Luciferase assay with the Id1 promoter in 32D cells. 32D cells were transfected either with an expression vector containing the C/EBPα, β or δ gene or with an empty expression vector. Additionally, the cells were transfected with a luciferase vector containing the Id1 promoter and the luciferase gene as well as with a renilla expression vector. After 24 hours of incubation, the cells were lysed and luciferase assays were performed. The diagrams show the ratio of luciferase and renilla values.

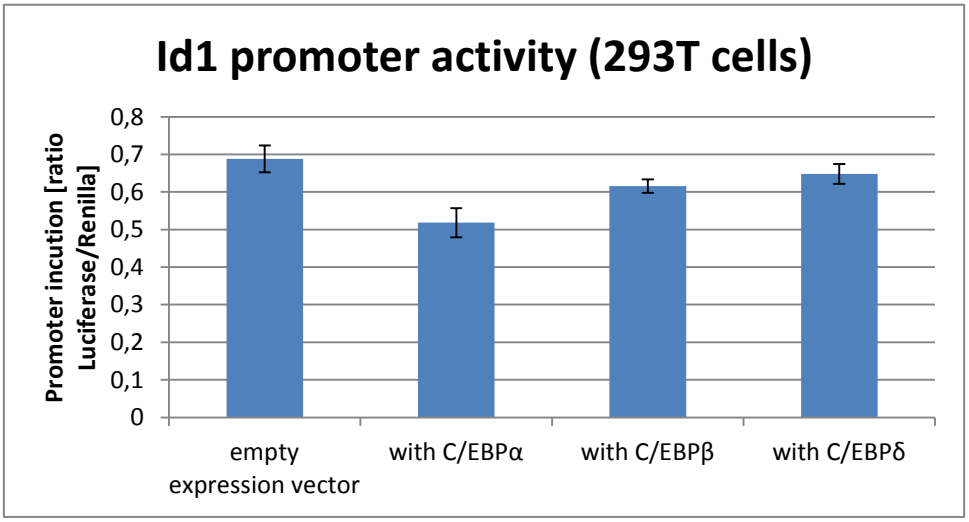


Figure 4.10: C/EBP proteins have no effect on the Id1 promoter in 293T cells. Luciferase assay with the Id1 promoter in 293T cells. 293T cells were transfected either with an expression vector containing the C/EBPα, β or δ gene or with an empty expression vector. Additionally, the cells were transfected with a luciferase vector containing the Id1 promoter and the luciferase gene as well as with a renilla expression vector. After 24 hours of incubation, the cells were lysed and luciferase assays were performed. The diagrams show the ratio of luciferase and renilla values.

5. Discussion

The success of differentiation therapy in APL brought forth the hope that targeted therapies can also be developed for other types of AML. As explained in section 1.6, the aim of this work was to increase the understanding of the connection between the differentiation-inducing drugs CDDO and ATRA, the C/EBP transcription factor family and the genes Id1 and Id2. The results allow us to reach four main conclusions which will be discussed in the following paragraphs.

5.1 Id1 and Id2 are direct target genes of C/EBPα

The results of the ChIPs performed in this thesis show that C/EBPα binds to several promoter binding sites in the Id1 and Id2 promoters and to the Id1 enhancer sequence "3PRE" in HL60 cells. Additionally, the performed luciferase assays revealed that C/EBPα activates the Id1 enhancer in 293T cells and the Id1 enhancer and the Id1 promoter in 32D cells. For the Id1 enhancer containing a substitution mutation, the activation by C/EBPα was less than half as strong as for the normal enhancer, which proves the validity of the findings. The results of the ChIPs and the luciferase assays are consistent with the findings of other researchers describing Id1 induction by CEBPα in other cell types, especially in human CD34+ cells (stem cells) (18) and K562 cells, which are human myelogenous leukemia cells (130). The Id1 enhancer referred to as "3 prime regulatory element" (3PRE), a 74 base pair 3' flanking sequence, was described in the year 1995 in pro-B-cells and activates the transcription of Id1 when it is placed in either direction and at either end of the gene (109). C/EBP binding to the Id1 enhancer has been detected in pro-B-cells by Saisanit and Sun in the year 1997 (110), although the enhancer was specific for this lineage stage and did not function in pre-B-cells or mature B cells. Enhancer activation by C/EBPα has subsequently also been observed in K562 cells (130).

This thesis presents the localization of C/EBPα binding sites in the Id1 and Id2 promoters, which were identified by the TFsearch program and validated by ChIP. It

has been shown previously that one sequence in the Id1 promoter called “5PCE” is no binding site for C/EBPα (130), which was confirmed in this thesis. However, two other regions each were tested for Id1 and Id2 and in each of these, C/EBPα binding could be shown.

It is an established theory that the differentiation block in AML is caused by an aberration of a transcriptional regulator (30, 135, 138). Mutations in the gene of the transcription factor C/EBPα occur in nearly 10% of the patients (94), and also down-regulation of C/EBPα mRNA and protein expression are often observed in AML cells (77, 93). These facts indicate that C/EBPα plays an important role in the development of AML, which is underlined by the finding that C/EBPα expression is crucial for differentiation of granulocytes (137). Following this concept, it has been shown that in mouse models, overexpression of C/EBPα can abolish leukemia and improve survival (65, 127) and that on the other hand, certain C/EBPα mutations can induce AML-like transformation of the granulocytic lineage in mice (101). The C/EBPα pathway is also relevant concerning the mode of action of the compounds CDDO and ATRA, which have differentiation-inducing effects. CDDO enhances C/EBPα function by induction of the active C/EBPα form “p42” in comparison to the inactive form “p30” on the translational level (59), and during ATRA induced granulocytic differentiation, C/EBPα protein is increased by induction of C/EBPα mRNA (104).

As C/EBPα regulates the expression of many genes which induce granulopoiesis and proliferation arrest (18), the finding that Id1 and Id2 are direct target genes of C/EBPα matches with the fact that Id1 and Id2 enhance neutrophil development (12) and inhibit erythroid differentiation (74). This fact indicates that they might play a role in C/EBPα induced differentiation. Interestingly, it has been described that up-regulation of Id1 and Id2 inhibits proliferation and induces a G0/G1 accumulation in NB4 cells, a human APL cell line, and in primary APL blasts (86), which may suggest that Id1 and Id2 represent capable target genes for differentiation therapies.

Nevertheless, it has been shown that overexpression of murine Id1 in human hematopoietic cells alone does not lead to a block in erythrocyte differentiation, which indicates that other genes cooperate with Id1 to shift the differentiation program away

from the erythroid lineage or that the level of Id1 expression must be within a certain range to generate a block in erythroid differentiation (130).

In conclusion, it has been shown in this thesis that Id1 and Id2 are direct target genes of C/EBPα, suggesting that they are associated with the differentiation of the neutrophil lineage, which is supported by the results of other publications (12, 86).

Paradoxically, literature generally describes Id proteins as positive regulators of cell growth and proliferation by direct interaction with other proteins (44, 64) as well as through the inactivation of tumor suppressors (4, 16, 89). Expression of Id1 and Id2 is down-regulated during the differentiation of a variety of cell types (51, 61, 121, 122). Id proteins are even considered potential proto-oncogenes due to the overexpression of Id1 that has been found in many types of human cancer (114). In general, overexpression of Id proteins in human cancer cells leads to induction of cell proliferation, invasion and protection of the cells against drug-induced apoptosis (20, 68, 71, 72, 91). Furthermore, Id1 is a common downstream target of constitutively activated oncogenic tyrosine kinases in leukemia cells such as BCR-ABL and FLT3/ITD (125). Down-regulation of Id1 translation by siRNA inhibits cell growth and increases apoptosis of Molm-14 and K562 leukemia cells which express the BCR-ABL and FLT3/ITD tyrosine kinases and exhibit high levels of Id1 expression (125). With this information, it is not astonishing that also in AML, a high Id1 expression is associated with both a shorter disease-free and overall survival and with lower complete remission rates in younger patients, even suggesting the use of Id1 as a molecular marker for risk classification of AML (126). Additionally, overexpression of Id1 leads to a myeloproliferative disease in mice (118).

As could be shown in the results of the ChIPs and luciferase assays performed for this thesis, Id1 and Id2 are direct, up-regulated target genes of C/EBPα, which is remarkable as C/EBPα is known as a tumor suppressor protein inducing differentiation and proliferation arrest, whereas the Id proteins are positive regulators of cell growth and proliferation by many different ways of action and overexpression of Id1 indicates a worse prognosis in many different types of human cancer including AML.

The explanation for this might be that, although Id1 induces proliferation, at the same time Id1 might be essential to inhibit erythroid differentiation (74) and open the way for

the neutrophil lineage. The fact that residual expression of C/EBPα is crucial to induce myeloid lineage identity in malignant hematopoietic progenitor cells and that down-regulation of Id1 by RNA interference impairs C/EBPα induced granulocytic differentiation (130) supports this statement. At the same time Id1 mRNA is expressed in 32D cells and the Id1 expression decreases during terminal differentiation into granulocytes (12, 61). This is compatible with the theory that at the start of hematopoiesis Id1 is required for the determination of the neutrophil cell line and exclusion of the erythroid cell line, which might be the reason why it is induced by C/EBPα, but it is not needed for the further process of granulopoiesis, when Id1 expression is decreased after the cell line is determined.

Identifying C/EBPα target genes and analyzing their regulation can maximize understanding of the development and prognosis of AML. The target genes can give insight into the mode of action of CDDO and ATRA and offer the chance to emerge new therapy approaches for AML. Especially genes like Id1 and Id2 are highly interesting in this context as their function is presumably associated with differentiation and proliferation in leukemic cells. The finding that Id1 and Id2 are direct target genes of C/EBPα is therefore a highly promising basis for further research.

5.2 ATRA increases Id1 and Id2 expression, CDDO increases Id1 expression

It has been shown here that by ATRA treatment, Id1 and Id2 mRNA are strongly increased in HL60 cells. These findings correlate with the existing literature reporting that ATRA induces Id1 and Id2 in the APL cell line NB4 as well as in primary patient cells (86, 139).

ATRA is already being applied very successfully for the treatment of APL. Remarkably, ATRA induces an up-regulation of C/EBPα on the transcriptional level (104). Considering the induction of Id1 by C/EBPα in HL60 cells, which has been shown here, it is likely that the up-regulation of Id1 by ATRA is mediated by the up-regulation of C/EBPα. This is supported by the finding that under ATRA treatment,

C/EBPα binding to the Id1 enhancer is stronger than with DMSO, demonstrated by the results of the ChIP which has been performed in this thesis. The different results concerning the enrichment for the Id1 enhancer with DMSO treatment in the first and in the second experiment (compare 4.2 and 4.3) probably arise from a different effect of the wash steps, a very sensitive factor which is a great disadvantage of ChIP experiments. It often limits the interpretation of the result to a comparison with samples from the same experiment run.

CDDO did not have a significant effect on Id1 mRNA, but it increased Id2 mRNA. Growth-suppressive effects of CDDO could be shown for many different types of human cancer, but its molecular effects still remain unclear. Interestingly, a probably very important mode of action of CDDO is the translational up-regulation of the active form of C/EBPα, p42 (59). However, CDDO treatment of HL60 cells does not induce Id1 mRNA but only Id2 mRNA after 24 hours as realtime PCR experiments in this work revealed, even though C/EBPα binding to the Id1 enhancer is strengthened by CDDO treatment as it is by ATRA treatment, which was shown by ChIPs.

For an explanation, it should be considered that the stimulation time of 24 hours might be too short for Id1 induction following C/EBPα induction. It is possible that CDDO needs longer time for C/EBPα induction than ATRA as it works on the translational level (59). Another possibility is that after CDDO treatment other transcription factors play a contradicting role to C/EBPα, so Id1 is not induced as it is after ATRA treatment. Furthermore, it is possible that Id1 is induced, but that at the same time contradicting proteins or miRNAs are increased to remove or degrade Id1 mRNA in a CDDO stimulated HL60 cell.

5.3 C/EBPβ and C/EBPδ also induce the Id1 enhancer, but C/EBPδ has a tenfold stronger effect, while C/EBPβ is only half as strong as C/EBPα

Although C/EBPα is the most widely studied transcription factor of the C/EBP family in issues of hematopoiesis and leukemia, other C/EBP family proteins do also play a role here.

In the luciferase assays performed in this work, induction of the Id1 enhancer 3PRE was not only achieved with C/EBPα but also with C/EBPβ and C/EBPδ in both 293T and 32D cells, which stresses the similar roles that these transcription factors play. Nevertheless, a different strength of the different C/EBP proteins in the induction was observed, which can have different reasons: First of all the binding of the transcription factors to the enhancer itself might be characterized by a different strength, but it is also possible that the concentration of the according C/EBP family member which was expressed from the expression vector was different in the cells, for example due to other receptors it bound to or because the proteins were degraded in a different rate during the time of expression. The fact that several C/EBP family members can form heterodimers with each other (19, 99, 131) might also play a role, as the other C/EBP proteins could be regulated differently in the cells, interfering with the C/EBP protein of interest and thus influencing the result of the experiment.

As described above, C/EBPα is essential for granulocytic differentiation (137), it is up-regulated by CDDO and ATRA (59, 104) and is often down-regulated in AML by protein-protein interactions or mutations (77, 93, 94).

Also C/EBPβ protein expression is induced rapidly by ATRA treatment of PML-RARα positive NB4 cells (29). Additionally, C/EBPβ induction is required for ATRA induced differentiation (29), correlating with the findings about C/EBPα induction by ATRA (104). C/EBPβ can drive immature cells into granulocytes (49, 100). Furthermore, it has been shown that expression of C/EBPβ from the C/EBPα gene locus is sufficient for

normal hematopoiesis in vivo (54), indicating that according to hematopoiesis, C/EBPβ and C/EBPα have a similar and at least in part interchangeable role.

C/EBPδ is not up-regulated by ATRA in human myeloid leukemia cells (47), but its expression in BCR-ABL positive cells is also sufficient to induce neutrophil differentiation (34), which indicates that C/EBPδ, too, plays an important role in granulopoiesis. This is supported by the fact that the C/EBPδ promoter is methylated in several AML cell lines, C/EBPδ expression is low in AML cells and C/EBPδ expression in vitro showed growth-inhibiting effects in primary progenitor cells (1).

In summary, it has been shown in this work that C/EBPα, β and δ all activate the Id1 enhancer, but not all with the same intensity. As described above, the three transcription factors play a role in granulopoiesis, inducing neutrophil differentiation and inhibiting the proliferation of hematopoietic cells. C/EBPα and C/EBPβ, but not C/EBPδ are induced by ATRA, but the results of this work demonstrate that C/EBPδ is the strongest inducer of Id1. As explained above, the reason can be a quantitative or a qualitative difference between the C/EBP proteins in the experiment, meaning that either there is an interference concerning the amount of the available transcription factor proteins or the quality of the binding to the enhancer is stronger for C/EBPδ and weaker for C/EBPβ compared to C/EBPα.

5.4 C/EBP proteins induce the Id1 promoter in 32D cells but not in 293T cells

The results of the luciferase assays in this work show that not only the Id1 enhancer 3PRE can be activated by C/EBPα overexpression in 32D cells, but also the Id1 promoter. As the enhancer might be active in the 32D cells also without artificial overexpression, this is not a proof of the independent function of the C/EBPα binding site in the Id1 promoter. However, it is at least an evidence for the importance of this newly found binding site, as the enhancer was thought to be the only source of C/EBPα induction of Id1 previously. The effects of overexpression of C/EBPβ and δ on the Id1 enhancer were also evident in the promoter: C/EBPβ had a weaker effect on the Id1

expression than C/EBPα, C/EBPδ was stronger than C/EBPα, which is consistent with the Id1 enhancer studies.

Nevertheless, C/EBPα, β or δ have no effect on the Id1 promoter in luciferase assays with 293T cells. The reason might be that 293T cells are no hematopoietic cells and the Id1 induction is in some way blocked. In contrast to the promoter, the Id1 enhancer could be activated by CEBPα in 293T cells, so another possibility is that the enhancer is needed to induce transcription and that it is not active on its own in 293T cells. Further experiments are needed to detect the exact way the promoter and the enhancer are cooperating.

5.5 Conclusion and perspectives

The aim of this thesis was to draw a connection between the transcription factor C/EBPα, the antileukemic substances ATRA and CDDO and the genes Id1 and Id2. The results of the ChIP-on-Chip technique were confirmed in this work by the verification of C/EBPα binding to the promoters of Id1 and Id2 and to the Id1 enhancer and the precise localization of the C/EBPα binding sites by ChIP. Binding was stronger when the cells were treated with ATRA and CDDO before the experiment. An induction of the Id1 enhancer was observed in 293T cells and 32D cells as a reaction to C/EBPα overexpression. Also C/EBPβ and C/EBPδ were able to induce the Id1 enhancer, although the effect of C/EBPβ was less strong, whereas C/EBPδ induction was stronger than C/EBPα induction.

This work can be seen as a basis for future experiments, which might take different directions. As explained above, Id1 and Id2 have been shown to play a role in granulocytic differentiation, proliferation of cell lines and development of a variety of human cancer types. The fact that Id1 expression is up-regulated by the transcription factor C/EBPα and by ATRA stimulation although it mostly promotes cell proliferation and eventually even tumorigenesis is intriguing. One possible explanation is the need for determination of the granulocytic lineage by Id1, but it must still be verified or denied by future experiments. In this context, Id1 and Id2 overexpression in different cell lines as well as an analysis of Id1 and Id2 expression levels during granulocytic

differentiation would be interesting experiments. Although Id1 knockout mice do not show any abnormalities (86), it is very likely that Id1 expression has an effect on the determination of the cell lineage in differentiation, on cell growth and cancer development. Analyzing the role of Id proteins in differentiation and proliferation is a promising approach to new, targeted cancer therapies because an intervention in the pathways which include Id proteins might be a key to disrupt uncontrolled proliferation and induce differentiation – an elegant therapy option which has been demonstrated by the introduction of ATRA.

The mechanisms of action of ATRA and CDDO are another interesting issue, potentially opening the way to new targeted therapies with similar ways of action like ATRA, which might work for other human cancers. A characterization of the working mechanisms would include the search for target genes as done in the ChIP-on-Chip experiment described above which was the basis for this work and an analysis of the target genes which the experiment reveals. It would also involve detecting if the effect of ATRA or CDDO is altered, enhanced or impaired when a certain gene is knocked out or down-regulated with siRNA. This would be particularly interesting as it has been shown that knockdown of Id1 with siRNA inhibits proliferation of Molm-14 cells and K562 cells (125), which is contradictive to the hypothesis that Id1 is important to mediate the antiproliferative effect of ATRA and CDDO. This may be due to cooperative factors of Id1 like the HLH proteins which are or are not present. A knockdown or knockout before treatment of the cells could show if Id1 and Id2 are really relevant for the differentiation therapy with ATRA and CDDO.

There are many publications concerning the transcription factor C/EBPα and its positive effect on granulopoiesis. In addition, up-regulation of C/EBPα after CDDO treatment has been shown (59). However, C/EBPα up-regulation under ATRA treatment has not been analyzed so exactly. It would be interesting to know if C/EBPα is essential for ATRA induced granulopoiesis. Another arising question is if it only works on the mRNA level or if it is also characterized by enhancement of the active C/EBPα form p42, like CDDO induction. Also a comparison of C/EBPα up-regulation by ATRA and by CDDO would be helpful, as well as a more exact examination of the pathway from the administration of ATRA to the induction of C/EBPα. Is C/EBPα directly induced by

ATRA or are there steps in between? Realtime PCRs after ATRA treatment and gel shift assays for C/EBPα DNA binding activity with or without ATRA treatment, using for example G-CSFR as a probe, as well as Western blot and Northern blot as it has been done for CDDO (59) might give response.

Additionally, the knowledge about the role of other C/EBP proteins than C/EBPα in hematopoiesis and leukemia is still vague, although at least for C/EBPβ and C/EBPε, an association to hematopoiesis has been shown (54, 134). It is known that C/EBP proteins can form dimers with each other (131), which might make it difficult to analyze each on its own account. However, it would be highly interesting to find out more about C/EBPδ in hematopoiesis, as this work showed that its induction of the Id1 promoter is much stronger than the one observed for C/EBPα. Expression levels during granulocytic differentiation would indicate the importance of the different C/EBP family members during the phases of differentiation. Another interesting question is if C/EBPβ and C/EPBδ bind to the same binding sites which were presented in this work as C/EBPα binding sites in the Id1 and Id2 promoters and the Id1 enhancer. This is an important information to understand if the roles of the C/EBP family members C/EBPα, β and δ are really interchangeable as former experiment results suggest (54) or if the effects are based on the induction of different target genes. An answer might be found with chromatin immunoprecipitations as it has been done in this work for C/EBPα, with specific antibodies for C/EBPβ and δ.

Figure 5.1 picks up figure 1.5 from the introduction and briefly shows some of the most important findings of this work.

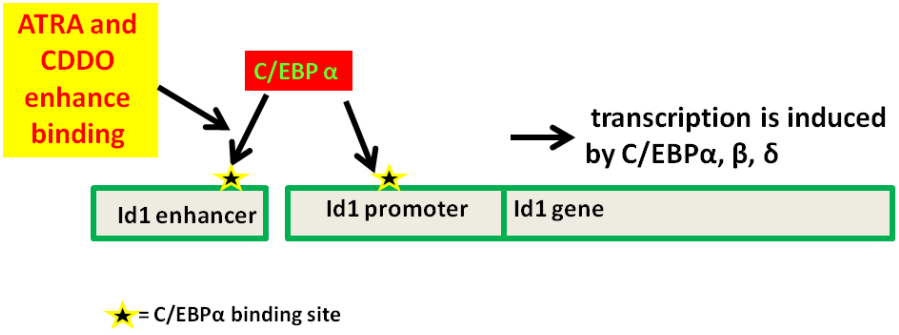


Figure 5.1: C/EBPα induction of the Id1 enhancer and promoter by binding to the localized binding sites and enhancement of C/EBPα binding by ATRA and CDDO stimulation.

Taken together, the results of this work have revealed several interesting findings which are valuable for further experiments concerning the gene and protein families of C/EBP and Id proteins. Furthermore, they have verified the results of the ChIP-on-Chip experiment, identified Id1 and Id2 as direct C/EBPα target genes and localized the C/EBPα binding sites in the Id1 enhancer and the Id1 and Id2 promoters.

As a future perspective, further research about the pathways regulated by ATRA and CDDO, the role of the C/EBP transcription factors in hematopoiesis and AML, and especially the regulation and effect of Id proteins on hematopoietic as well as on leukemic cells, are highly promising topics to find points of action for targeted differentiation therapies.

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