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Investigating the mechanism underlying modulation
of ATRA response by the transcription factor EVI1

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Abbreviations

aa	amino acids
AB	antibody
ac or AR	acidic region
ADH	alcohol dehydrogenase
AML	acute myeloid leukemia
amp	ampicillin
APL	acute promyelocytic leukemia
ATRA	all- <i>trans</i> retinoic acid
BM	bone marrow
bp	base pair
BSA	bovine serum albumin
CBP	CREB binding protein
CGD	chronic granulomatous disease
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
CtBP	C-terminal binding protein
CYP26	cytochrome P450 family 26
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxid
DPBS	Dulbecco's phosphate buffered saline
dpc	days post coitum
DR	direct repeats
DTT	dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EVI1	ecotropic viral integration site 1
FBS	fetal bovine serum
FD	fast digest
GATA1	GATA element DNA binding factor 1
HAT	histone acetyltransferase
HDAC	histone deacetylase
HSCs	hematopoietic stem cells
ICAM1	intercellular adhesion molecule 1
IF	immunofluorescence
JNK	c-jun N-terminal kinase
kb	kilo bases
LB	Luria Broth
MBI	Montreal Biotech
MDS	myelodysplastic syndrom
MEL1	MDS1/EVI1 like gene 1
MLL	mixed-lineage leukemia
M-MVL	moloney murine leukemia virus
NEB	New England Biolabs
O/N	over night
oligos	oligonucleotides
OS	overall survival
p.a.	pro analysi

P/CAF	p300/CBP associated factor
PB	peripheral blood
PBS-T	phosphate buffered saline-Tween
PCR	polymerase chain reaction
PFA	paraformaldehyde
PLA	proximity ligation assay
PPAR γ	peroxisome proliferator-activated receptor γ
PRDM16	PR-domain containing 16
PSG	Penicillin-Streptomycin-Glutamine
qRT-PCR	quantitative reverse transcription-PCR
RA	retinoic acid
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptors
RARE	retinoic acid receptor element
RBP	retinol binding protein
rcf	relative centrifugal force
RD	repression domain
rpm	rounds per minute
RT	room temperature
RXR	retinoid X receptors
SEM	standard error of the mean
STRA6	stimulated by retinoic acid 6
TBE	Tris/Borate/EDTA
TEM	transendothelial migration
TF	transcription factor
tk	thymidin kinase
TSS	transcription start site
TTR	transthyretin
ZF	zinc finger

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1. Introduction

1.1. Hematopoiesis

Hematopoiesis is the development of blood cells. It starts in the human embryo in the yolk sac and progresses to the fetal liver and further into the bone marrow (BM)¹. As mature blood cells have a limited life span they need to be replaced continuously from hematopoietic stem cells (HSCs). In adults, mature blood cells are produced in the BM¹. Asymmetric cell division of HSCs enables them to simultaneously maintain their own compartment (self-renewal) and produce differentiated blood cells². Cells which are committed to differentiate can either enter the myeloid or the lymphoid cell lineage. Progenitor cells of these two lineages differentiate irreversibly into various mature blood cell types. Lymphoid progenitor cells differentiate into B- and T-cells, whereas myeloid progenitor cells differentiate into granulocytes, monocytes, platelets and erythrocytes (Fig. 1). The process of differentiation is highly restricted and controlled by a number of transcription factors (TFs), among others PU1, GATA1, GATA2 and c-fos³.

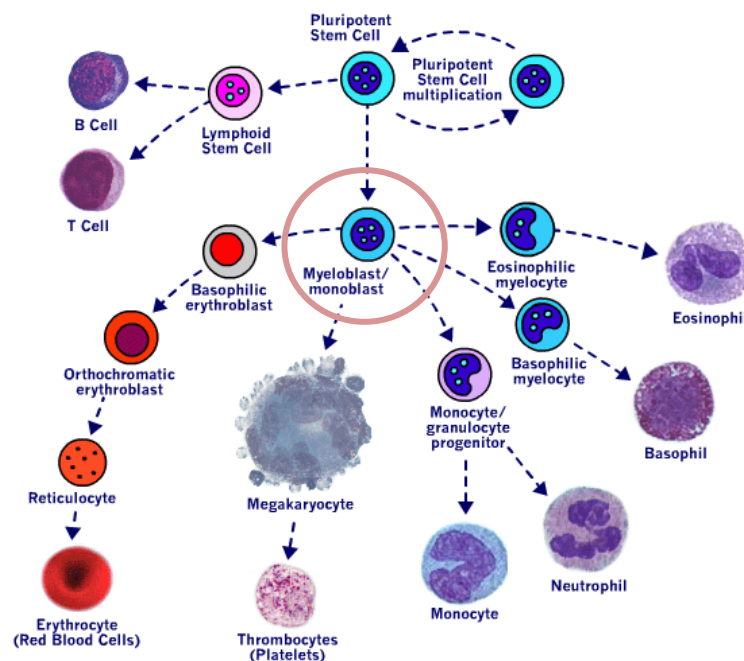


Fig. 1 Hematopoiesis: development of mature blood cells from pluripotent hematopoietic stem cells (HSCs). Pluripotent HSCs divide asymmetrically to repopulate their own compartment and produce mature blood cells. HSCs can differentiate into two lineages, the myeloid and the lymphoid lineage. In acute myeloid leukemia (AML), myeloblasts (encircled in red) accumulate at the expense of other hematopoietic cells. (Figure taken from <http://lymphoma.about.com/od/glossary/g/What-Is-Hematopoiesis.htm>, © McGill Molson Medical Informatics Project)

1.2. Acute myeloid leukemia (AML)

AML is a malignant disease which affects the myeloid blood cell lineage. It is characterized by accelerated proliferation and the lack of differentiation of immature myeloid cells (myeloblasts) which accumulate in the BM and peripheral blood (PB)⁴. This accumulation leads to a dysfunction in the production of mature blood cells with the consequence of a decrease in the numbers of red blood cells, platelets and normal white blood cells⁴.

Even though significant progress was made in understanding the biological mechanisms leading to this disease in the past three decades, AML is still characterized by high mortality and a median 5-year overall survival (OS) rate after diagnosis of only 24%⁵. Statistical surveys name it as a disease of the elderly, with a median age of 64 years⁶. The age is one of the most important prognostic factor in AML, which can be observed regarding the variation in the 5-year OS rate. This rate can differ from 38% for patients younger than 60 years and only 12% for patients older than 60 years⁷.

The WHO classification divides AML patients into three major biological subgroups: AML with recurrent genetic abnormalities (Table 1), AML with multi-lineage dysplasia, and therapy-related AML⁸. The overall percentage of the myeloid cell lineage accounts for approximately 85% of the cells in the BM, of which less than 5% are myeloblasts in healthy individuals. According to the WHO classification, a person suffers from AML if the percentage of myeloblasts lies at 20% or more of total cells counted in the PB or BM⁸. However, in some cases with recurrent genetic abnormalities like t(8;21)(q22;q22), inv(16)(p13.1q22), or t(15;17)(q22;q12) AML may be diagnosed regardless of the blast percentage in the BM and PB^{9,10}.

AML with recurrent genetic abnormalities

AML with t(8;21)(q22;q22); RUNX1-RUNX1T1

AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11

Acute promyelocytic leukemia with t(15;17)(q22;q12); PML-RARA

AML with t(9;11)(p22;q23); MLLT3-MLL

AML with t(6;9)(p23;q34); DEK-NUP214

AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1

AML with mutated NPM1*

AML with mutated CEBPA*

*Provisional entities.

Table 1: WHO classification of AML with recurrent genetic abnormalities¹⁰.

In the last decades several different biological subtypes of AML could be classified by varying cytogenetic characteristics. Cytogenetic aberrations in AML can be divided into three prognostic groups: favorable, intermediate, and unfavorable⁶. Studies showed correlations between favorable cytogenetic aberrations and young age whereas unfavorable aberrations were more often found in older patients⁶.

1.3. Ecotropic viral integration site 1 (EVI1)

1.3.1. Identification of *EVI1* and its biological functions

Evi1 was first identified in 1988 as a common integration site for ecotropic proviruses¹¹. The insertion of retroviruses into this locus caused transcriptional activation of *Evi1* leading to emergence of myeloid leukemia in a murine model system¹².

Soon thereafter, the human *EVI1* gene was found to be located in chromosome band 3q26¹³ which is often rearranged in patients with myeloid diseases¹⁴. Translation of the mRNA of *EVI1* gives rise to a predominant protein variant of 1051 amino acids (aa), with an apparent molecular weight of 145 kDa¹³. The protein consists of an N-terminal seven zinc finger (ZF) domain¹⁵, referred to as ZF1, a repression domain (RD)¹⁶, a second three ZF domain (ZF2)¹⁵, and an acidic region (ac)¹⁷ (Fig. 2 A). Besides various alternative splice isoforms¹⁷, there are also different 5' variants, one of which gives rise to the MDS1/EVI1 protein. This is a full length EVI1 protein with additional 188 aa at the N-terminus, including a PR domain (Fig. 2 B)¹⁸. Immunostaining of EVI1 showed that it is located in the nucleus¹⁹.

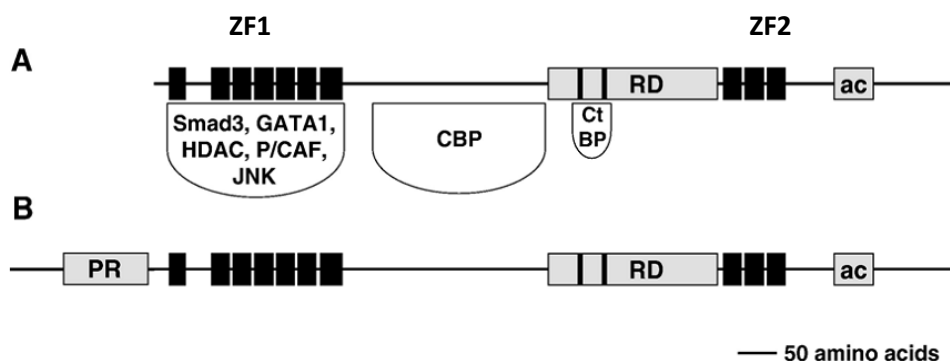


Fig. 2 Protein domains of EVI1 and MDS1/EVI1.

The EVI1 protein consists of an N-terminal zinc finger (ZF) domain (ZF1), a repression domain (RD), a second ZF domain (ZF2) and an acidic region (ac). The protein can interact with various transcription factors (TFs), cofactors and other proteins, among others Smad3, GATA element DNA binding factor 1 (GATA1), histone deacetylase (HDAC), p300/CBP associated factor (P/CAF), c-jun N-terminal kinase (JNK), CREB binding protein (CBP) and C-terminal binding protein (CtBP) (A). The MDS1/EVI1 fusion protein additionally contains a PR-domain at the N-terminus (B). Reprinted from Wieser R. 2007¹⁷ with permission from Elsevier.

Besides its location in the nucleus, the presence of ZF domains, and the observation that EVI1 possesses the ability to directly bind to distinct DNA sequences with each of its two ZF domains^{20,21}, strengthen the assumption that EVI1 acts as a TF. *In vitro* studies showed that ZF1 bound a GATA-like DNA motif with the consensus sequence GA(C/T)AAGA(T/C)AAGATAA²⁰ whereas ZF2 bound an ETS-like DNA site with the consensus sequence GAAGATGAG²¹.

EVI1 was reported to exert a dual role in transcriptional regulation and acted as an activator²² as well as a repressor of transcription¹⁶. However, only few direct target genes could be identified until recently. A ChIP-seq analysis published in 2012 by Bard-Chapeau *et. al*²³ identified 5308 EVI1 bound genes (peak -10 to +5 kilo bases (kb) from the transcription start site (TSS)) in the ovarian carcinoma cell line SKOV3. Gene expression microarray analysis revealed that 236 of these EVI1 bound genes were also regulated by EVI1, and therefore might be direct EVI1 target genes. This study also confirmed the dual role of EVI1 in transcription regulation, as 65% of these genes were up- and 35% down-regulated in the presence of EVI1. Further *in silico* analysis revealed that the EVI1 binding sites of 62% of the EVI1 regulated genes contained an ETS-like DNA motif, while a GATA-like motif was present in only 24% of the cases. Although these results were not obtained in hematopoietic cells, 34% of the EVI1 bound genes are named by the Cancer Gene Census database to be implicated in human myeloid leukemia. Furthermore the EVI1 bound genes were shown to significantly correlate with the gene expression signature of AML patients with abnormal *EVI1* expression but not with other gene expression profiles identified in AML patients²³.

One biological role of EVI1 as a TF is to transactivate transcription of target genes. For example, ectopically expressed Evi1 was shown to induce *Gata2* expression in hematopoietic cell lines and *in vivo* studies revealed that Evi1 bound the promoter of *Gata2* with its ZF1 domain²⁴. Besides acting as a TF, EVI1 is also able to exert its biological function by interacting with a variety of other proteins (Fig. 2)²⁵. It was shown, for example, that EVI1 can interfere with the TGF- β signaling by interacting with Smad3²⁶ or the corepressor CtBP²⁷, with both interactions leading to the inhibition of TGF- β induced growth arrest. Furthermore, interaction of EVI1 with GATA1 prevents the same from binding to the DNA, thereby inhibiting erythroid differentiation²⁸. Another study showed that EVI1 did not block all-*trans* retinoic acid (ATRA) induced granulocytic differentiation of the human acute promyelocytic leukemia cell line NB4²⁹, suggesting that EVI1 plays various roles in differentiation, depending on cell type and expression levels. Moreover, EVI1 can interact with c-jun N-terminal kinase (JNK),

thereby disturbing the interaction with JNK substrates, resulting in inhibition of apoptosis³⁰. All in all *EVI1* is involved in many cellular processes such as proliferation, differentiation, and apoptosis.

1.3.2. *EVI1* in normal development and in hematological malignancies

In mouse embryos, *Evi1* was shown to be expressed at high levels in the urinary system, Mullerian duct, nasal cavities, lung and heart. In adult mice, *Evi1* was expressed at high levels in ovaries and kidneys³¹. Other studies showed predominant expression of *Evi1* in embryonic as well as in adult HSCs but down regulation during hematopoietic differentiation^{24,31}. In human tissue, highest expression was detected in stomach, kidney, lung, prostate, and uterus, as well as lower expression in the testis, placenta, small intestine, colon, thymus, heart, spleen, and brain³². That *Evi1* is important for normal development is not only supported by its very restricted spatiotemporal expression pattern, but also by knockout models. Mice with a homozygous disruption of the *Evi1* locus were phenotypically normal until 9 days post coitum (dpc) but showed a number of aberrations including hemorrhaging, hypocellularity, and disruption of the paraxial mesenchymal development at 10.5 dpc. All mice died before 11.5 dpc³³. These observations indicate that *Evi1* is important for normal cell proliferation, vascularization, and cell-specific developmental signaling at midgestation³³. Another *Evi1*^{-/-} mouse model showed that mice died between 13.5 and 16.5 dpc and had a reduced number of HSCs in comparison to wild type mice³⁴. In addition *Evi1*^{+/-} mice showed an intermediate phenotype suggesting a gene dosage requirement for *Evi1*³⁴.

Besides its role in development, *EVI1* was found to be overexpressed in human malignancies, e.g., ovarian, prostate, breast, and lung cancer³⁵. The role of *EVI1* in malignant diseases has been most intensively investigated, however, in the context of myeloid diseases as AML and myelodysplastic syndrome (MDS).

1.3.2.1. Clinical importance of *EVI1* overexpression in myeloid malignancies

EVI1 overexpression can be detected in about 10% of human AML patients and is associated with a poor prognosis^{36,37}. AML with rearrangements in the *EVI1* locus is furthermore associated with increased platelet counts and dysmegakaryopoiesis³⁸. These rearrangements in chromosome band 3q26 include t(3;21)(q26;q22) and

t(3;12)(q26;p13) and lead to the expression of the fusion genes *AML1/MDS1/EVI1* and *ETV6/MDS1/EVI1*, respectively¹⁷. In contrast, inv(3)(q21q26) and t(3;3)(q21;q26) can result in elevated expression of an unaltered *EVI1* mRNA. Furthermore, *EVI1* can also be overexpressed in a normal cytogenetic background or in patients with cytogenetic aberrations not affecting chromosome 3, but the mechanism of activation in these cases still remains unclear³⁹.

As mentioned previously, chromosomal rearrangements in AML can be divided into those associated with favorable, intermediate, or poor prognosis. Among others, the latter group includes rearrangements of the *EVI1* locus such as the inv(3)(q21q26) or the t(3;3)(q21;q26)⁴⁰.

In the murine system it was shown that *Evi1* overexpression in hematopoietic cells leads to development of MDS whereas onset of AML needs further genetic events, eg. overexpression of *Hoxa9/Meis1*⁴¹ or mutated *AML1*⁴². Besides studies in the murine system there is also evidence that *EVI1* overexpression can trigger development of MDS in humans. In a gene therapy trial for chronic granulomatous disease (CGD) two patients were treated with autologous stem cells modified with a therapeutic retroviral vector. Integration of the retroviral vector also took place at the *MDS1/EVI1* locus. This integration lead to overexpression of *EVI1* and to predominant expansion of these affected cells. Although initially symptoms of CGD improved, both patients developed MDS after periods of 18 and 28 months, respectively. The dominant clone, showing integration at *MSD1/EVI1* and overexpression of *EVI1*, also showed monosomy 7^{43,44}, which in AML patients is often associated with *EVI1* overexpression⁴⁵.

Although *EVI1* overexpression itself does not seem to be sufficient for AML development, it predicts very poor OS for the affected patients^{46,7}. *EVI1* overexpression is frequently associated with mixed-lineage leukemia (MLL)-rearranged AML, and among patients suffering from MLL-rearranged AML those with additional *EVI1* overexpression had a significantly lower 5-year OS compared to those without *EVI1* overexpression⁴⁷. A study distinguishing between *MDS1/EVI1* and *EVI1* expression showed that AML patients with 3q26 rearrangements almost mainly overexpressed *EVI1* whereas *MDS/EVI1* was more often activated in patients without 3q26 rearrangements⁴⁸. However, analysis including 319 *de novo* AML patients attested that especially *EVI1* but not *MDS/EVI1* expression is responsible for the worse OS³⁶. Hence, there is great need to understand the mechanism of *EVI1* in myeloid diseases in order to find proper treatment for this subtype of leukemia.

1.4. Transcriptional regulation by retinoic acid (RA)

1.4.1. Transcriptional regulation by ATRA

RA is the biologically active form of vitamin A. Vitamin A cannot be synthesized by the body and has to be taken in by diet as retinyl esters or β -carotenes. Vitamin A is converted by alcohol dehydrogenase (ADH) to retinaldehyde, which is further processed by retinaldehyde dehydrogenase (RALDH) to RA (Fig. 3)⁴⁹. However, not all cells are able to convert vitamin A to RA as some miss this machinery⁴⁹.

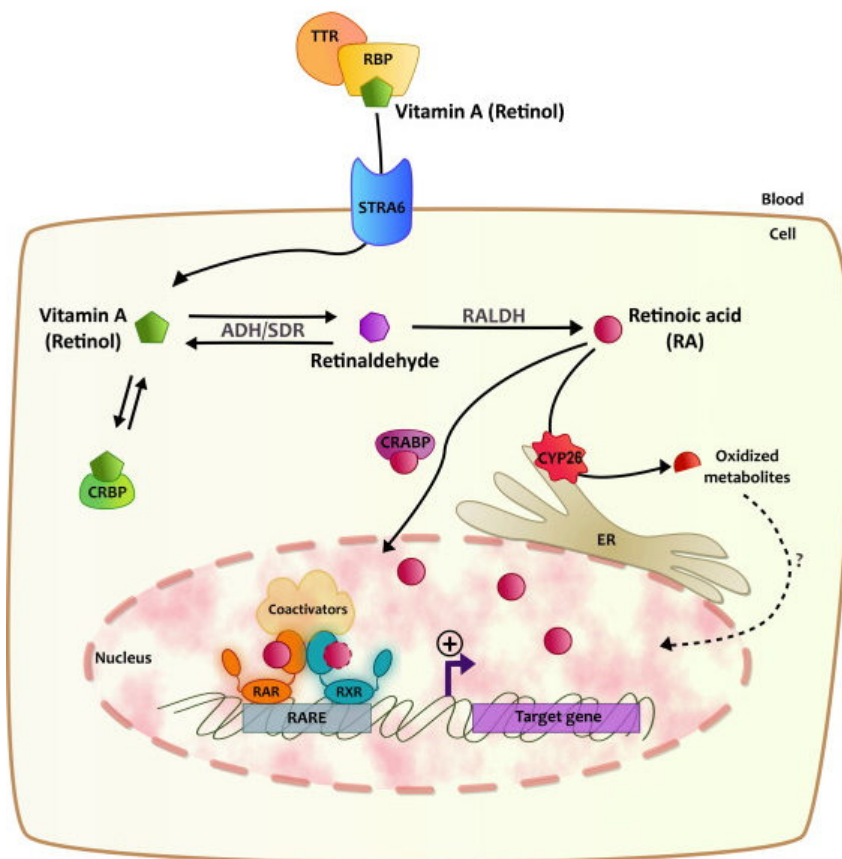


Fig. 3 Vitamin A metabolism and retinoic acid (RA) signaling.

Vitamin A is transferred to target tissues via retinol binding protein (RBP) and stimulated by retinoic acid 6 (STRA6) proteins. In the cell, vitamin A is oxidized in two steps into RA by alcohol dehydrogenase (ADH) and retinaldehyde dehydrogenase (RALDH). RA in the form of ATRA (red circle, full line) then binds to retinoic acid receptors (RAR) in the nucleus and regulates expression of its target genes. Another isoform, 9-cis RA (red circle, broken line) can bind to RAR and retinoid X receptors (RXR). cellular retinoic acid binding protein (CRABP), cellular retinol binding protein (CRBP), retinoic acid receptor element (RARE), transthyretin (TTR), cytochrome P450 family 26 (CYP26). Reprinted from Gutierrez-Mazariegos *et al*⁴⁹ with permission from Elsevier.

ATRA is the most abundant of the six RA isoforms in vertebrates. It is important in development, proliferation, differentiation, and apoptosis⁵⁰. ATRA was shown to play a role in development of several compartments in early embryogenesis by spatiotemporally restricted production and also in fertility and normal vision in adulthood⁵¹. In early development it was found to regulate the anterior-posterior axis through some of its target genes, the homeobox genes (Hox genes)⁵¹. In addition, ATRA was found to play a role in the development of the heart, hindbrain, lungs, kidneys, eyes, and pancreas in different animal models⁵¹. Furthermore, ATRA plays an important role in normal hematopoiesis as it is involved in granulocytic differentiation and increases the number of hematopoietic precursor cells *in vitro*⁵². The regulatory ability of ATRA is mainly carried out via binding to a nuclear receptor and thereby ATRA directly influences transcription of its target genes⁵⁰.

1.4.2. Nuclear retinoid receptors

Nuclear receptors are a superfamily of ligand dependent transcriptional regulators⁵³. For RA there are two families of nuclear receptors; the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs)⁵³. Both of them exist as three different isotypes (α , β and γ), encoded by different genes. They bind to DNA as RAR/RXR heterodimers or RXR/RXR homodimers⁵⁴. The receptors bind to specific DNA sequences, so called retinoic acid response elements (RAREs). The classical RARE, also found in the *RAR β* promoter, consist of two direct repeats (DR) of the sequence PuG(G/T)TCA, separated by a 5 base pair (bp) spacer (DR5). In addition, RAREs with spacers of 1 and 2 bp were also described in the literature (DR1 and DR2, respectively)⁵⁴. Vertebrate RARs can bind ATRA and 9-*cis* RA, another isoform of RA, whereas RXRs were shown to only bind 9-*cis* RA *in vitro*⁵⁴. In the absence of ligand, the receptors are bound to DNA and repress transcription of target genes by attracting corepressors that contribute to the formation of heterochromatin. In the presence of RA, a conformational change of the receptor takes place, resulting in the activation of transcription as repressive cofactors are released and replaced by activating cofactors. Additionally, ligand binding enhances the DNA affinity of the receptors⁵⁴. More precisely, RAR/RXR heterodimers bind the NCoR/SMRT complex which leads to transcriptional repression of RAR/RXR regulated genes through its associated histone deacetylase (HDAC) activity. In the presence of ligand, these cofactors are exchanged for the histone acetyltransferase (HAT) complexes p160 and p300/CBP and the ATP-dependent SWI/SNF chromatin remodeling complex. Due to the ensuing chromatin remodeling, general TFs as well as

RNA polymerase II are attracted to the site of action, leading to the activation of gene expression⁵⁵.

Regulation of RA target genes can be modulated by cooperation of RAR and/or RXR with other TFs. IL-3 enhanced RAR mediated gene transcription by facilitating the binding of STAT5 to RAR/RXR, which enhanced the DNA binding ability of the heterodimer⁵⁶. Furthermore, it was shown that TNF α synergistically effects the ATRA mediated induction of the *DIF2* promoter by facilitating binding of NF- κ B to the promoter⁵⁷.

1.4.3. Treatment of acute promyelocytic leukemia (APL) with ATRA

APL is a form of leukemia where promyelocytic progenitor cells accumulate due to a block of normal granulocytic differentiation. This type of leukemia is almost always associated with a disrupted RAR α protein. The by far most frequent rearrangement causing a disrupted protein version is the t(15;17), which leads to the formation of a PML-RAR α fusion protein. The aberrant protein PML-RAR α was shown to have a higher DNA-binding capacity⁵⁸ and inhibits the physiological function of RA signaling as it has a higher avidity to corepressors than the wild-type protein RAR α , thereby acting as a constitutive transcriptional repressor⁵². This inhibition can be revoked with pharmacological concentrations of ATRA, which leads to the degradation of the malformed PML-RAR α protein mostly through the proteasome-mediated pathway⁵⁹. Additionally, repressive cofactors are dissociated and cells are stimulated to differentiate again⁶⁰. The pharmaceutical potential of ATRA was realized already in 1987⁶¹, when it was used the first time for the treatment of APL, without even knowing about the molecular cause for the loss of differentiation due to the formation of PML-RAR α . ATRA was then approved as a drug for treatment of APL by the FDA in 1995.

Further studies on ATRA treatment revealed that it does not only have benefits in APL treatment but also in other kinds of tumors. Indeed, ATRA is also included in anti-tumor therapeutical schemes for the treatment of various other malignancies such as Kaposi's sarcoma, head and neck squamous cell carcinoma, ovarian carcinoma, bladder cancer, and neuroblastoma⁵⁰. Furthermore, it showed anti-angiogenic effects in

several systems, leading to inhibited proliferation in vascular smooth muscle cells and anti-inflammatory effects in rheumatoid arthritis⁵⁰.

1.4.4. ATRA as a regulator of *EVI1* expression

ATRA was shown to induce the transcription of *EVI1*. Xi *et al.* first recognized that expression of *EVI1* was activated upon ATRA therapy in some patients suffering from APL²⁹. Further *in vitro* studies showed that ATRA treatment could enhance *EVI1* mRNA levels in NB4 cells, an APL cell line expressing PML-RAR α ²⁹. Additionally, it was observed that *Evi1* was upregulated upon ATRA induced neuronal differentiation in P19 teratocarcinoma cells⁶². *EVI1* was also induced by ATRA in the human teratocarcinoma cell line NTERA-2⁶³.

Reporter gene assays in NTERA-2 cells showed that the transcriptional regulation of *EVI1* upon ATRA treatment was mediated by a functional DR5 RARE with the consensus sequence CGACCTTTTGTGACCT, which was located in exon 1a of *EVI1*, and was bound by RAR and RXR⁶³.

1.5. Modulation of the ATRA response by *EVI1*

1.5.1. *EVI1* enhances the ATRA induction of the *RAR β* gene and represses the ATRA induction of the *EVI1* gene

EVI1 is not only regulated by ATRA but is also able to modulate the ATRA response of other genes. The first two genes which were shown to be induced by ATRA and modulated by *EVI1* were the *RAR β* gene and the *EVI1* gene itself⁶³. The *RAR β* gene was previously shown to be a direct target gene of ATRA regulation as its transcription is highly and rapidly upregulated upon ATRA treatment⁶⁴. Reporter gene assays in NTERA-2 cells revealed that the ATRA regulation of the *RAR β* RARE was enhanced by cotransfection of an *EVI1* expression vector, whereas ATRA induction from the *EVI1* RARE was repressed under these conditions⁶³. Furthermore, the ATRA induction of the endogenous *RAR β* mRNA was enhanced by *EVI1* in U937 cells. Hence, it was postulated that *EVI1* acts as a dual modulator of the ATRA response⁶³.

1.5.2. Further genes whose ATRA response was modulated by *EVI1*

Previously to this work, a microarray analysis was carried out by Steinmetz *et al* (unpublished data) to analyze the potential of *EVI1* to modulate the ATRA response on a genome-wide level. U937 cells stably infected with an *EVI1* expression vector (U937_ *EVI1*) or the respective empty vector (U937_vec) were treated either with ATRA or its solvent dimethylsulfoxide (DMSO). Genes that were induced or repressed by at least two-fold in the presence of ATRA, and whose ATRA response was further modulated by *EVI1* by a factor of at least 2, were considered as candidate genes. Analysis of the microarray data resulted in 44 candidate genes which were divided into three groups according to their mode of regulation. One group consisted of 7 genes whose repression by ATRA was enhanced by *EVI1*. The second group consisted of 3 genes that were repressed by ATRA and *EVI1* counteracted this repression. Most of the genes, 34 in total, were assembled in the third group, which comprised genes whose expression was induced upon ATRA treatment and further enhanced by *EVI1* (Fig. 4). The type of effect observed at the *EVI1* promoter itself (*EVI1* repressing ATRA induction)⁶³, was not detected in other genes in the microarray.

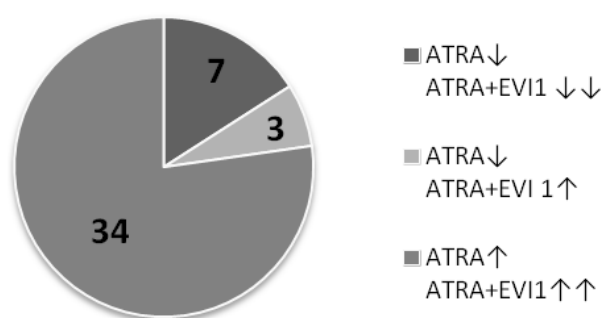


Fig. 4 Microarray analysis revealed 44 genes whose ATRA response was modulated by *EVI1*. U937 cells with or without stable ectopic expression of *EVI1* were treated with DMSO or ATRA and differentially regulated genes were analyzed (Steinmetz et al, unpublished results).

As the third group contained the biggest number of genes I concentrated my attention to those genes, where *EVI1* could enhance the ATRA induction of gene expression. In order to understand the mechanism behind the *EVI1* induced modulation of the ATRA response, two candidate genes revealed by the microarray analysis, *HOXA1* and

ICAM1, were chosen for further studies (Fig. 5). This selection was based on the fact that both of these genes contain previously described functional RAREs^{65,66}.

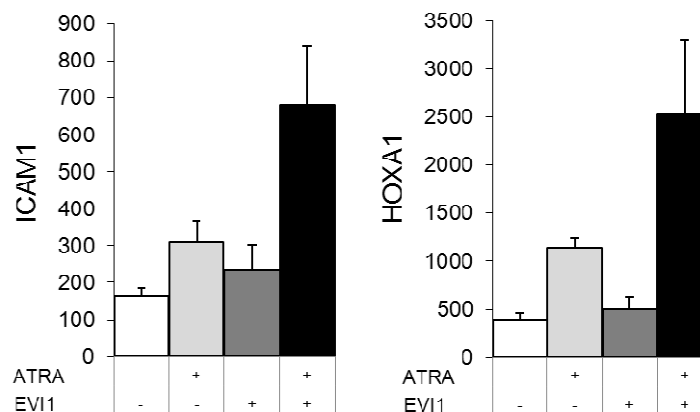


Fig. 5 EVI1 enhances the ATRA dependent induction of *ICAM1* and *HOXA1* on the mRNA level.

U937_EVI1 and U937_vec cells were treated with DMSO or ATRA for 24 hours. RNA was extracted and subjected to microarray analysis. Results are shown as the mean of three biological replicates + standard error of the mean (SEM). Data obtained by Steinmetz *et al* (unpublished).

1.5.2.1. Intercellular adhesion molecule 1 (ICAM1)

ICAM1 was identified as a member of the immunoglobulin superfamily. The protein consists of five extracellular immunoglobulin like domains, one transmembrane domain, and a short cytoplasmic domain⁶⁷. Since ICAM1 is the main receptor for β 2-Integrins of leukocytes and neutrophils, it plays an important role in the immune response⁶⁸. When the immune cells observe ICAM1 on the endothelial cell surface, it allows them to migrate through the endothelium to the target tissue in a process called transendothelial migration (TEM)⁶⁹.

ICAM1 expression is present in low levels in various cells and is upregulated upon exposure to inflammatory mediators such as $\text{TNF}\alpha$, IL-1^{70} , IL6 , and $\text{IFN}\gamma^{71}$. Its expression is also enhanced upon ATRA treatment, especially in various tumors such as melanoma, glioma, teratocarcinoma, neuroblastoma⁷², and breast cancer⁷³. Furthermore, *ICAM1* was shown to be upregulated by ATRA in myeloid cells from leukemia patients as well as in the cell line NB4, where extravasation took only place after treatment with ATRA⁷⁴. It was proposed that increased expression of *ICAM1* in endothelial cells, bound by CD18, facilitate the mediated migration of neutrophils through the endothelial barrier⁷⁵. Furthermore it was also shown that the induction of

ICAM1 expression by ATRA is responsible for increased accessibility of tumor cells to monocyte-mediated lysis⁷⁶.

Investigations of the promoter region of the human *ICAM1* gene identified a functional DR5 RARE which mediates upregulation of its expression upon ATRA treatment⁶⁶. The RARE consists of the core sequence GGGTCATCGCCCTGCCA, and is located at -270/-178 bp relative to the translational start site⁶⁸, corresponding to position +54/+70 bp relative to the TSS.

Aoudjit *et al.* showed that the activity of the *ICAM1* RARE was induced by ATRA treatment ~8-fold in COS1 cells⁶⁸. Furthermore, it was found that the isotypes RAR β and RXR α were involved in the transcriptional regulation, as cotransfection of one or both expression plasmids for these receptors increased the ATRA responsiveness of the *ICAM1* RARE to a great extent⁶⁸. The RAR α /RXR heterodimer bound the described RARE *in vitro*⁷⁷.

1.5.2.2. HOXA1

The mammalian Hox genes consist of two classes. Class I comprises the 39 clustered *HOX* genes. These genes are organized in 4 clusters (A,B,C,D) which are located on four different chromosomes. Class II family genes are non-clustered or non-*HOX* genes which are located throughout the whole genome⁷⁸. *HOX* genes were first identified by their homology to the *Drosophila Homc* genes. They act as TFs, playing an important role in development. The *Hox* genes are expressed in mouse development in a spatiotemporal pattern and determine the anterior-posterior axis⁷⁹. Furthermore, *HOX* genes are involved in murine and human hematopoiesis, as clusters A-C are mostly expressed in immature hematopoietic cells and down-regulated during differentiation⁷⁸. These genes regulate not only normal hematopoietic differentiation, but some of them are also associated with the development of leukemia. HOXA9 and MEIS1, a cofactor for HOX genes, are often found to be elevated in AML patient samples and their overexpression is associated with poor prognosis⁷⁸.

As *Hox* genes are expressed in a very strict spatiotemporal pattern, regulation of expression is very important⁷⁹. A number of TFs regulating the HOX promoters were identified, e.g. Mll, Cdx, or PcG proteins⁷⁹. Another potent regulator of the HOX cluster is ATRA⁸⁰. However, despite decades of studying the factors that govern the *Hox* gene expression, the detailed mechanisms of tight regulation are still not fully understood.

In the microarray experiment carried out by Steinmetz *et al.*, *HOXA1* was identified as a target gene of the *EVI1* modulated ATRA response. *Hoxa1* had already previously

been studied for its ATRA response^{81,82} and an active DR5 RARE in the 3'enhancer region of the gene has been reported. The *HOXA1* 3' RARE was first identified in the murine *Hoxa1 gene*⁸³. Later the RARE was also found in the 3' sequence of the human *HOXA1 gene*⁸⁴. The sequence of this DR5 RARE is identical to that of the *RAR β* RARE and was shown to activate its promoter in response to ATRA⁸³. The RARE was described to be located at +4279 bp relative to the TSS and +1732 bp from the 3' *HOXA1* transcript end. Furthermore, it was shown that the paralog *RAR γ* is essential for the gene activation by ATRA⁸⁵.

1.6. Possible mechanisms for *EVI1* modulation of the transcriptional regulation by ATRA

This thesis is part of a study that addresses the molecular mechanism underlying the modulation of the ATRA response by *EVI1*. The most straight forward explanation for this phenomenon is that *EVI1* binds directly to *RAR* and/or *RXR*, and/or that *EVI1* binds to DNA sequences in the vicinity of the RAREs.

1.6.1. *PPAR γ* , an interaction partner of the *EVI1* paralog *PRDM16*

The hypothesis that *EVI1* might interact with one of the retinoid receptors was supported by a recent publication by Seale *et al*⁸⁶. In this study it was shown that PR-domain containing 16 (*PRDM16*) coactivated the transcriptional activity of peroxisome proliferator-activated receptor (*PPAR γ*), and that *PPAR γ* and *PRDM16* interacted in a direct manner. The coactivation of *PPAR γ* in cellular assays was ligand dependent whereas the *in vitro* interaction of the two proteins was ligand independent⁸⁶. The protein domains ZF1 and ZF2 of *PRDM16* were shown to be necessary for the interaction with *PPAR γ* . *PRDM16* also bound and coactivate *PPAR α* , another paralog of the receptor⁸⁶.

PRDM16 is also called MDS1/*EVI1* like gene 1 (*MEL1*) as it is highly homologous to MDS1/*EVI1*. Like MDS1/*EVI1*, *PRDM16* consists of a PR domain, two ZF domains with 7 (ZF1) and 3 (ZF2) ZFs, an RD, and an Ac⁸⁷. Furthermore, *PRDM16* is also involved in AML as it is overexpressed in patients with the rearrangement t(1;3)(p36;q21)⁸⁷.

The second protein, *PPAR γ* , belongs to the same superfamily of nuclear receptors as *RAR* and can also forms heterodimers with *RXR*⁸⁸.

1.7. Objectives

EVI1 is an oncogene that is overexpressed in up to 10% of AML due to rearrangements in chromosome band 3q26 or other, unknown molecular mechanisms³⁷. *EVI1* overexpression is correlated with a poor prognosis and shortened OS^{46,7}. Hence, it is important to further investigate the mechanism of *EVI1* action in myeloid diseases.

Bingemann *et al*⁶³ showed that *EVI1* modulates the ATRA dependent transcriptional regulation of the *RAR β* gene as well as of the *EVI1* gene through their respective RAREs. Further unpublished studies from the host lab displayed that this regulation could be a more general phenomenon and that *EVI1* might be able to enhance the ATRA regulation of a number of genes.

The objective of this study was to investigate the mechanism through which the ZF protein EVI1 modulates the ATRA response. First, I tested whether EVI1 modulated the ATRA responses of genes that had emerged as candidates from the microarray analysis through their RAREs, as had been shown for *RAR β* and *EVI1*⁶³. To this end, reporter gene assays with two candidate genes, *ICAM1* and *HOXA1*, were carried out in already described ATRA responsive systems. Furthermore, I investigated the hypothesis that EVI1 binds to the retinoid receptors RAR and/or RXR. This question was addressed by using the very sensitive Duolink *in situ* interaction assay.

2. Methods

2.1. Cloning

2.1.1. Primer Design

RAREs:

Primers for amplifying RARE sequences from human genomic DNA with conventional polymerase chain reaction (PCR) were designed by using the free software “Primer 3” (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The Sequences and relative position of the respective primers are shown in Table 2.

Primer name	Sequence 5' → 3' coding strand	Relative position from TSS
ICAM1-P-fwd1	catctcgagAGCTAGGTGTGGACGTGACC	-277/-258
ICAM1-P-fwd2	catctcgagACCGTGATTCAAGCTTAGCC	-9/+11
ICAM1-P-rev1	TTTAGCTTGGAAATTCGGAagatctatg	+86/+105
ICAM1-P-rev2	CTGAGCTCCTCTGCTACTCAGAagatctatg	+287/+299
Hoxa1-P-fwd1	CAGAGTGTTCCCAAAGTAGGC	+4058/+4079
Hoxa1-P-fwd2	TGGGGCAATCAGATTCAAAC	+4175/+4195
Hoxa1-P-rev1	CCATCCCTCTTTGGAAAAGGagatct	+4384/+4403
Hoxa1-P-rev2	AGTCCCAGCAGTTTATCTGAGCagatct	+4335/+4356

Table 2: Sequences and relative position of primers to generate *ICAM1* and *HOXA1* RARE constructs.
Capital letters: genomic sequence, small letters: engineered restriction sites, forward (fwd), reverse (rev).

RXR α -HA:

To generate an expression plasmid containing an HA tagged version of RXR α , the RXR α sequences was amplified from an existing expression plasmid with conventional PCR using the primers shown in Table 3. The reverse primer contained an engineered SacII restriction site upstream of the stop codon in order to be able to insert a 3xHA tag into this site.

Primer name	Sequence 5' → 3' coding strand	Annealing temperature
RXR α -HA fwd	taggatatcATGGACACCAAACATTTCTG	63.9
RXR α -HA rev	cgatctagaCTAaccgcggAGTCATTTGGTGCGGCG	66.6

Table 3: Sequence of primers to generate the RXR α -HA construct.
Capital letters: RXR sequence, small letters: engineered restriction sites, fat letters: stop codon, forward (fwd), reverse (rev).

2.1.2. Phusion PCR

For amplifying RARE sequences from genomic DNA from a healthy human donor, a PCR with Phusion polymerase, possessing a proofreading function, was carried out as described below.

50 ng	genomic DNA
1.6 µl	Each dNTP [2.5 mM]
1 µl	Primer fwd [10 µM]
1 µl	Primer rev [10 µM]
4 µl	Phusion Buffer HF
0.2 µl	Phusion® High-Fidelity Polymerase
	ddH ₂ O
20 µl	TOTAL

A gradient PCR was carried out in order to find the best cycle conditions. Melting temperatures of primers were calculated on <http://www.thermoscientificbio.com/webtools/tmc/> which gives the correct annealing temperature for Phusion polymerase reactions, as the Phusion Polymerase works better with slightly higher annealing temperatures than other standard polymerases as e.g. Taq polymerase. According to these temperatures a temperature gradient PCR with four reactions per sample was carried out using the following cycling parameters:

98°C - 30"	
98°C - 10"	
60-66°C - 30"	} 30x
72°C - 40"	
72°C - 10'	

The most suitable annealing temperatures, at which no unspecific products were amplified and the efficiency was best, are listed below.

Amplified region*	Annealing Temperature	Primers used for amplification
ICAM1(-277/+299)	64.9°C	ICAM1 fwd1, ICAM1 rev2
ICAM1(-9/+105)	63.9°C	ICAM1 fwd2, ICAM1 rev1
HOXA1(+4085/+4403)	60.5°C	HOXA1 fwd1, HOXA1 rev1
HOXA1(+4175/+4356)	64.0°C	HOXA1 fwd2, HOXA1 rev2

*Regions are given relative to the TSS

Table 4: Optimal annealing temperatures for amplification of *ICAM1* and *HOXA1* RAREs from genomic DNA.

For subsequent amplification, optimal annealing temperatures were used with the above described cycle conditions.

Materials:

- dNTPs: Montreal Biotech (MBI)
- Phusion® High-Fidelity Polymerase: New England Biolabs (NEB)
- Phusion® Buffer HF: NEB
- UltraPure DNase/RNase-Free Distilled Water: Invitrogen

2.1.3. Preparative digest of plasmid DNA and PCR products

For preparative digests between 1 and 10 µg plasmid DNA or PCR products, depending on the length of the desired DNA fragment, were digested with enzymes (1 µl/µg DNA) from Fermentas or NEB for 2 h in manufacturer supplied buffers at 37°C.

Materials:

- BglII, fast digest (FD): Fermentas
- XhoI, FD: Fermentas
- SmaI: NEB
- KpnI: NEB
- EcoRV: NEB

2.1.4. Ethanol precipitation of DNA

When DNA was cut with two enzymes requiring different buffers, it had to be precipitated after the first digest in order to be able to change the buffer. For ethanol precipitation, DNA was mixed with sodium acetate and ethanol in the following volumes:

50 µl	DNA
5 µl	Sodium acetate (300 mM)
125 µl	96% EtOH

All components were combined in a tube, vortexed, and centrifuged for 30' at 4°C and 14000 rounds per minute (rpm). After centrifugation the supernatant was taken off, the DNA was washed with 1 ml 70% Ethanol and centrifuged again for 5'. The supernatant was now completely removed and the DNA pellet was dried at 37°C for around 5-10'. The DNA was then dissolved in DNase/RNase free H₂O and used for the second enzymatic digestion.

Materials:

- Sodium acetate: Sigma
- 96% EtOH pro analysi (p.a.): Merck
- UltraPure DNase/RNase-Free Distilled Water: Invitrogen

2.1.5. Agarose gel electrophoresis

For preparation of an agarose gel the amount for the required concentration of agarose (1-2%) was dissolved in 0.5x Tris/Borate/EDTA (TBE) buffer and boiled in a microwave oven until all solid particles were dissolved. The agarose mixture was cooled down until it was hand-warm and 0.006% GelRed were added for the detection of DNA. The agarose solution was poured into a gel tray and a comb was applied in a manner avoiding air bubbles. The gel was either used directly after solidifying or stored at 4°C up to four weeks. For preparative agarose gel electrophoresis, chambers, trays, and combs were first cleaned with Milli-Q H₂O and combs with wider slots (20 mm) were used for gel preparation. Preparative gels were run at 100 Volt, non-preparative gels were run at 135 Volt. Two different DNA markers were used for comparing the DNA fragment sizes. Depending on the size range needed, 100 ng of either λ DNA EcoRI/HindIII marker or GeneRuler 100 bp DNA ladder were applied on the gel.

Materials:

- Agarose: Peqlab
- GelRed: Biotum

- λ DNA EcoRI/HindIII: Promega
- GeneRuler 100 bp DNA ladder: Fermentas
- 10x TBE (1 Liter):

108 g	Trizma [®] Base: Sigma
55 g	Boric acid: Sigma
0.02 M	EDTA (pH 8.0): Sigma
	Milli-Q H ₂ O
1 Liter	TOTAL

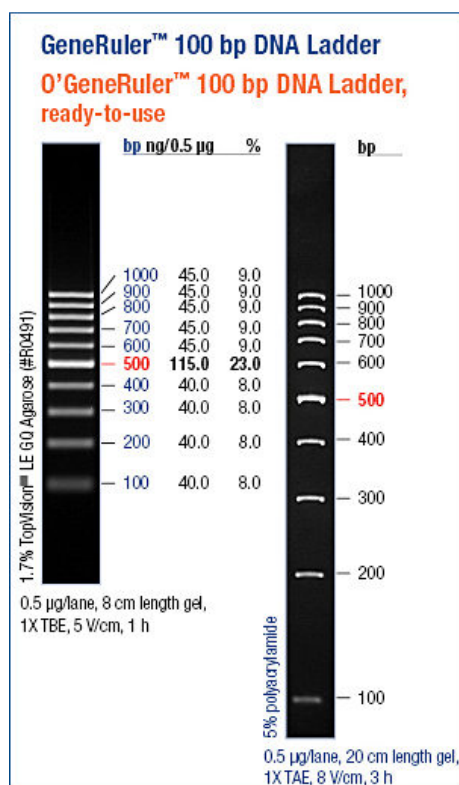


Fig. 6 GeneRuler 100 bp DNA ladder
Figure taken from:
<http://www.thermoscientificbio.com/nucleic-acid-electrophoresis/generuler-100-bp-dna-ladder-100-to-1000-bp/>

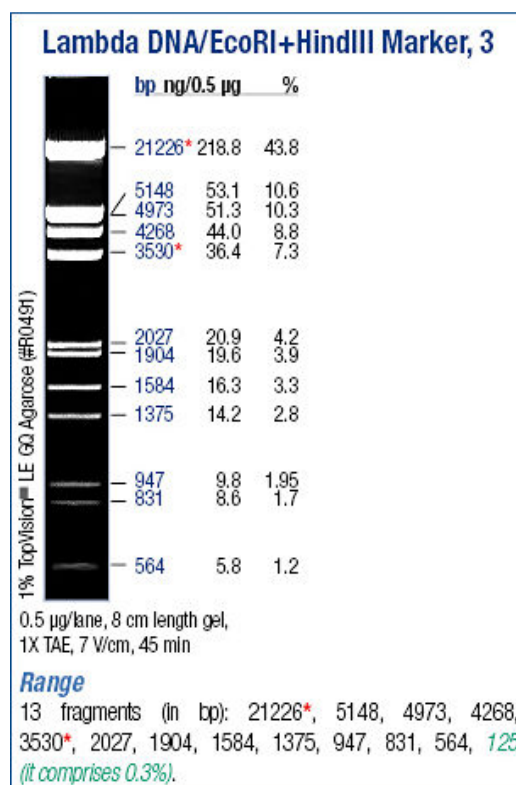


Fig. 7 Lambda DNA EcoRI/HindIII Marker
Figure taken from:
<http://www.thermoscientificbio.com/nucleic-acid-electrophoresis/lambda-dna-ecori-plus-hindiii-marker/>

2.1.6. DNA Isolation from agarose gel

The DNA bands of interest, thoroughly separated from the other bands, were cut out from preparative gels with a clean scalpel under UV light. In order to prevent thymidine dimer formation, it was taken care that DNA was not exposed to UV light for longer than absolutely necessary. Enzymatically restricted DNA fragments were extracted from agarose gels with the QIAquick gel extraction kit (QIAGEN) according to the manufacturer's instructions, in short: The piece of gel was melted in 3 gel volumes extraction buffer at 50 °C for about 10'. One gel volume of Isopropanol was added and

mixed. 800 µl of the mixture were applied to a QIAgen quick spin column and centrifuged at 13000 rpm for 1'. In case of a bigger volume the step was repeated as often as necessary. Flow through was discarded and the spin column was washed twice with 750 µl PE buffer (5' at 13000 rpm). To get completely rid of the alcohol, the spin column was centrifuged again without addition of buffer. 30 µl elution buffer were pipetted directly onto the membrane of the spin column, incubated for 1' at room temperature (RT), and DNA was eluted by centrifugation for 1' at 13000 rpm.

Materials:

- Isopropanol, p.a.: Roth
- QIAquick gel extraction kit: QIAgen

2.1.7. Estimation of DNA amount

To estimate how much DNA had been recovered from the preparative gel, 1 and/or 3 µl of the eluted DNA were loaded onto an agarose gel. In addition, various amounts (40-140 ng) of marker DNA, either λ marker EcoRI/HindIII or 100 bp GeneRuler, were loaded next to the eluted DNA. The amount of the DNA of interest was estimated by comparing the intensities of the corresponding bands to similarly intense marker bands, whose DNA content, in turn, was calculated based on the total amount of marker loaded in that lane and information given by the manufacturer about the relative intensities of the marker bands (see section 2.1.5).

2.1.8. Ligation of vector and insert DNA

Ligation of vector DNA and insert DNA was carried out with the Quick ligation Kit (NEB). The required amount of insert DNA was calculated with the following formula.

$$\left(\frac{\text{amount}(\text{vector}) \times \text{size}(\text{insert})}{\text{size}(\text{vector})} \right) \times 4 = \text{amount}(\text{insert})$$

15 – 25 ng of vector DNA were used for each ligation.

According to the manufacturer's instructions a total DNA volume of 10 µl was used for the ligation. The ligation was carried out as described below.

10 µl	DNA
10 µl	2x Quick Ligation Buffer
1 µl	Quick T4 DNA ligase

The reaction was mixed well and incubated at RT for 5 to 10'. The ligation was either stored at -20°C or directly used for transformations.

Materials:

- Quick ligation Kit: NEB

Vectors used for ligation:

Vector	Luciferase	Promoter	Provider
pGL3Promoter (pGL3P)	firefly	SV40	Promega
pGL3basic (pGL3)	firefly	none	Promega
pGlucbasic (pGlucB)	<i>Gaussia</i>	none	NEB

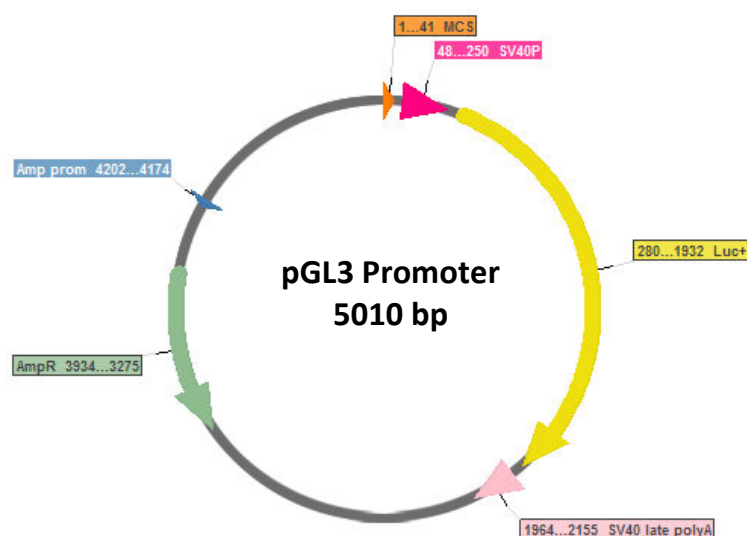


Fig. 8 pGL3 Promoter (pGL3P) vector.

Luciferase reporter vector containing an SV40 promoter upstream of a firefly luciferase coding sequence. multiple cloning site (MCS), firefly luciferase (Luc+), ampicillin (Amp).

multiple

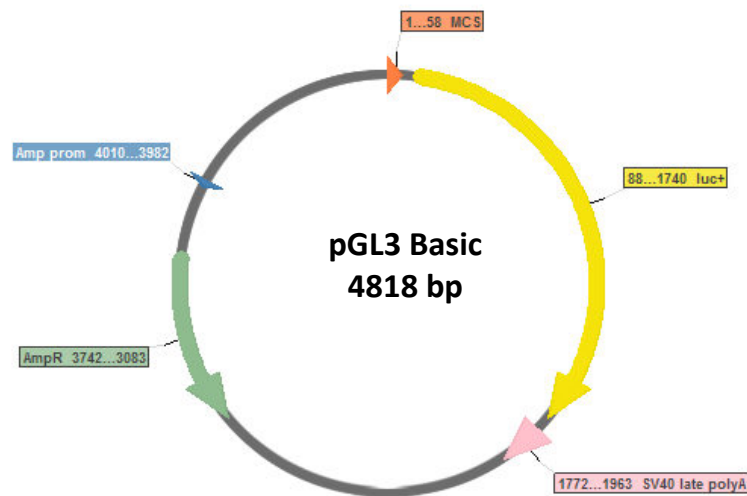


Fig. 9 pGL3 Basic (pGL3) vector.

Luciferase reporter vector containing a firefly luciferase coding sequence but lacking an eukaryotic basal promoter sequence. multiple cloning site (MCS), firefly luciferase (Luc+), ampicillin (Amp).

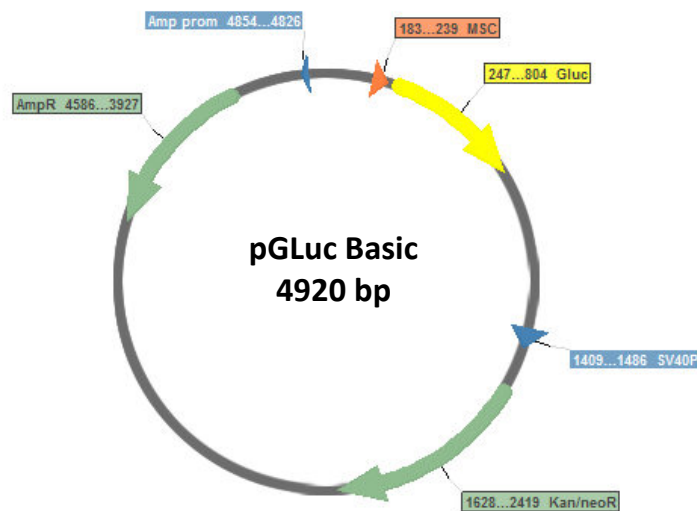


Fig. 10 pGluc Basic (pGlucB) vector.

Luciferase reporter vector containing a *Gaussia* luciferase coding sequence. multiple cloning site (MCS), *Gaussia* luciferase (Gluc), ampicillin (Amp), kanamycin (Kan), neomycin (neo).

2.1.9. Transformation of competent *Escherichia coli* (*E.coli*)

Transformation with ligation reactions:

The transformation of ligation reactions was carried out with 10-beta competent *E.coli*. Immediately after thawing bacteria on ice, 2.5 µl of the ligation reaction were added to 25 µl of bacteria and the tube was gently flicked 5 times. The bacteria suspension was placed on ice for 30'. A heat shock was carried out at 42°C for 30". The tube was cooled down again on ice for 5'. 470 µl SOC-media were added and the tube was incubated on a thermo shaker for 1 h (300 rpm, 37°C). 100 µl of the suspension were spread onto a Luria Broth (LB) plate supplemented with ampicillin (amp) with a sterile spatula and the remaining solution was spread onto a second LB-amp plate. The plates were incubated upside down at 37°C ON.

Materials:

- 10-beta competent *E.coli*: NEB
- SOC-media: NEB (comes with *E.coli*)
- LB-amp plates (1 Liter):

10 g	Bacto-Tryptone: Merck
5 g	Yeast Extract: Merck
5 g	NaCl: Sigma
15 g	Agar-Agar: Roth
1mM	NaOH: Sigma
	Milli-Q H ₂ O
1 Liter	TOTAL

LB was autoclaved, cooled down to ~50-60°C, and ampicillin (stock solution 50 mg/ml in H₂O) was added to a final concentration of 50 µg/ml.

- ampicillin, Na-salt: Sigma

Re-transformation of plasmids:

For retransformation of plasmids, home-made competent DH5α *E.coli* were used. The bacteria were thawed on ice. 100 ng plasmid DNA were added to 100 µl *E.coli* in a 1.5 ml reaction tube and it was gently tapped 5 times in order to mix the suspension. The mixture was incubated on ice for 30', followed by a heat shock at 42°C for 1'. The bacteria slurry was cooled down on ice for 2'. 1000 µl SOC media were added to the

bacteria and the mixture was placed at 37°C for 1 h under gentle agitation (300 rpm). 100 µl suspension were spread on a pre-warmed LB-amp plate and incubated O/N at 37°C.

Materials:

- SOC media, home-made

0.5 %	Yeast Extract: Merck
2 %	Bacto-Tryptone: Merck
10 mM	NaCl: Sigma
2.5 mM	KCl: Merck
10 mM	MgCl ₂ : Merck
10 mM	MgSO ₄ : Merck
20 mM	Glucose: Sigma
To pH 7.0 with	NaOH: Sigma

2.1.10. Colony PCR

To check whether bacterial colonies contained the correct plasmid, a colony PCR was carried out. Single colonies were picked with a sterile tip, the tip was immersed into a PCR tube containing 5 µl DNase/RNase free H₂O and then the remaining bacteria were streaked onto a fresh LB-Amp plate in a marked area. For each ligation, between 24 and 35 colonies were picked. A positive control containing the insert and a negative control containing only DNase/RNase free H₂O were also prepared. A PCR master mix for all reactions was prepared as described below. The same primers as for amplification of the insert (see section 2.1.1) could be used, as the cloning strategies were based on directed ligations and the inserts were only a few hundred bp long.

2 µl	10X PCR-buffer
1.2 µl	MgCl ₂ [25 mM]
1 µl	each dNTP [2.5 mM]
1 µl	Primer fwd [10 µM]
1 µl	Primer rev [10 µM]
0.2 µl	Taq polymerase
8.6 µl	DNase/RNase free H ₂ O
15 µl	TOTAL

15 µl of the mastermix were added to each PCR tube. Cycle conditions are listed below.

94°C - 2'	
94°C - 15"	} 30x
60°C - 15"	
72°C - 20"	
72°C - 5'	

4 µl of each PCR reaction were loaded onto a 2 % agarose gel next to a marker, separated by gel electrophoresis, and visualized with a transilluminator.

Materials:

- dNTPs: MBI
- Taq polymerase: Invitrogen
- 10X PCR Buffer: Invitrogen
- MgCl₂: Invitrogen
- Ultrapure DNase/RNase free H₂O: Invitrogen

2.1.11. Small scale preparation of plasmid DNA from bacteria (Mini-prep)

Colonies tested positive for the presence of the plasmid in the colony PCR were inoculated into 3 ml LB-Amp in a sterile bacteria tube and were incubated overnight (O/N) at 37°C under agitation (~200 rpm) in a thermo shaker. The next day, 1.5 ml of bacterial culture were transferred into a sterile 1.5 ml reaction tube and bacteria were pelleted (13000 rpm, 1' at 4°C). The supernatant was removed and the rest of the culture was pelleted in the same tube. This time the supernatant was completely removed. 200 µl of lysis buffer were added and bacteria were fully resuspended. 400 µl of freshly prepared alkaline SDS were added and the tube was inverted 5 times and incubated on ice for 5'. 300 µl 3 M potassium acetate were added, the tube was inverted and incubated for 5' on ice. The mixture was centrifuged at 13000 rpm for 10' at 4°C. After the centrifugation a white precipitate should be seen on the wall of the tube. Carefully 700 µl supernatant (without any white precipitate) were transferred into a new 1.5 ml reaction tube. 700 µl of 96% EtOH were added and DNA was precipitated for 15' at -20°C. DNA was pelleted by centrifugation for 15' at 13000 rpm (4°C), the

supernatant was removed, the DNA was washed with 70% EtOH (5', 13000 rpm, 4°C) and dried at 37°C for around 10-15'. The DNA was mixed with 40 µl DNase/RNase free H₂O supplemented with 2 µl RNase A (10 mg/ml in H₂O), and dissolved for 20' under gentle agitation at 37°C.

Materials:

- Lysis buffer

50 mM	Glucose: Sigma
25 mM	Trizma® Base: Sigma
10 mM	EDTA (stock solution with pH 8): Sigma

has to be autoclaved before use

- Alkaline SDS solution (freshly prepared)

1%	SDS (w/v): Sigma
0.2M	NaOH: Roth

- LB-amp (1 Liter)

10 g	Bacto Tryptone: Merck
5 g	Yeast extract: Merck
5 g	NaCl: Sigma
1mM	NaOH: Roth
Milli-Q H ₂ O	
1 liter	TOTAL

LB was autoclaved and ampicillin (stock solution: 50 mg/ml in H₂O) was added for a final concentration of 50 µg/ml.

- RNase A: Sigma
- ampicillin, Na-salt: Sigma

2.1.12. Control digestion of plasmid DNA

In order to check if the prepped plasmid DNA is correct and if the DNA fragment was inserted into the vector in the right direction the DNA was digested using at least three different enzyme combinations. If possible, digests were designed so that one cutting site was in the insert and another in the vector backbone. For control digests, 200 ng

plasmid DNA were digested by single or double enzymatic restriction. Enzymatic digests were set up as described below and was carried out at 37°C for 20'.

0.75 µl	each enzyme
1 µl	DNA (200 ng/µl)
2 µl	Buffer
	DNase/RNase free H ₂ O
20 µl	TOTAL

Used restriction enzymes:

Fermentas (FD):

- HindIII
- NheI
- EcoRI
- XhoI
- SalI
- NotI
- NcoI

- StuI

- SacI

NEB:

- SmaI
- BglII
- SacII
- HindIII

2.1.13. Medium scale preparation of plasmid DNA (Midi-prep)

For Midi-preps, 100 ml LB-amp were inoculated with a bacteria clone from one smear. Bacteria were grown O/N at 37°C and 200 rpm in a thermo shaker. Subsequently, DNA was isolated with the PureYield Plasmid Midiprep Kit (Promega) according to the manufacturer's instructions. In short, bacteria were centrifuged for 10' at 5000 g in 300 ml centrifuge tubes in a Sorvall centrifuge. Bacteria were resuspended in 3 ml cell resuspension solution. 3 ml cell lysis solution were added, the tube was inverted until the solution was getting viscous and a 3' incubation at RT followed. 5 ml neutralization solution were added and the tube was shaken until cloudy white precipitate was visible. The lysate was centrifuged for 15' at 15 000g. After centrifugation a blue PureYield™ clearing column was placed on top of a white PureYield™ binding column and the column stack was placed onto a vacuum manifold. The whole lysate was poured into the clearing column and vacuum was applied until the liquid had passed through both columns. The clearing column was discarded and the binding column was washed with

5 ml endotoxin removal wash by applying vacuum. 20 ml column wash solution were added and allowed to be pulled through the column completely by applying vacuum. Vacuum was applied for another minute, and the column was tapped onto paper towels to get rid of all the alcohol contained in the washing buffers. The column was put onto an eluator vacuum elution device, into which a 1.5 ml reaction tube was placed before. 500 µl nuclease-free water were applied to the membrane, incubated for 1' and liquid was sucked through the membrane into the reaction tube by applying vacuum. The total yield of Midi-preps was between 75-200 µg of plasmid DNA.

Material:

- PureYield Plasmid Midiprep Kit (Promega)

2.1.14. High yield preparation of plasmid DNA (Maxi-prep)

For often used plasmids, Maxi-preps were carried out with the QIAfilter Plasmid Maxi Kit (QIAGEN). A bacteria clone from one smear on an LB-amp plate was inoculated in 3 ml LB-amp and incubated for around 6 h at 37°C under gentle agitation (200 rpm). This pre-culture was then transferred into 250 ml LB-amp and was incubated O/N under the same conditions as above. The next day, cells were centrifuged at 4°C, with 5000g in a 300 ml tube in a Sorvall centrifuge. The supernatant was discarded and bacteria were resuspended in 10 ml pre-chilled buffer P1. 10 ml buffer P2 were added, mixed well and incubated for 5' at RT. 10 ml pre-chilled buffer P3 were added to the lysate, the tip of the QIAfilter cartridge was sealed with the provided screw-cap and the lysate was poured quickly into the cartridge. The mixture was incubated for 10' so that the white precipitate could concentrate at the upper part of the cartridge. Meanwhile the QIAGEN-tip 500 was equilibrated with 10 ml buffer QBT. The lysate was pushed through the cartridge into the QIAGEN-tip and the tip was washed in two steps with 30 ml buffer QC. In a 50 ml falcon tube 10.5 ml Isopropanol were prepared and the DNA was eluted with 15 ml buffer QF into the very same tube. DNA precipitation was carried out in an allegro X12R centrifuge for 60' with Umax at 4°C. Supernatant was discarded, 1 ml 70% EtOH was added to the DNA pellet and all together was transferred into a 1.5 ml reaction tube and centrifuged at 13000 rpm for 5' at 4°C. EtOH was removed and the pellet was dried at 37°C and dissolved in 100 µl DNase/RNase free H₂O. Subsequently DNA concentration was measured with Nanodrop 8000. The yield of Maxi-Preps was between 150-500 µg of plasmid DNA.

Materials:

- 96% EtOH, diluted to the needed concentration in Milli-Q H₂O: Merck
- Isopropanol: Roth
- QIAfilter Plasmid Maxi Kit: QIAGEN

2.2. Cell Culture

COS1 and MCF7 cells were cultured in Dulbeccos's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1X Penicillin-Streptomycin-Glutamine (PSG), further referred to as DMEM/FCS/PSG. Adherent cells were cultured in T75 cell culture flasks. U937 cells and derivatives as well as HL60 cells and derivatives were cultured in RPMI supplemented with 10% FBS and 1X PSG, further referred to as RPMI/FCS/PSG. Suspension cells were cultured in 10 cm cell culture dishes. All cells were cultured in a humidified incubator with 5% CO₂ at 37°C. Adherent cells were grown to around 80% confluence and suspension cells were grown to a density of around 1000-1500 cells/ml and split every second or third day. For passaging, adherent cells were washed with 1X Dulbecco's phosphate buffered saline (DPBS) and then 1 ml trypsin solution was added. Cells were kept on 37°C until all cells were detached from the culturing flask (~5'-10'). Cells were then resuspended in DMEM/FCS/PSG and MCF7 cell were split 1:5 whereas COS1 cells were split 1:10. U937 cells were split 1:10 by simply diluting the cells with RPMI/FCS/PSG. Counting of cells for all experiments was carried out with a Casy Counter TT according to the manufacturer's protocol.

U937T_EVI1-HA cells contain a EVI1-HA/pUHD10S plasmid which allows tetracyclin-controllable expression of EVI1-HA. The respective vector control cells U937T_vec contain the empty vector pUHD10S. These cells were cultured in RPMI supplemented with 10 % FCS, 0.5 µg/µl puromycin, 500 µg/ml hygromycin and 1 µg/ml tetracycline⁸⁹. In order to induce expression of EVI1-HA, cells were washed three times with sterile 1X DPBS and resuspended in RPMI supplemented with 10 % FCS only, as the system is a tet-off expression system. Antibiotics were not added as the cells were used 48 h after washing anyways. Cells containing the empty vector were treated in the same manner.

Materials:

- FBS: Invitrogen
- 100X PSG: Invitrogen

- DMEM, high glucose, pyruvate: Invitrogen
- RPMI-1640: Invitrogen
- Puromycin: Sigma
- Hygromycin: Roche
- Tetracycline: Sigma
- 1X DPBS, no calcium, no magnesium: Invitrogen
- Trypsin from porcine pancreas: Sigma
- T75 cell culture flask, with filtercap: Nunc
- 10 cm cell culture dishes: Greiner

2.2.1. ATRA treatment

ATRA was dissolved in dimethylsulfoxid (DMSO) to a final concentration of 10 mM and aliquoted to be stored at -20°C for a maximum of three month. As ATRA is light sensitive all steps using ATRA were carried out under low-light conditions. For the treatment of cells, an aliquot was thawed, diluted 1:100 in the needed cell culture media, and cells were treated by adding 1/100 of the total volume in the well. Hence, a final concentration of 1 μ M ATRA was used in all experiments, except when indicated otherwise. An equivalent amount of the solvent DMSO was added to control cells. Media was mixed by gently rotating the cell culture plates directly after adding ATRA or DMSO.

Materials:

- ATRA, $\geq$ 98% HPLC: Sigma
- DMSO, cell culture grade: AppliChem

2.3. Quantitative Reverse Transcription-PCR (qRT-PCR)

2.3.1. RNA isolation

For all steps working with RNA, separate pipettes, tips, and reagents were used to ensure that everything is RNase free.

500 µl of TRIzol® reagent were added directly to adherent cells (80% confluent), cultured in a 10 cm dish. Cells were harvested by scraping and repetitive pipetting. Suspension cells (5 ml of cell suspension, ~600-800 cells/ml) were pelleted for 5' at 300 rcf and lysed in 500 µl TRIzol® reagent. Lysed cells were transferred to a 1.5 ml reaction tube and incubated for 5' at RT before 100 µl chloroform were added and the mixture was vortexed vigorously. Samples were incubated for 2-3' at RT and were centrifuged for 15' at 4°C, 12000 g. The upper clear and aqueous phase was carefully transferred into a fresh tube. 250 µl Isopropanol were added and vortexed and after a 10' incubation at RT, the RNA precipitate was pelleted by centrifugation for 10', 4°C at 12000g. The supernatant was removed and the pellet was washed with 1 ml 75% EtOH. The supernatant was carefully removed and the RNA pellet was dried at 37°C until it was transparent. RNA was dissolved in 20 µl DNase/RNase free H₂O for 10' at 55°C. Subsequently the concentration was measured with Nanodrop 8000. RNA was either stored at -80°C or directly used for cDNA synthesis.

Materials:

- TRIzol® Reagent: Invitrogen
- Chloroform, analysis grade: Merck
- Isopropanol, p.a.: Roth
- Ultrapure DNase/RNase free H₂O: Invitrogen

2.3.2. cDNA synthesis

500 ng RNA were diluted into a volume of 11 µl with DNase/RNase free H₂O. A mastermix was prepared using 1.8 µl 10mM dNTPs and 1.25 µl random hexamer primer (50 ng/µl) for each reaction. The mastermix was distributed to each RNA tube, and heated for 3' at 65°C, spun down briefly, and placed on ice immediately. Thereafter for each reaction 4 µl 5X first strand buffer, 2 µl 0.1 M dithiothreitol (DTT) and 0.75 µl moloney murine leukemia virus (M-MVL) reverse transcriptase were mixed and added to each sample. Tubes were incubated at 37°C for 1 h. The reaction was heat-inactivated by placing samples at 85°C for 10' and cDNA was stored at -20°C or directly used for control PCR to check the cDNA quality. Therefore the housekeeping gene, β-2-microglobulin, was amplified with conventional PCR.

Materials:

- dNTPs: MBI
- hexamer primer: Invitrogen
- 5x first strand buffer: Invitrogen
- DTT: Invitrogen
- M-MVL reverse transcriptase: Invitrogen
- Ultrapure DNase/RNase free H₂O: Invitrogen

2.3.3. qRT-PCR

Filter-tips were used to prepare the samples for the qRT-PCR. For quantification of mRNA levels, TaqMan® assays were performed in a StepOnePlus™ cyclor. TaqMan® assays for the genes of interest, *ICAM1* and *HOXA1*, were used and a β 2-microglobulin assay was performed as endogenous control. Before cDNA was used for qRT-PCR it was diluted 1:8 in RNase free H₂O. All samples were measured in technical triplicates for each probe set and also a no-template control with only H₂O instead of cDNA was carried out for each mastermix. For each probe set a mastermix was prepared for all samples according to the following table (volume given per well).

0.5 μ l	Taqman assay (<i>ICAM1</i> , <i>HOXA1</i> , <i>RARβ</i> , or β 2-microglobulin)
5 μ l	Taqman mastermix
0.5 μ l	H ₂ O
6 μ l	TOTAL

After the mastermix was prepared it was vortexed briefly and spun down. The following step was carried out on ice: 4 μ l cDNA or 4 μ l H₂O were transferred into the wells of an optical qPCR 96-well plate, and 6 μ l mastermix were added to each well. The plate was sealed with optical sealing tape and spun down briefly. Samples were then placed in a StepOnePlus™ cyclor and analyzed with the settings: StepOnePlus™ Instrument (96-wells), quantification-comparative CT ($\Delta\Delta$ Ct), TaqMan® reagents and standard ramp speed. Results were analyzed with the quantification-comparative $\Delta\Delta$ Ct method⁹⁰. For data presentation when one Ct value varied more than 0.5 from the two others, the one was omitted from calculation. When all three values varied more than 0.5 from each other the results have to be repeated. In this thesis, in the data showing *HOXA1* mRNA analysis in MCF7 cells one Ct value was omitted.

Materials:

- ICAM1 TaqMan® probe Hs00164932_m1: Applied Biosystems
- HOXA1 TaqMan® probe Hs00939046_m1: Applied Biosystems
- RARβ TaqMan® probe Hs00977140_m1*: Applied Biosystems
- Human b2-microglobulin Endogenous Control TaqMan® probe, #4333766F: (Applied Biosystems)
- TaqMan® Mastermix: Applied Biosystems
- Polyolefin Sealing Tape, optically transparent: Sarstedt
- Fast PCR Plate 96 well half skirt: Sarstedt

2.4. Dual-luciferase® reporter gene assays

Dual-luciferase® reporter gene assays were used for analyzing reporter gene activity in MCF7 and COS1 cells. This system is based on the use of two luciferase plasmids, a firefly luciferase plasmid containing the suspected regulatory region of the gene of interest upstream of a firefly luciferase coding sequence, and a *renilla* plasmid containing a *renilla* luciferase coding sequence which is constitutively active. The advantage of this system is the possibility to normalize firefly luciferase to renilla luciferase and thereby excluding variations in results due to variation in transfection efficiency or cell density.

2.4.1. Transfection of MCF7 cells with JetPei for dual-luciferase® reporter assays

60 000 MCF7 cells were seeded per well of a 24-well plate in a volume of 500 µl DMEM/FCS/PSG. 24 h after seeding, cells were transfected with 400 ng EVI1 effector plasmid (pEFzeo/EVI1-HA or pEFzeo), 200 ng reporter plasmid (firefly luciferase plasmids) and 6 ng p*Renilla*. Each plasmid DNA was added to 25 µl of 150 mM sodium chloride (NaCl), and 2 µl JetPei were added to 25 µl 150 mM NaCl. Tubes were vortexed gently and spun down. The diluted JetPei was added to the diluted DNA mixture and tubes were vortexed briefly and spun down. The mixture was incubated for 30' at RT. 52 µl of the prepared transfection mixture was added dropwise to each well.

200 ng	reporter plasmid
400 ng	EVI1 effector plasmid
6 ng	pRenilla
606 ng	TOTAL

Materials:

- JetPei: Invitrogen
- 150 mM NaCl (is provided with JetPei): Invitrogen
- Reporter plasmids (firefly luciferase plasmids):
constructed during this thesis except when indicated otherwise
 - RAR β –RARE/pGL3P
 - RAR β –RARE-tkP/pGL3
 - HOXA1(+4085/+4403)/pGL3P
 - HOXA1(+4175/4356)/pGL3P
 - pRARE-tk-luc: kindly provided by Dr. Hanna Harant, Ingenetix, Vienna, Austria
- Effector plasmids
 - pEFzeo/EVI1-HA: constructed by Bingemann S.⁹¹
 - pEFzeo: kindly provided by Ao.Univ.Prov.Dr. Pavel Kovarik, University Vienna, Austria

2.4.2. Transfection of COS1 cells with FuGene for dual reporter luciferase assays

40 000 COS1 cells were seeded per well of a 24-well plates in a volume of 500 μ l DMEM/FCS/PSG. 24 h after seeding, cells were transfected with 400 ng EVI1 effector plasmid (pEFzeo/EVI1-HA or pEFzeo), 200 ng reporter plasmid (firefly luciferase plasmids) and 6 ng p*Renilla*. 2 μ l FuGene 6 reagent were added to 20 μ l DMEM. The tube was vortexed briefly and incubated for 5' at RT. DNA was pipetted onto the walls of the tube and everything was vortexed briefly and spun down. The mixture was incubated for 15' at RT and then added dropwise to one well.

200 ng	reporter plasmid
400 ng	EVI1 effector plasmid
6 ng	pRenilla
606 ng	Total

The total amount of DNA per transfection was kept constant throughout all reporter gene experiments. As in the experiments without cotransfection of retinoid receptors no additional empty vector was added, the amounts of effector had to be diminished when additional retinoid receptors were cotransfected, in order to keep the DNA/JetPei ratio even. A scheme of DNA amounts used is shown below.

Expression plasmids used:	EVI1 only	EVI1 + one nuclear receptor	EVI1 + two nuclear receptors
EVI1	400 ng	200 ng	200 ng
reporter plasmid	200 ng	200 ng	200 ng
1st nuclear receptor		200 ng	100 ng
2nd nuclear receptor			100 ng
pRenilla	6 ng	6 ng	6 ng

Materials:

- FuGene 6: Roche
- DMEM, high glucose, pyruvate: Invitrogen
- Reporter plasmids (Firefly luciferase plasmids):
constructed during this thesis except when indicated otherwise
 - RAR β -RARE/pGL3P
 - RAR β -RARE-tkP/pGL3
 - ICAM1(-277/+299)/pGL3P
 - ICAM1(-9/+105)/pGL3P
 - ICAM1(-277/+299)tkP/pGL3
 - pRARE-tk-luc: kindly provided by Dr. Hanna Harant, Ingenetix, Vienna, Austria

- Effector plasmids:
 - pEFzeo/EVI1-HA: constructed by Bingemann S.⁹¹
 - pEFzeo: kindly provided by Ao.Univ.Prov.Dr. Pavel Kovarik, University Vienna, Austria
 - pCMV-hRXR α : kindly provided by Makoto Makichima PhD, Department of Biomedical Sciences, Nikon, Japan
 - pcDNA3.1-hRAR γ : kindly provided by Youn Hoon Han PhD, Korea Institute of Radiological and Medical Sciences

2.4.3. Measuring firefly and renilla luciferase activity

For reporter gene assays in MCF7 and COS1 cells the dual-luciferase® reporter assay (Promega) was used. 48 h after transfection cells were washed with 1x PBS and lysed in 100 μ l 1x passive lysis buffer for 15' under agitation (600 rpm) at RT. After incubation, the lysate was pipetted up and down to resuspend cell clumps. 30 μ l of the lysate were transferred into a clean white walled 96-well plate. 50 μ l of the luciferase assay substrate were added to each well manually and the firefly luciferase activity was measured in a luminometer with a retention time of 10 sec. The plate was taken out again and 50 μ l 1x Stop&Glo Substrate were added to each well in order to measure renilla luciferase activity with the same program. For normalization, firefly values were divided by renilla values and multiplied by 1000.

Materials:

- 10x PBS (diluted to 1x PBS with MilliQ-H₂O)

1.37 M	NaCl
27 mM	KCl
80 mM	Na ₂ HPO ₄ x7H ₂ O
15 mM	KH ₂ HPO ₄
	Milli-Q H ₂ O

- Dual-luciferase reporter assay Kit (Promega)
 - Passive lysis buffer
 - Luciferase assay substrate
 - Stop&Glo Substrate

2.5. *Gaussia* luciferase assay

Gaussia luciferase is a reporter luciferase that is naturally secreted into the cell medium. Hence, lysis of cells is not necessary but the culture media can be analyzed directly. U937 cells are hard to lyse with the passive lysis buffer, which is why I used the *Gaussia* luciferase and accepted the lack of a normalizer in this system.

2.5.1. Transfection of U937 cells with JetPei for *Gaussia* luciferase assays

300 000 U937 cells were seeded in 3 ml RPMI/FCS/PSG per well of a 6-well cell culture plate. 665 ng effector plasmid (EVI1/pEFzeo or pEFzeo) and 335 ng reporter plasmid (pGluc) were added to 50 μ l 150 mM NaCl, 4 μ l JetPei were diluted in 50 μ l NaCl, vortexed briefly, and spun down. Diluted JetPei was added to the DNA, the tube was vortexed briefly, spun down, and incubated for 30' at RT. 100 μ l of JetPei-DNA mix was added drop-wise to each well.

665 ng	Effector plasmid
335 ng	Reporter plasmid
1000 ng	Total

Materials:

- JetPei
- 150 mM NaCl (comes with JetPei): Invitrogen
- Effector plasmids:
 - pcDNA3-codophthEvi1: constructed in the host lab, contains the human codon optimized EVI1 sequence
 - pcDNA3: Invitrogen
- Reporter plasmids:
constructed during this thesis
 - ICAM1(-277/+299)/pGluc
 - HOXA1(+4085/+4403)SV40P/pGluc

2.5.2. Measuring *Gaussia* luciferase activity

Gaussia luciferase activity was measured with the BioLux*Gaussia* luciferase assay kit (NEB). 48 h after transfection, cell suspensions were transferred into a 1.5 ml micro-centrifuge tube and centrifuged for 5' at 300 g. 50 µl supernatant were transferred into a white walled 96-well plate. BioLuxGluc substrate was diluted 1:100 in assay buffer. 50 µl of the diluted substrate were added to each well containing supernatant and luminescence was measured with a retention time of 1 sec.

Materials:

- BioLuc® *Gaussia* luciferase assay kit (NEB)
 - BioLuxGluc substrate
 - Assay buffer

2.6. Immunofluorescence (IF) techniques

2.6.1. Preparation of cells for IF and Duolink *in situ* assay

Preparation of adherent cells

Adherent MCF7 cells were grown either on autoclaved cover slips (60 000 cells/13 mm $\odot$) or on tissue culture coated 8-well chamber slides (30 000 cells/well). Chamber slides were used especially for Duolink assays as they provided the possibility to wash the specimen in bigger buffer volumes to diminish background signal. Transfection of the cells was carried out with a total DNA amount of 500 ng. 24 h after seeding, cells were transfected with JetPei by adding the indicated expression plasmids to 25 µl of 150 mM NaCl, and 2 µl JetPei to 25 µl 150 mM NaCl. The diluted JetPei was added to the DNA mixture and tubes were vortexed briefly and spun down. The mixture was incubated for 30' at RT, and 52 µl of the prepared transfection mixture were added dropwise into one well. Cells were either left untreated for 48 h, or treated with ATRA or DMSO 24 h after transfection as described in section 2.2.1 and grown for another 24 h, before they were fixed and stained as described in section 2.6.2.

Materials:

- Sterile Lab-Tek Chamberslides, 8-well, Permax: Thermo Scientific Nunc, #177445
- Cover slips 13 mm $\odot$: VWR

- JetPei®: Invitrogen
- 150 mM NaCl (provided with JetPei): Invitrogen
- Expression plasmids

Experimentally expressed protein	Vector
EVI1-HA	pEFzeo/EVI1-HA
RXR α	pCMV-hRXR α
RXR α -HA	pcDNA3-RXR α 3xHA
RAR α -mycHIS	pcDNA3.1 RAR α -mycHIS
none	pEFzeo
none	pcDNA3

- pEFzeo: kindly provided by Ao.Univ.Prov.Dr. Pavel Kovarik, University Vienna, Austria
- pcDNA3: Invitrogen
- pcDNA3.1 RAR α -mycHIS: kindly provided by Youn Hoon Han PhD, Korea Institute of Radiological and Medical Sciences
- pCMV-hRXR α : kindly provided by Makoto Makichima PhD, Department of Biomedical Sciences, Nikon, Japan

Preparation of suspension cells

Cytospin preparation:

Suspension cells had to be immobilized onto slides before IF staining could be carried out. For this purpose, 200 000 cells were counted and washed once with PBS. Microscopic slides were cleaned and sterilized by wiping them with 80% ethanol, and allowed to dry completely. A cleaned, pre-labeled glass slide together with a paper pad and a reusable sample chamber were fixed in a cytoclip slide clip (Fig. 11). Cells were gently resuspended in 70 μ l PBS and pipetted onto the bottom of the cuvette. Cells were spun down with a Shandon Cytospin centrifuge 3 for 5' at 700 rpm. The cuvettes and the paper pads were carefully removed and cells were encircled with a fat pen. After the usage the sample chambers were cleaned with dH₂O and 80% ethanol and dried at 60°C.

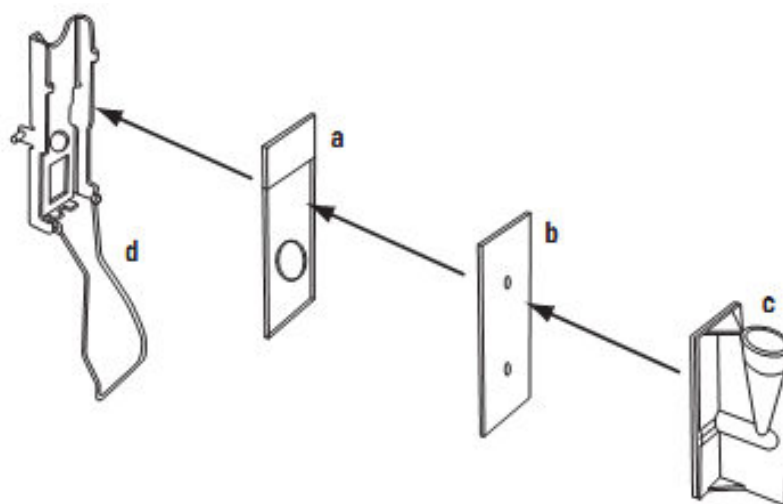


Fig. 11 Schematic set-up for cytospin with Shandon Cytospin Centrifuge 3.

A cleaned slide (a), a filter pad (b), and a reusable sample chamber (c) are positioned in a cytoclip slide clip (d). Figure taken from: <http://www.thermoscientific.com/ecommerce/servlet/techresource?storeId=11152&langId=-1&taxonomy=4&resourceId=102223&contentType=Product+Instructions&productId=11956307#>

Materials:

- Shandon Filter Pads: Thermo Scientific, #5991022
- Shandon cytoclip™: Thermo Scientific, #59910052
- Glass slides: VWR
- Shandon Cytospin 3: Thermo Scientific
- Fat pen: Sigma
- 80% Ethanol, denatured:

Cell-Tak™ adhesion

Another method to immobilize suspension cells is the usage of Cell-Tak™, a tissue adhesive. With this substance, suspension cells can be immobilized on glass or plastic slides. According to the manufacturer's instruction the suggested amount of Cell-Tak™ is 3.5 µg/cm² surface. The cover slip had a surface of 1.3 cm² and one chamber of an 8-well chamber slide had a surface of 0.8 cm². The stock solution was supplied at a concentration of 1.49 µg/µl. The calculated amount of Cell-Tak™ was dissolved in 200 µl 0.1 M Sodium bicarbonate. This procedure could also be carried out for more than one well at the same time. As Cell-Tak™ immediately adheres to any surface when the pH changes from acidic to neutral upon addition of sodium bicarbonate, the solutions were combined in a 1.5 ml reaction tube, vortexed briefly, and pipetted on the surface to be coated (cover slip or chamber slide) immediately. Cover slips were first placed into a well of a 24-well plate. After an incubation of 60' at RT, the liquid was

aspirated and the coated surface was washed twice with sterile DNase/RNase free H₂O. The slides or cover slips were used directly but can also be stored for up to 1 week at 4°C wrapped in wetted tissues. 200 000 cells/well were washed in PBS, pipetted onto the coated surface, and incubated for up to 30'. Under the microscope it could be observed whether the cells had attached by very gently moving the object around. Next cells were fixed and stained as described below.

This method was used preferentially for the Duolink in situ assay with suspension cells as the interactions could not be detected after cytopsin centrifugation.

2.6.2. IF staining

When cells, grown or immobilized in chamber slides were used, the chambers were removed gently so that the silicon fringe, framing each well remained on the slides. Cells immobilized by cytopsin were encircled with a fat pen in order to minimize the reaction volume. Slides were always kept in a humidified chamber consisting of an empty tip box where the bottom part was filled with water and the slide was placed on the tip inset. The reaction volume for slides was 40 µl per reaction. Cover slips were placed in 24-well plates and 500 µl reaction volume were used, except when indicated otherwise. During all experiments cells were either fixed with freshly prepared 4% paraformaldehyde (PFA) in PBS for 15' at RT, or with 100% ice cold methanol for 3' on ice. Next, cells were washed 3 times for 5' with PBS. All washing steps were carried out under gentle agitation either in a volume of 70 ml for slides and chamber slides, or in 1 ml in 24-well plates for cover slips. For permeabilization, cells were incubated with 1% TritonTM-X 100/PBS for 1' at RT and again cells were washed 3x 5' with PBS. Blocking of unspecific binding sites was carried out either with 3% bovine serum albumin (BSA) in PBS-Tween (PBS-T) or with oLink blocking buffer for 1 h at RT. Subsequently, cells were incubated with primary antibodies (ABs) diluted in 1% BSA/PBS-T or in oLink AB dilution buffer, respective to the blocking solution, for 1 h at 37°C or O/N at 4°C in the humidified chamber. For cover slips, this step was carried out as follows: Wetted tissues were placed into a petri dish, 80 µl AB dilution were pipetted onto a piece of parafilm, and the parafilm was placed into the petri dish. Following the cover slips were placed upside down onto the parafilm. The petri dish was closed and cells were incubated for 1 h at 37°C or O/N at 4°C. After the incubation, cover slips were returned to the 24-well plates. To remove the remaining AB, cells were washed 3x 5' with PBS. ALEXA 586, FITC or TRITC conjugated secondary ABs were applied in the same manner as described for the primary ABs with the modification that all steps

from now on where carried out in dimmed light conditions, as the fluorophor-conjugated secondary ABs are sensitive to light. Cells were incubated for 1 h at RT in a humidified chamber. Unbound ABs were removed by washing cells 2x for 5' with PBS. Specimens were counterstained with 4',6-Diamidin-2-phenylindol (DAPI) (1 µg/ml) in PBS for 3' followed by 2 final wash steps for each 5' with PBS. Specimens were mounted with pre-warmed PermaFluor (viscous at 4°C but liquid at RT) mounting media. Slides were stored at 4°C for up to 2 months.

All steps except for the washing steps were carried out directly on the slides. During the whole procedure the specimens were never allowed to dry out.

The ABs were used for preliminary tests with PFA fixation and BSA blocking. Later it was switched to methanol fixation and olink blocking buffer. Results were comparable for all four ABs.

Materials:

Primary ABs used:

Rabbit anti-RXR AB ΔN197: Santa Cruz	1:100
Rabbit anti-myc AB 71D10: Cell signaling	1:200
Mouse anti-RARα 2C91F8: Sigma Aldrich	1:100
Mouse anti-HA 16B12: Covance	1:200

Secondary ABs used:

ALEXA 568 conjugated anti rabbit secondary AB #A21069: Invitrogen	1:1000
FITC conjugated anti mouse secondary AB: Jackson Laboratories	1:400
TRITC conjugated anti mouse secondary AB: Jackson Laboratories	1:400

- 10x PBS (diluted to 1x PBS with Milli-Q H₂O)

1.37 M	NaCl
27 mM	KCl
80 mM	Na ₂ HPO ₄ x7H ₂ O
15 mM	KH ₂ HPO ₄
	Milli-Q H ₂ O

- PBS-T
 - 1x PBS with 0.1% Tween-20: Sigma
- PFA, 36.5-38% in H₂O: Sigma
- Methanol, ≥99,9 %, UV/IR-Grade: Roth
- TritonTM-X 100 for molecular biology: Sigma

- BSA, lyophilized powder, ≥96%: Sigma
- DAPI: Roche

Confocal microscopy:

IF stained or Duolink *in situ* treated specimens were examined with an LSM700 (Zeiss) confocal microscope using the ZEN2009 software.

2.6.3. Duolink *in situ* assay

All washing and incubation steps were carried out as described for IF staining (2.6.2) respectively to the surface on which the cells were immobilized. Furthermore all incubations were performed at 37°C, e.g. in an incubator.

Cells were fixed, permeabilized, and stained with primary ABs according to the IF procedure (2.6.2). For all Duolink *in situ* samples, oLink blocking buffer (provided with Duolink in situ assay) and oLink AB diluent (provided with Duolink in situ assay) were used. After the incubation with primary ABs, cells were washed 3 times for 5' in PBS under gentle agitation. Meanwhile PLA probes anti-rabbit PLUS and PLA probes anti-mouse MINUS were diluted 1:5 in oLink AB dilution buffer. A total volume of 40 µl was needed per surface area of up to 1 cm³. Washed slides were incubated with 40 µl PLA probes in a pre-warmed (37°C) humidified chamber at 37°C for 1 h as described in section 2.6.2. After incubation, slides were washed twice in pre-warmed (RT) oLink buffer A for 5' under gentle agitation. Meanwhile the ligation stock (5x) was thawed at RT and diluted 1:5 in DNase/RNase free H₂O. Shortly before adding the ligation solution to the slides, ligase (1 µl/reaction), which was always kept on ice, was added to the diluted ligation stock and slides were incubated with 40 µl ligation solution in a pre-warmed humidified chamber at 37°C for 30'. The following steps are light sensitive and were carried out under low-light conditions. After ligation, slides were washed 2x 2' in buffer A under gentle agitation. The amplification stock (5x) was diluted 1:5 in DNase/RNase free H₂O. Shortly before adding amplification the solution onto the slides, polymerase (0.5 µl/per reaction), which was always kept on ice, was added onto the diluted amplification stock. The solution was vortexed briefly, 40 µl amplification solution were added to the cells and slides were incubated in a pre-warmed humidified chamber at 37°C for 100'. After amplification, slides were washed 2x 10' in pre-warmed (RT) 1x oLink buffer B under gentle agitation. Slides were washed once again in 0.01x buffer B for 1', the silicon was removed from chamber slides, and the remaining solution was tapped off. Slides were dried in the dark for around 10-15'. Mounting for microscopy was carried out with the oLink DAPI mounting media. Cover slips were

placed onto the slides in a way that no bubbles were enclosed. When cells were immobilized on cover slips, they were placed upside down onto mounting media on an object tray. If bubbles occurred they could be gently pressed to the edge e.g. using forceps. The remaining mounting media was soaked up with a tissue and the slides were sealed with nail polish. For storage for longer than two weeks slides were kept at -20°C.

Materials:

- primary ABs according to IF staining (see section 2.6.2)
- Methanol, ≥99,9 %, UV/IR-Grade: Roth
- TritonTM-X 100 for molecular biology: Sigma
- Duolink in situ assay: oLink
 - Duolink *in situ* PLA probe anti-mouse MINUS #92004
 - Duolink in situ PLA probe anti-rabbit PLUS #92002
 - Duolink in Situ Detection Reagents Orange #92007
 - Ligation stock
 - Ligase
 - Amplification stock
 - Polymerase
 - Duolink in Situ Wash Buffers for Fluorescence #82049
 - Buffer A
 - Buffer B

3. Equipment

Function	Equipment	Supplier
Measurement of nucleic acid concentration and purity	NanoDrop 8000	Thermo Scientific
Sorvall centrifuge	Sorvall RC6 Plus	Thermo Scientific
Thermoshaker for bacterial cultures	KS 4000 i control	IKA
Cytospin centrifuge	Cytospin 3	Shandon
Luminometer	Tristar LB 941	Berthold Tech
UV transilluminator	E-Box VX2	Paqlab
Microcentrifuge	Microfuge 22R	BeckmanCoulter
Thermomixer	Thermomixer comfort	Eppendorf
Thermocycler	Vapo.protect	Eppendorf
qRT-PCR cycler	StepOnePlus™	Applied Biosystems
Vacuum manifold	Vac-Man®	Promega
Plate shaker	Titromax 1000	Heidolph
Confocal microscopy	LSM 700	Zeiss
Laminar flow cabinet	Herasafe KS	Thermo Scientific
Cell incubator	BBD620	Thermo Scientific

4. Results

4.1. Validation of *ICAM1* and *HOXA1* as target genes of the *EVI1* modulated ATRA response

Microarray analysis performed prior to this study by B. Steinmetz, unpublished, indicated that *EVI1* is able to modulate the ATRA response not only of the *RAR β* and *EVI1* genes, but that this is a more general phenomenon affecting 44 genes in U937 cells. Bingemann *et al.*⁶³ showed that the ATRA response of the *RAR β* and *EVI1* genes was modulated by EVI1 through DR5 RAREs located in the promoter region or the first exon, respectively. To investigate whether that mechanism is also valid for other genes. Two of the genes whose ATRA response was enhanced by *EVI1* according to the microarray results, *ICAM1* and *HOXA1*, were chosen for further studies. These two genes were selected because their regulatory regions have been reported to contain functional RAREs^{66,77,83,92}. Before the genes were tested in reporter assays the microarray results were validated by qRT-PCR.

4.1.1. Regulation of the candidate genes *ICAM1* and *HOXA1* by ATRA and *EVI1* in human hematopoietic cell lines

The mRNA expression levels of the candidate genes, *ICAM1* and *HOXA1*, were investigated with qRT-PCR after treatment with ATRA (1 μ M) or its solvent DMSO (equivalent amount) for 24 h. The first cell lines to be investigated were U937 cells stably infected with an *EVI1* expression vector or the respective empty vector⁹³. These cells are referred to as U937_*EVI1* and U937_vec, respectively.

mRNA levels for *ICAM1* (Fig. 12 A) showed similar regulation as the microarray results (Steinmetz *et al.*, unpublished) had revealed (Fig. 5). In fact, ATRA induced the expression of *ICAM1* and experimental expression of EVI1 significantly enhanced this effect 2-fold. However also basal expression (DMSO treated) was increased significantly when EVI1 was expressed. Looking at the mRNA levels of *HOXA1* (Fig. 12 B), ATRA treatment upregulated the expression more than 2-fold. However, a >2-fold enhancement of the ATRA regulation by *EVI1*, as seen in the microarray, could not be reproduced. Instead, EVI1 enhancement (induction from ATRA treated cells without EVI1 expression to ATRA treated cells with EVI1 expression) was only 1.4-fold, and did not reach statistical significance.

To see whether upregulation of *ICAM1* by ATRA and enhancement of this effect by *EVI1* could be reproduced, and whether possibly *HOXA1* was regulated more strongly by *EVI1* in other cell lines, expression levels of the two candidate genes were also evaluated in HL60 cells, a promyelocytic cell line. HL60 cells had been infected with either *EVI1* expression vector or the empty vector to obtain the respective cell lines HL60_ *EVI1* and HL60_vec (B. Steinmetz, unpublished). In these cells, upregulation of *ICAM1* expression (Fig. 12 C) upon ATRA treatment resembled the regulation in U937 cells, and experimental expression of *EVI1* enhanced the ATRA response significantly. Again, basal expression of *ICAM1* was increased with *EVI1* expression but the upregulation of *ICAM1* expression in presence of *EVI1* and ATRA was not only an additive but a potentiation effect as the effect of both substances is higher than the sum of the single effects. For *HOXA1* (Fig. 12 D) increased expression upon ATRA treatment could be reproduced, however in the presence of *EVI1* the effect was not enhanced.

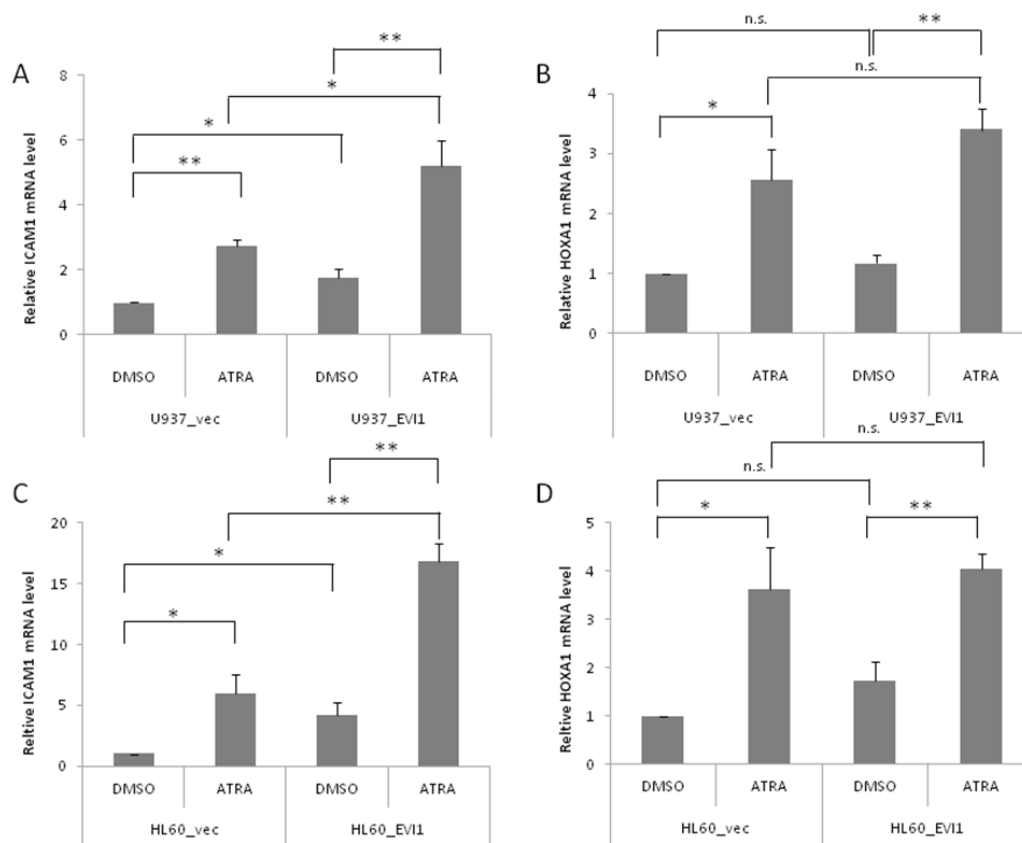


Fig. 12 *ICAM1*, but not *HOXA1*, microarray results could be validated by qRT-PCR in the human hematopoietic cell lines U937 and HL60 expressing or not expressing *EVI1*.

U937 cells and HL60 cells stably infected with empty vector or *EVI1* expression vector, termed U937_vec and U937_ *EVI1* or HL60_vec and HL60_ *EVI1* respectively, were analyzed for the expression levels of *ICAM1* and *HOXA1* mRNA. Relative mRNA levels were analyzed after treatment with 1 μ M ATRA or an equivalent amount of solvent (DMSO) for 24 h. Data were analyzed with StepOnePlus® software using the $\Delta\Delta C_t$ method with beta-2-microglobulin expression as an endogenous control and U937_vec or HL60_vec cells treated with DMSO as calibrator. **A, B**, U937; **C, D**, HL60. **A,C**, *ICAM1*; **B,D**, *HOXA1*. Results are shown as the mean + SEM of three biological experiments. Significance was analyzed with the Student's t-test (* p ≤0.05, ** p ≤0.01).

4.1.2. Regulation of the candidate genes *ICAM1* and *HOXA1* by ATRA in MCF7 cells

As reporter gene assays were planned to first be carried out in easy transfectable adherent cells, two adherent cell lines were chosen for which the ATRA response of the candidate genes was already described. These cells were MCF7 cells, a human breast cancer cell line for *HOXA1*, and COS1 cells, a simian fibroblast like cell line, for *ICAM1*. As for COS1 cells no TaqMan probes were available mRNA levels were not analyzed in this cell line but only in MCF7 cells. MCF7 cells express EVI1 in an endogenous way at a moderate level and no derivatives of this cell line with overexpression or knock down of EVI1 were available. Therefore, only ATRA responsiveness but not the modulation of this regulation by EVI1 could be investigated with qRT-PCR. Cells were treated with 1 μ M ATRA or the equivalent amount of DMSO for 24 h and RNA was extracted. Analysis of *HOXA1* mRNA expression in MCF7 cells confirmed the results reported in the literature: *HOXA1* expression was induced upon ATRA treatment (Fig. 13 B) in MCF7. In contrast, *ICAM1* showed only a minor response to ATRA (Fig. 13 A) in this cell line.

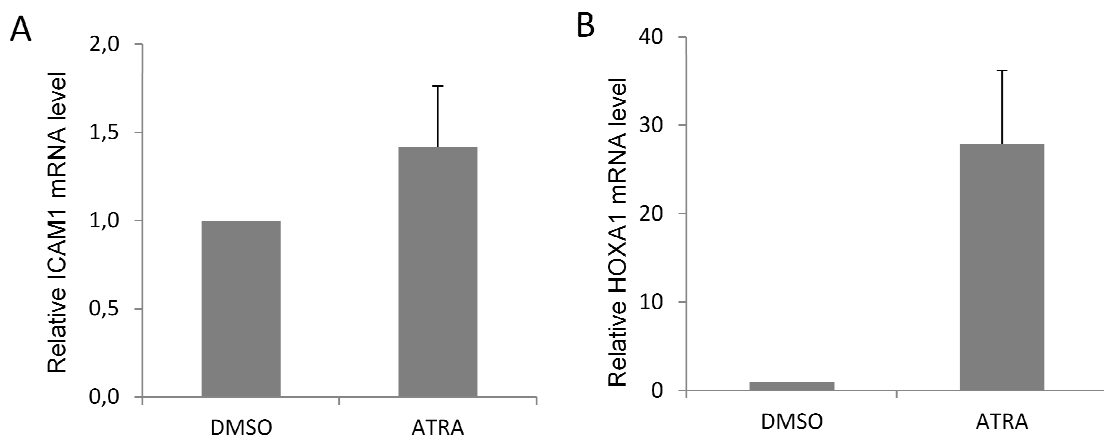


Fig. 13 *HOXA1*, but not *ICAM1*, mRNA levels are strongly upregulated by ATRA in MCF7 cells.

MCF7 cells were analyzed for the expression of *ICAM1* and *HOXA1* mRNA. Relative mRNA levels were measured after treatment with 1 μ M ATRA or an equivalent amount of solvent (DMSO) for 24 h. Data were analyzed with StepOnePlus® software using the $\Delta\Delta$ Ct method with β 2-microglobulin expression as endogenous control and cells treated with DMSO as calibrator. **A**, *ICAM1*; **B**, *HOXA1*. Results are shown as the mean of two independent experiments + SEM. Significance was not analyzed as data was achieved from two experiments only.

4.2. Reporter gene studies of genes whose ATRA response was enhanced by *EVI1*

Bingemann *et al*⁶³ showed with reporter gene assays that the modulation of the ATRA responses of the *RAR β* and *EVI1* genes by *EVI1* was mediated via DR5 RAREs. Based on these results the aim of this study was to investigate if the modulation of the ATRA response by *EVI1*, of the two chosen candidate genes *ICAM1* and *HOXA1*, was mediated through the same mechanism. To this end, various cell lines, vectors, and conditions were tested. First experiments were carried out in easily transfectable adherent cells and later the regulation was also tested in hematopoietic cells. Assays yielding negative results for *ICAM1* and/or *HOXA1*, even though positive controls measured in parallel showed the expected behavior, were not repeated. Hence the results shown in this section were carried out only once except when indicated otherwise.

4.2.1. Reporter gene assays with the human *ICAM1* regulatory region

According to the literature a functional DR5 RARE with the sequence GGGTCATCGCCCTGCCA is present at position +54/+70 bp relative to the human *ICAM1* TSS⁶⁶. The activity of this RARE was shown with reporter gene assays in COS1 cells⁶⁸ as well as in human melanoma cells (Colo38 cells)⁷⁷. In order to investigate if this RARE mediates the enhancement of ATRA induction by *EVI1*, a ~600 bp and a ~110 bp fragment of the human *ICAM1* regulatory region, both including the RARE, were amplified from genomic DNA and cloned into various luciferase vectors. The two fragments including the RARE span from -277 to +299 bp and -9 to +105 bp relative to the TSS (Fig. 14).

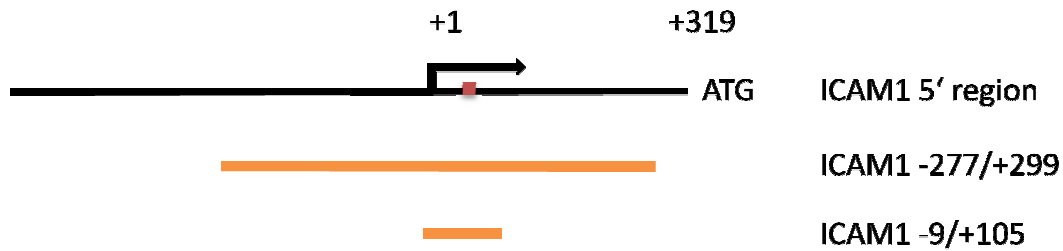


Fig. 14 Relative position of two *ICAM1* fragments containing the DR5 RARE sequence.

For reporter gene assays two *ICAM1* fragments varying in length were amplified from genomic human DNA. Here, the position of the two fragments *ICAM1*(-277/+299) and *ICAM1*(-9/+105) (orange) relative to the TSS (right angle arrow) and the RARE (red) is shown.

4.2.1.1. Testing the *ICAM1* and the *RARβ* RARE in the pGL3P backbone in reporter gene assays in COS1 cells

The two *ICAM1* fragments described above (section 4.2.1) were cloned into the pGL3P backbone which contains an SV40 promoter (SV40P) (Fig. 15). The identities of the newly generated constructs were confirmed by control digests and sequencing (see section 8.2106).

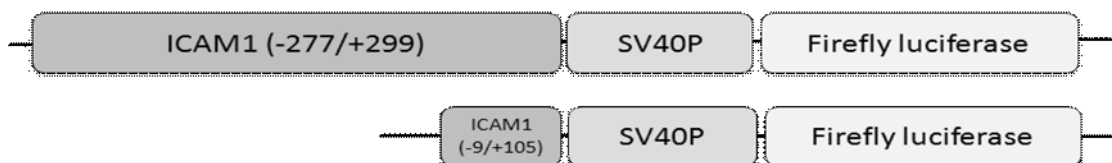


Fig. 15 Scheme of the *ICAM1*(-277/+299)/pGL3P and the *ICAM1*(-9/+105)/pGL3P reporter constructs.

The *ICAM1*(-277/+299) and *ICAM1*(-9/+105) DNA fragments were cloned into pGL3P, which contains an SV40 promoter upstream of a firefly luciferase coding sequence. The constructs were designated as *ICAM1*(-277/+299)/pGL3P and *ICAM1*(-9/+105)/pGL3P, respectively.

In order to investigate the activity of the cloned promoter sequence, dual-luciferase reporter assays were carried out. The *ICAM1* RARE, which was cloned into pGL3P, had been described to be activated by RA in COS1 cells in absence as well as in presence of additionally cotransfected retinoid receptors⁶⁸. Therefore the adherent cell line COS1 was also used for the following experiments. As test system a dual-luciferase assay was used, meaning that cells were also cotransfected with pRenilla (pRL), a plasmid constitutively expressing *Renilla* luciferase. This system allows normalization of specific firefly luciferase activity to the constitutively active *Renilla* luciferase signal. This makes it possible to eliminate false effects due to varying cell numbers or transfection efficiencies. Luciferase assays were performed after transfection of cells with the indicated luciferase reporter plasmid (1/3 of total DNA amount), pRL (1/100 of total DNA amount) and an EVI1 expression plasmid

(EVI1/pEFzeo) as effector plasmid or the respective empty vector (pEFzeo) (2/3 of total DNA amount). As positive control a reporter plasmid was used which contains two copies of the human *RARβ* RARE upstream of a thymidin kinase (tk) promoter (tkP) followed by a firefly luciferase gene in the pGL2 backbone, designated as pRARE-tk-luc. This luciferase reporter plasmid had shown ATRA responsiveness as well as enhancement of that regulation by *EVI1* in NTERA-2 cells⁶³. Pilot experiments in COS1 cells (data not shown) showed that the *ICAM1* RARE was not responsive to ATRA in this system. In order to increase the responsiveness of the luciferase reporter constructs in COS1 cells, cotransfection of different RXR and RAR expression plasmids was tested. Preliminary tests were carried out with the positive control reporter, pRARE-tk-luc, only. Results showed that cotransfection of RXRα expression plasmid could increase the ATRA response of the *RARβ* RARE compared to no coexpression of RXRα. This is also in accordance with previously reported data from Aoudjit *et al*⁶⁴. To determine whether cotransfection of RXRα would also affect the *EVI1* enhancement, an *EVI1* expression plasmid or the respective empty vector were cotransfected. For cotransfection of retinoid receptors the amount of the *EVI1* expression plasmid was lowered to half (compared to standard assay conditions as shown in the four most left panels) in order to keep the total amount of DNA constant (see section 2.4.2). The induction factor from DMSO treated cell and ATRA treated cells both not cotransfected with an *EVI1* expression plasmid is referred to as ATRA response or ATRA induction throughout the thesis. The induction factor from ATRA treated cells (light grey) to ATRA treated cells expressing *EVI1* (black) is referred to as *EVI1* enhancement throughout the thesis. The ATRA response in cells cotransfected with an RXRα expression plasmid is higher than in cells without RXRα cotransfection. The *EVI1* enhancement with cotransfection of an RXRα expression plasmid was slightly lower than in cells not cotransfected with RXRα (Fig. 16).

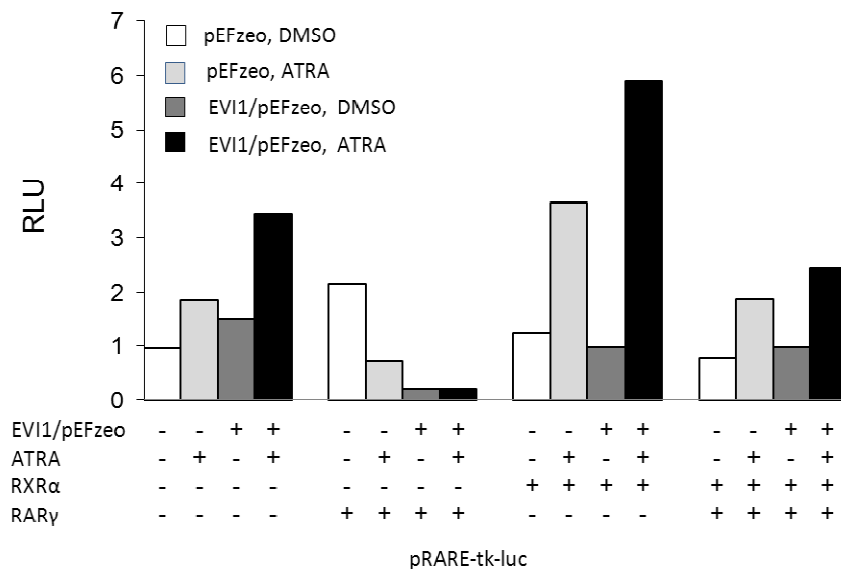


Fig. 16 RXRα coexpression increased the ATRA response, but not the enhancement by *EVI1*, of pRARE-tk-luc.

COS1 cells were transiently transfected with the reporter plasmid pRARE-tk-luc along with the normalizer plasmid pRL, the retinoid receptor expression plasmids RXRα/pCMV or RARγ/pCDNA3.1, and the effector plasmid EVI1/pEFzeo or the empty vector pEFzeo. In order to keep the total amount of transfected DNA constant throughout the whole experiment the amount of effector plasmid was adjusted considering the number of cotransfected retinoid receptor plasmids as described in section 2.4.2. Cells were treated with 1 μM ATRA or the equivalent amount of DMSO for 24 h, lysed, and firefly and *Renilla* luciferase activities were measured. Results are expressed as firefly counts normalized to *Renilla* counts in relative light units (RLU).

Since these results showed that ATRA responsiveness and *EVI1* enhancement of pRARE-tk-luc without as well as with cotransfection of RXRα (Fig. 16), these conditions were also used for tests with the *ICAM1* promoter constructs. Luciferase reporter assays showed that ATRA increased the activity of ICAM1(-277/+299)/pGL3P and ICAM1(-9/+105)/pGL3P slightly with or without cotransfection of RXRα/pCMV (Fig. 17). Ectopic *EVI1* coexpression lead to enhanced activity of ICAM1(-277/+299)/pGL3P even without addition of ATRA when no RXRα/pCMV was cotransfected. However, *EVI1* expression without ATRA treatment shows high activity that does not fit the previous findings, regarding the regulation on mRNA levels. With cotransfection of RXRα/pCMV ectopic *EVI1* expression did not enhance the ATRA response. For the pRARE-tk-luc, which was used as positive control, the *EVI1* enhancement could be indeed reproduced with the standard conditions but with cotransfection of RXRα/pCMV, in contrary to the previous results (Fig. 16), ectopic *EVI1* expression lead to a decreased ATRA response. An enhancement of basal luciferase activity could be detected for ICAM1(-277/+299)/pGL3P and pRARE-tk-luc when RXRα/pCMV was cotransfected, indicating that there is also a ligand independent way of activation as previously described⁹⁵. Although moderate regulation by ATRA and *EVI1* could be detected for ICAM1(-277/+299)/pGL3P and ICAM1(-9/+105)/pGL3P, repetitions of the experiment showed not consistent results. In summary, reporter gene assays with the

pGL3P backbone in transiently transfected COS1 cells did not yield reproducible results for pRARE-tk-luc and the ATRA induction of the ICAM1 RARE had been shown in literature⁹⁴ could not be reproduced with the indicated assay conditions.

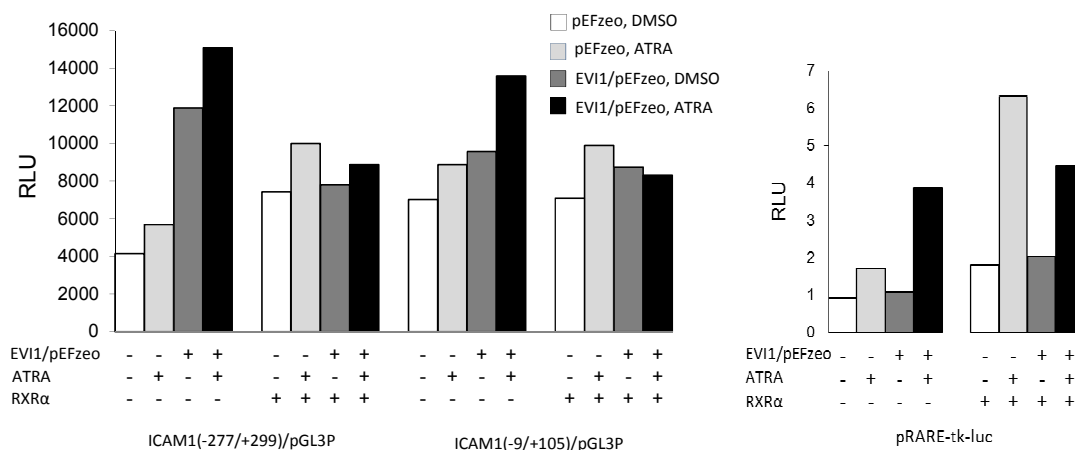


Fig. 17 The ICAM1 RARE responded only moderately to ATRA and EVI1 in COS1 cells.

COS1 cells were transiently transfected with the reporter plasmids ICAM1(-277/+299)/pGL3P, ICAM1(-9/+105)/pGL3P, or pRARE-tk-luc, along with the normalizer plasmid pRL, the retinoid receptor expression plasmid RXRα/pCMV, and the effector plasmid EVI1/pEFzeo or the empty vector pEFzeo. In order to keep the total amount of transfected DNA constant throughout the whole experiment the amount of effector plasmids were adjusted considering the number of cotransfected retinoid receptor plasmids as described in section 2.4.2. Cells were treated with 1 μM ATRA or an equivalent amount of DMSO for 24 h, lysed, and firefly and *Renilla* luciferase activities were measured. Results are expressed as firefly counts normalized to *Renilla* counts in RLU. All samples were part of one experimental setup. High variation in responsiveness between the different firefly luciferase constructs comes from different vector backbones and therefore results are shown in two distinct panels.

The results above, showed that pRARE-tk-luc basically functioned in the expected manner in COS1 cells, but ICAM1(-277/+299)/pGL3P and ICAM1(-9/+105)/pGL3P, both of which contained the well described functional ICAM1 RARE, did not. Hence, the possibility was considered that this failure was due to the vector backbone rather than to the inserted regulatory sequences. To address this possibility, the RARβ RARE was also subcloned into pGL3P in order to test the backbone for functionality in COS1 cells.

The two copies of the RARβ RARE were cut out from pRARE-tk-luc and cloned into pGL3P to obtain the reporter plasmid RARβ-RARE/pGL3P (Fig. 18). In order to be able to clone the sequence into the pGL3P backbone, 25 bp of the tk promoter, but not the TATA box, had to be maintained. After ligation, transformation and plasmid preparation, the identity of the obtained construct was confirmed with control digests (see section 8.2).



Fig. 18 Scheme of the RAR β -RARE/pGL3P reporter construct

Two copies of the RAR β RARE were cut out from the plasmid pRARE-tk-luc and cloned into the pGL3P backbone, which contains an SV40 promoter upstream of a firefly luciferase coding sequence. The construct was designated as RAR β -RARE/pGL3P.

Dual-luciferase reporter assays were carried out in COS1 cells with the reporter constructs RAR β -RARE/pGL3P and pRARE-tk-luc. Results in COS1 cells showed that the RAR β -RARE/pGL3P reporter construct did not show higher activity than the empty vector (Fig. 19 A). To investigate whether the construct RAR β -RARE/pGL3P is generally not active, or whether the system does not work in COS1 cells, the same experiment was carried out in MCF7 cells. In contrast to COS1 cells, in MCF7 cells the RAR β RARE showed responsiveness to ATRA and *EVI1* with both reporter constructs, RAR β -RARE/pGL3P and pRARE-tk-luc (Fig. 19B). However the effects were smaller with RAR β -RARE/pGL3P. Comparing the reporter gene assays with the pRARE-tk-luc construct in COS1 and MCF7 cells showed that the responsiveness of the luciferase constructs to ATRA and *EVI1* in COS1 cells was weaker than in MCF7 cells.

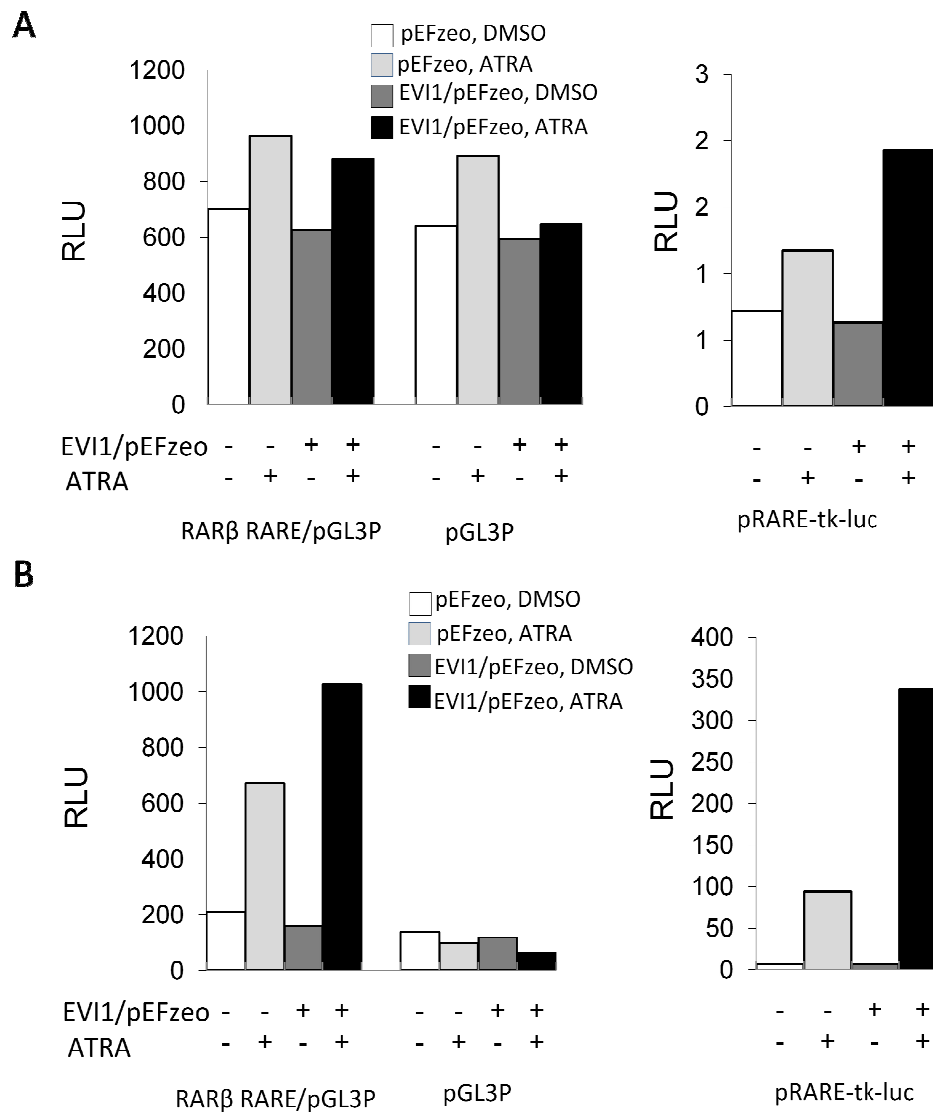


Fig. 19 RARβ-RARE/pGL3P was only active in MCF7 cells but not in COS1 cells.

COS1 cells (A) and MCF7 cells (B) were transiently transfected with the reporter plasmids RARβ-RARE/pGL3P or pRARE-tk-luc along with the normalizer plasmid pRL and the effector plasmid EVI1/pEFzeo or the empty vector pEFzeo. Cells were treated with 1 μM ATRA or an equivalent amount of DMSO for 24 h, lysed, and firefly and *Renilla* luciferase activities were measured. Results are expressed as firefly counts normalized to *Renilla* counts in RLU.

4.2.1.2. Reporter gene assays with the *ICAM1* and the *RARβ* RARE with tk promoter constructs in COS1 cells

The data shown above suggest that the pGL3P backbone, which contains an SV40 promoter, may not be suitable to observe the expected ATRA responsiveness in COS1 cells but that the construct pRARE-tk-luc, containing the tk promoter, does show the expected behavior. Furthermore, in MCF7 cells the *EV11* enhancement was bigger with the tk promoter than with the pGL3P backbone. Based on these results, the construct ICAM1(-277/+299)tkP/pGL3 was established by cloning ICAM1(-277/+299) and the tk promoter into the pGL3 basic (pGL3) backbone, which is identical to pGL3P except that the SV40 promoter is missing. In order to be able to directly compare the construct to the positive control, which was cloned into a pGL2 backbone, the *RARβ* RARE was also subcloned into the pGL3 backbone together with the tkP to establish the *RARβ*-RARE-tkP/pGL3 construct (Fig. 20). After ligation, transformation and plasmid preparation, the identity of the obtained construct was confirmed with control digests (see section 8.2).



Fig. 20 Scheme of the ICAM1(-277/+299)tkP/pGL3 and the *RARβ*-RARE-tkP/pGL3 reporter constructs

The ICAM1(-277/+299) fragment, or two copies of the *RARβ* RARE, together with the tkP from the construct pRARE-tk-luc were cloned into the pGL3 backbone upstream of the firefly luciferase coding sequence. The constructs were designated ICAM1(-277/+299)tkP/pGL3 and *RARβ*-RARE-tkP/pGL3, respectively.

Luciferase assays with the newly established constructs ICAM1(-277/+299)-tkP/pGL3 and *RARβ*-RARE-tkP/pGL3 showed again that in COS1 cells neither the *ICAM1* nor the *RARβ* RARE revealed the expected regulation pattern (Fig. 21). However, in MCF7 cells *RARβ*-RARE-tkP/pGL3 was activated by ATRA, and EV11 elevated this effect (Fig. 22), although the fold changes were smaller than with the original vector pRARE-tk-luc. As the *ICAM1* expression was not induced by ATRA in MCF7 cells (Fig. 13) and ICAM1(-277/+299)/pGL3P or ICAM1(-9/+105)/pGL3P were not regulated in the expected manner in MCF7 cells (data not shown) no further analysis with ICAM1 in MCF7 cells were carried out.

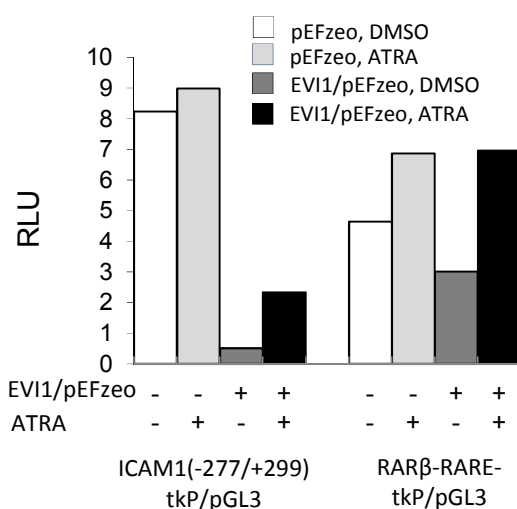


Fig. 21 ICAM1(-277/+299)tkP/pGL3 and RARβ-RARE-tkP/pGL3 were not regulated in the expected manner in COS1 cells.

COS1 cells were transiently transfected with the reporter plasmids ICAM1(-277/+299)tkP/pGL3 or RARβ-RARE-tkP/pGL3, along with the normalizer plasmid pRL, and the effector plasmid EVI1/pEFzeo or the empty vector pEFzeo. Cells were treated with 1 μM ATRA or an equivalent amount of DMSO for 24 h, lysed, and firefly and *Renilla* luciferase activities were measured. Results are expressed as firefly counts normalized to *Renilla* counts in RLU.

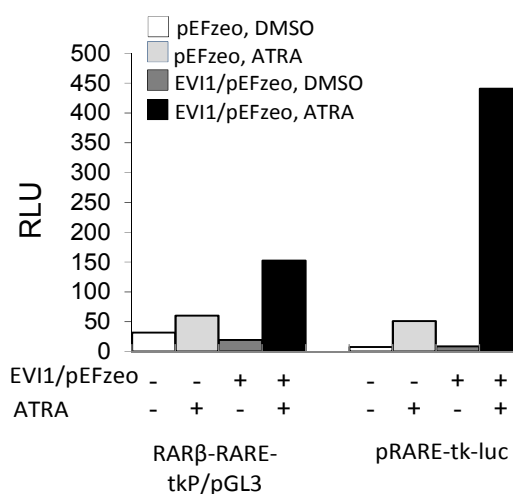


Fig. 22 RARβ-RARE-tkP/pGL3 was responsive to ATRA and EVI1 in MCF7 cells but pRARE-tk-luc showed higher effects.

MCF7 cells were transiently transfected with the reporter plasmids RARβ-RARE-tkP/pGL3 or pRARE-tk-luc, along with the normalizer plasmid pRL, and the effector plasmid EVI1/pEFzeo or the empty vector pEFzeo. Cells were treated with 1 μM ATRA or an equivalent amount of DMSO for 24 h, lysed, and firefly and *Renilla* luciferase activities were measured. Results are expressed as firefly counts normalized to *Renilla* counts in RLU.

In summary, these results showed that the ATRA and *EVI1* responsiveness of the human *RARβ* RARE is not an artifact observed only with a single plasmid, but can be reproduced in MCF7 cells also with a different backbone and the different promoters SV40P or tkP. COS1 cells show weaker responses of pRARE-tk-luc to ATRA than MCF7 cells and they do not show the expected regulation of the *RARβ* RARE in the pGL3P backbone. Hence, COS1 cells might not be sensitive enough to ATRA which is supported by the fact that in most publications, where reporter gene assays are shown in COS1 cells, nuclear receptors were cotransfected^{68,96}.

4.2.1.3. Reporter gene assays with the *ICAM1* RARE in U937 cells

As luciferase reporter assays in COS1 cells did not show the expected results, not even for the positive control, another test system was used. The microarray analyses, which had led to the identification of genes whose ATRA response was modulated by *EVI1*, had been carried out with RNA from U937 derivative cell lines. These results were confirmed, at least for *ICAM1*, by qRT-PCR. Hence, U937 cells were next used for reporter assays. Hematopoietic cells are known to be hard to transfect, which is the reason that tests were carried out first in easy transfectable COS1 cells. Previous tests in the host lab had shown that U937 cells are also hard to lyse with the passive lysis buffer. Therefore, a system was used which allows to measure secreted luciferase activity from the culture supernatant. The plasmid pGluc-basic (pGlucB), containing a *Gaussia* luciferase gene, which encodes a secreted luciferase enzyme, was used for luciferase assays in U937 cells. A drawback of this system is that it lacks a control for the transfection efficiency.

The ICAM1(-277/+299) fragment was cut out from the respective pGL3P constructs (section 4.2.1.1) and cloned into the pGlucB backbone (Fig. 23). A minimal promoter sequence is contained in the ICAM1(-277/+199) fragment. The identity of the newly generated construct ICAM1(-277/+299)/pGlucB was confirmed by control digests (8.2).



Fig. 23 Scheme of the ICAM1(-277/+299)/pGlucB reporter construct.

The ICAM1(-277/+299) fragment was cut out from the ICAM1(-277/+299)/pGL3P plasmid and cloned into the pGlucB backbone, which contains a *Gaussia* luciferase gene. The construct was designated as ICAM1(-277/+299)/pGlucB.

Gaussia luciferase reporter assays were carried out with the reporter constructs ICAM1(-277/+299)/pGlucB or pGlucB, and the effector plasmid EVI1/pEFzeo or the empty vector pEFzeo. *Gaussia* luciferase activity was measured from the supernatant. Experiments revealed that the basal activity was increased in cells transfected with ICAM1(-277/+299)/pGlucB compared to cells transfected with empty pGlucB. However, no increase in activity was detected in the presence of ATRA and/or *EVI1* (Fig. 24).

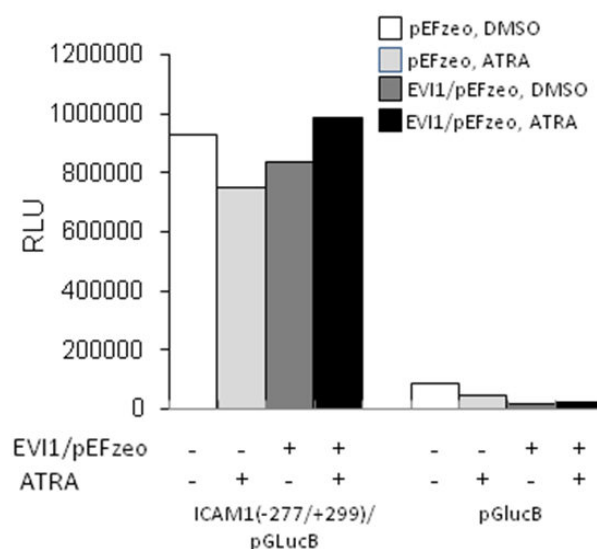


Fig. 24 The *ICAM1* RARE showed no responsiveness to ATRA or *EVI1* in U937 cells.

U937 cells were cotransfected with ICAM1(-277/+299)/pGlucB or pGlucB and the expression plasmid EVI1/pEFzeo or the empty vector pEFzeo. Cells were treated with 1 μ M ATRA or an equivalent amount of DMSO for 24 h. *Gaussia* luciferase activity was measured from the supernatant and the results are expressed in RLU.

4.2.2. Reporter gene assays with the human *HOXA1* regulatory region

HOXA1 is another gene whose induction by ATRA was enhanced by *EVI1* according to microarray analysis in U937 cells. The literature states that a functional DR5 RARE with the sequence GGTTCACCGAAAGTTCA is present 3' of the coding region of the *HOXA1* gene at position +4279/+4298 relative to the TSS⁸³. The sequence of this RARE is identical to that of the *RARβ* RARE and had been found to mediate ATRA responsiveness for example in MCF7 cells⁹⁷. In a similar manner as for the *ICAM1* RARE, a larger (~350 bp) and a smaller (~180 bp) fragment of the 3' non-coding region of the human *HOXA1* gene, both of which included the described RARE, were cloned into the pGL3P backbone. The bigger fragment extends from +4058 to +4403 bp and the smaller one from +4175 to +4356 bp relative to the human *HOXA1* TSS (Fig. 25).

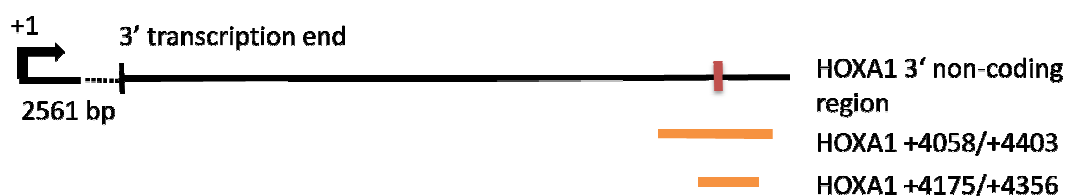


Fig. 25 Relative position of two *HOXA1* fragments containing the DR5 RARE sequence.

For reporter gene assays two *HOXA1* fragments varying in length were amplified from genomic DNA. Here, the position of the two fragments (orange) relative to the TSS (right angle arrow) and the RARE (red) is shown.

4.2.2.1. Reporter gene assays with the *HOXA1* RARE in the pGL3P backbone in MCF7 cells

The two *HOXA1* fragments described above (section 4.2.2) were cloned into the pGL3P backbone. The identities of the newly generated constructs were confirmed by control digests and sequencing (8.2).

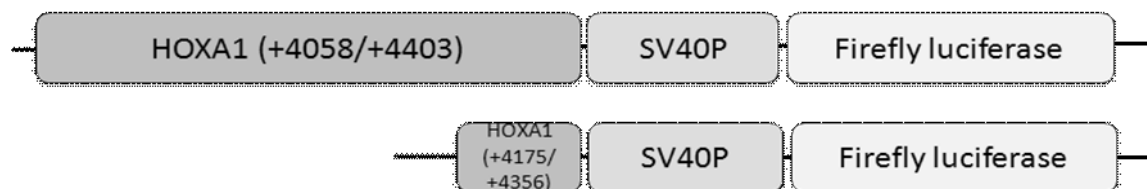


Fig. 26 Scheme of *HOXA1*(+4058/+4403)/pGL3P and *HOXA1*(+4175/+4356)/pGL3P.

The two fragments *HOXA1*(+4058/+4403) or *HOXA1*(+4175/+4356) were cloned into the pGL3P backbone containing an SV40 promoter upstream of a firefly luciferase coding sequence. The constructs were designated as *HOXA1*(+4058/+4403)/pGL3P and *HOXA1*(+4175/+4356)/pGL3P, respectively.

The *HOXA1* mRNA was strongly induced by ATRA in MCF7 cells (Fig. 13), and the *HOXA1* RARE had been previously shown to be functional in this cell line. Hence, luciferase assays with *HOXA1*(+4058/+4403)/pGL3P and *HOXA1*(+4175/+4356)/pGL3P were carried out in MCF7 cells after cotransfecting the indicated luciferase reporter gene, pRL, and the effector plasmid *EV11*/pEFzeo or the empty vector pEFzeo. Preliminary tests showed that *HOXA1*(+4058/+4403)/pGL3P as well as *HOXA1*(+4175/+4356)/pGL3P constructs are strongly responsive to ATRA treatment in MCF7 cells (Fig. 27). However, when cells were cotransfected with *EV11*/pEFzeo, the ATRA response was not enhanced. In contrast, the positive control pRARE-tk-luc, which was assayed in parallel, showed the expected *EV11* enhancement (Fig. 27).

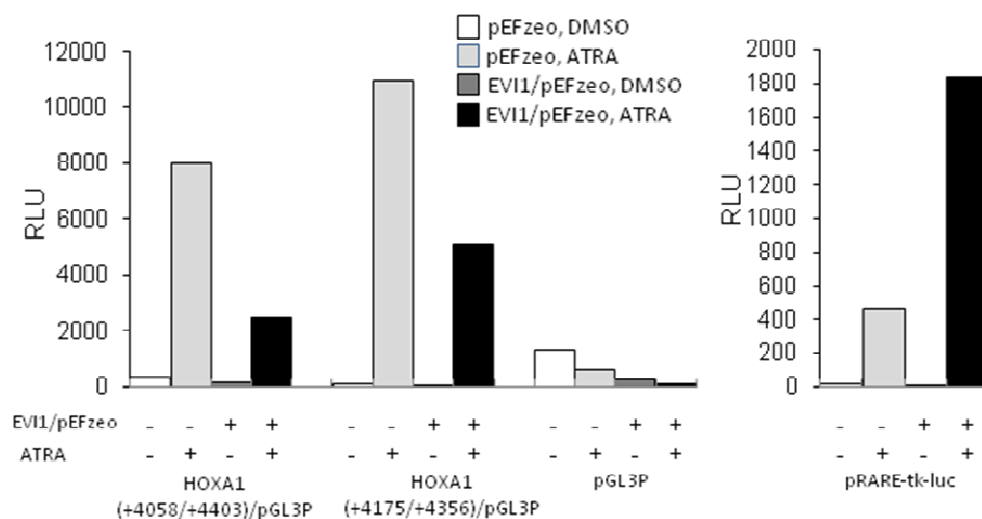


Fig. 27 The *HOXA1* DR5 RARE was responsive to ATRA but not to *EV11* in MCF7 cells.

MCF7 cells were transiently cotransfected with the reporter plasmids *HOXA1*(+4058/+4403)/pGL3P, *HOXA1*(+4175/+4356)/pGL3P, pGL3P or pRARE-tk-luc, along with the normalizer plasmid pRL, and the effector plasmid *EV11*/pEFzeo or the empty vector pEFzeo. Cells were treated with 1 μ M ATRA or an equivalent amount of DMSO for 24 h, lysed, and firefly and *Renilla* luciferase activities were measured. Results are expressed as firefly counts normalized to *Renilla* counts in RLU.

As the increase in activity of *HOXA1*(+4058/+4403)/pGL3P and *HOXA1*(+4175/+4356)/pGL3P after treatment with 1 μ M ATRA was tremendous, the possibility was considered that the dosage of ATRA was too high, so that there was no space for *EV11* to further enhance the effect. Therefore another luciferase experiment with *HOXA1*(+4058/+4403)/pGL3P was set up with ATRA concentrations ranging from 10 nM to 1000 nM. However, the results from the previous experiment were reproduced. The *HOXA1* RARE responded to ATRA in a dose dependent manner, reaching saturation at 500 nM. In the presence of *EV11* the ATRA response was not

enhanced, but rather decreased. The positive control vector pRARE-tk-luc again revealed the expected pattern.

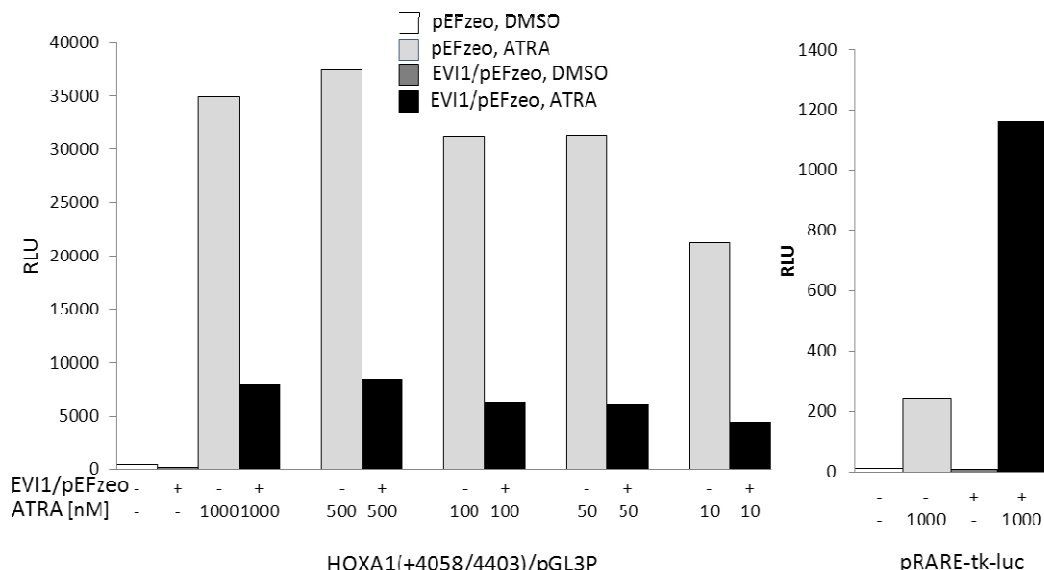


Fig. 28 HOXA1(+4058/+4403)/pGL3P responded to ATRA in a dose dependent manner, but *EVI1* decreased the level of activity.

MCF7 cells were transiently cotransfected with the reporter plasmids HOXA1(+4058/+4403)/pGL3P or pRARE-tk-luc, along with the normalizer plasmid pRL and the effector plasmid EVI1/pEFzeo or the empty vector pEFzeo. Cells were treated with ATRA concentrations ranging from 1000 to 10 nM or DMSO (equivalent amount corresponding to 1000 nM ATRA) for 24 h, lysed, and firefly and *Renilla* luciferase activities were measured. Results are expressed as firefly counts normalized to *Renilla* counts in RLU.

4.2.2.2. Reporter gene assays with the *HOXA1* RARE in U937 cells

The constructs HOXA1(+4058/+4403)/pGL3P and HOXA1(+4175/+4356)/pGL3P tested in MCF7 cells, did show ATRA responsiveness but not the expected *EVI1* enhancement. Therefore, the *HOXA1* RARE was transferred into the pGlucB vector to test the *HOXA1* RARE for *EVI1* responsiveness in U937 cells. For further studies only the HOXA1(+4058/+4403) fragment was used. As HOXA1(+4058/+4403) lacks a minimal promoter region and the vector pGlucB does not contain one, the *HOXA1* fragment had to be cloned into that vector together with a promoter sequence. Hence, the *HOXA1* RARE was amplified from HOXA1(+4058/+4403)/pGL3P together with the downstream located SV40 promoter by PCR, and the resulting DNA fragment was cloned into the pGlucB backbone. Control digests of the obtained construct HOXA1(+4058/+4403)/pGlucB (Fig. 29) were carried out to check the identity of the plasmid (8.2).



Fig. 29 Scheme of the HOXA1(+4058/+4403)-SV40P/pGLucB construct

The HOXA1(+4058/+4403) fragment together with the downstream located SV40 promoter was amplified from the HOXA1(+4058/+4403)/pGL3P plasmid and cloned into the pGLucB backbone, which contains a *Gaussia* luciferase gene. The construct was designated as HOXA1(+4058/+4403)-SV40P/pGLucB.

HOXA1(+4058/+4403)-SV40P/pGLucB or pGLucB were transiently cotransfected with EVI1/pEFzeo or pEFzeo into U937 cells and these cells were treated with ATRA or DMSO for 24 h. HOXA1(+4058/+4403)-SV40P/pGLucB displayed increased basal activity compared to the empty control vector (pGLucB), which possibly was due to the integrated SV40P sequence, the *HOXA1* RARE sequence, or both. Neither ATRA responsiveness, nor an *EVI1* enhancement, was detected for HOXA1(+4058/+4403)-SV40P/pGLucB (Fig. 30).

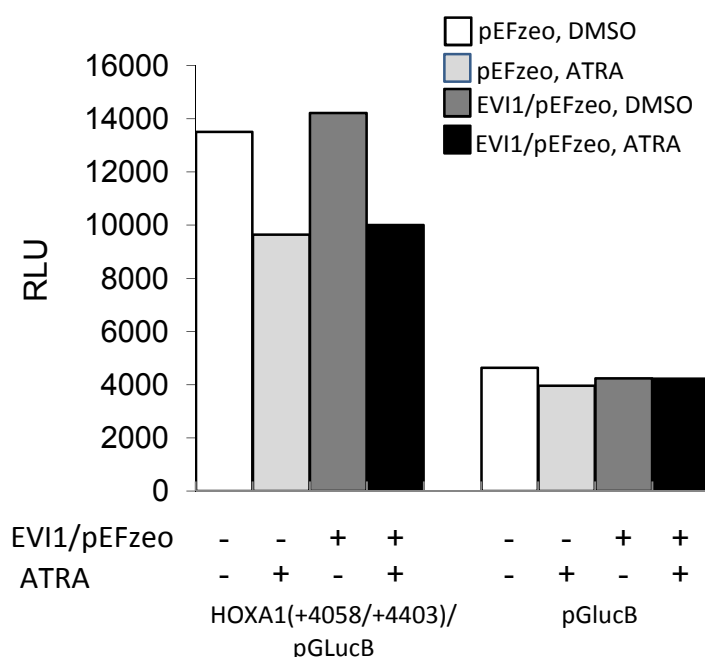


Fig. 30 The *HOXA1* RARE shows no responsiveness to ATRA or *EVI1* in U937 cells.

U937 cells were cotransfected with the HOXA1(+4058/+4403)-SV40P/pGLucB construct or pGLucB and the effector plasmid EVI1/pEFzeo or the empty vector pEFzeo. Cells were treated with 1 μ M ATRA or an equivalent amount of DMSO for 24 h. *Gaussia* luciferase activity was measured from the supernatant. Results are expressed in RLU.

4.3. Investigation of possible interactions between EVI1 and the retinoid receptors RAR and/or RXR

Bingemann *et al*⁶³ have shown that the modulation of the ATRA response by *EVI1* for the two genes *EVI1* and *RAR β* is mediated via their DR5 RAREs. The most simple mechanistic explanation for this regulation would be that *EVI1* interacted directly with one or both of the retinoid receptors, RAR and RXR, and thereby attracted cofactors that either enhance or repress the ATRA response. Seale *et al*⁶⁶ have shown that PRDM16, a paralog of *EVI1*, directly interacts with PPAR γ , a nuclear receptor which belongs to the same superfamily as the RARs. This fact encourages our hypothesis and therefore I investigated the possibility that *EVI1* interacts with the retinoid receptors using the Duolink *in situ* assay.

4.3.1. Principle of the Duolink *in situ* assay

The Duolink *in situ* assay (Olink Bioscience) is based on proximity ligation assay (PLA) probes. PLA probes are secondary antibodies (ABs) conjugated with short single stranded DNA molecules. To generate a fluorescent signal, primary ABs for the two proteins of interest, raised in different species, are necessary. After hybridization of these ABs to their respective target proteins, PLA probes with different DNA oligonucleotides (oligos) raised against the two species of the primary ABs, are added. If the two different PLA probes are in spatial proximity, the DNA strands can form a circular DNA molecule after addition of partially complementary DNA oligos. Once these oligos hybridized to the PLA probes the linker oligos can be ligated to a circular molecule which then can be amplified starting on the only existing double stranded 3' end which is formed with one of the PLA probes and the linker oligo. By rolling circle amplification, the circular DNA molecule can be amplified and still remains connected to one PLA probe. Next, fluorescently labeled DNA probes are hybridized to the amplified DNA sequence, leading to the emergence of one fluorescent dot for each pair of interacting molecules (Fig. 31). Since the binding sequence for the fluorescent probe is amplified by rolling circle amplification, the intensity of the signal does not give information about the affinity of the interaction of the target proteins. Advantages of this method are that interactions can be investigated *in situ* and in small numbers of cells. Furthermore, as the signal is amplified, the assay can be used for investigating interactions of endogenous proteins that may not be detectable with other methods.

However, the assay detects interaction between proteins that are up to 40 nm apart from each other, and possible interactions should be confirmed using other methods.

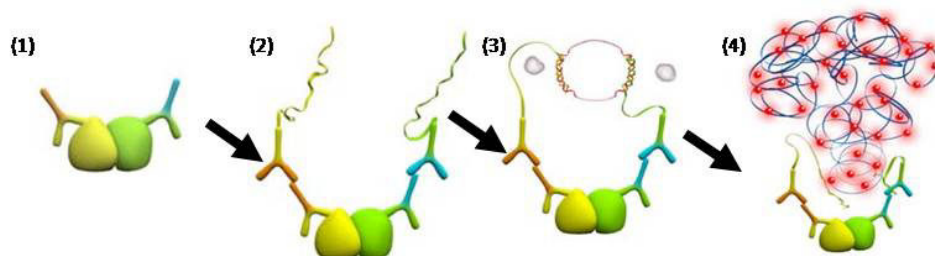


Image courtesy of Olink Bioscience

Fig. 31 Principle of the Duolink *in situ* assay

The Duolink *in situ* assay is an immuno-based assay used to detect protein interactions *in situ*. **(1)** Primary antibodies (ABs) raised in different species are hybridized to the proteins of interest. **(2)** Secondary ABs conjugated with different short single-stranded DNA molecules are hybridized to the primary ABs. **(3)** Linker oligonucleotides connect the two DNA molecules from the PLA probe and a circular DNA molecule is formed by ligation. **(4)** The circular DNA molecule is amplified by rolling circle amplification and fluorescent probes are hybridized and can be detected by microscopy.

4.3.2. Testing of primary antibodies by immunofluorescence (IF) analysis

The first step of the Duolink *in situ* assay is specific binding of primary ABs to proteins of interest in fixed cells. Hence, to be able to carry out these interaction studies, suitable primary ABs against the target proteins RXR, RAR, and EVI1 had to be identified. As for the Duolink *in situ* assay one primary AB had to be raised in mouse and the other one in rabbit, the number of possible ABs was restricted.

A rabbit anti-RXR AB Δ N197 (Santa Cruz), already shown to be specific in chromatin immunoprecipitation (Bingemann S., unpublished) and immunoblotting in transiently transfected cells (data not shown), was tested for application in IF. Even though the AB had been raised against RXR α , the manufacturer states that RXR β and RXR γ would also be recognized by that AB. Preliminary tests carried out in MCF7 and COS1 cells transiently transfected with RXR α /pCMV revealed that the AB showed the expected predominant nuclear staining in both cell lines (MCF7 Fig. 32, and COS1 data not shown). When U937 cells were stained to detect endogenous RXR with the same AB, a nuclear staining with few dots in the cytoplasm could be observed (Fig. 33).

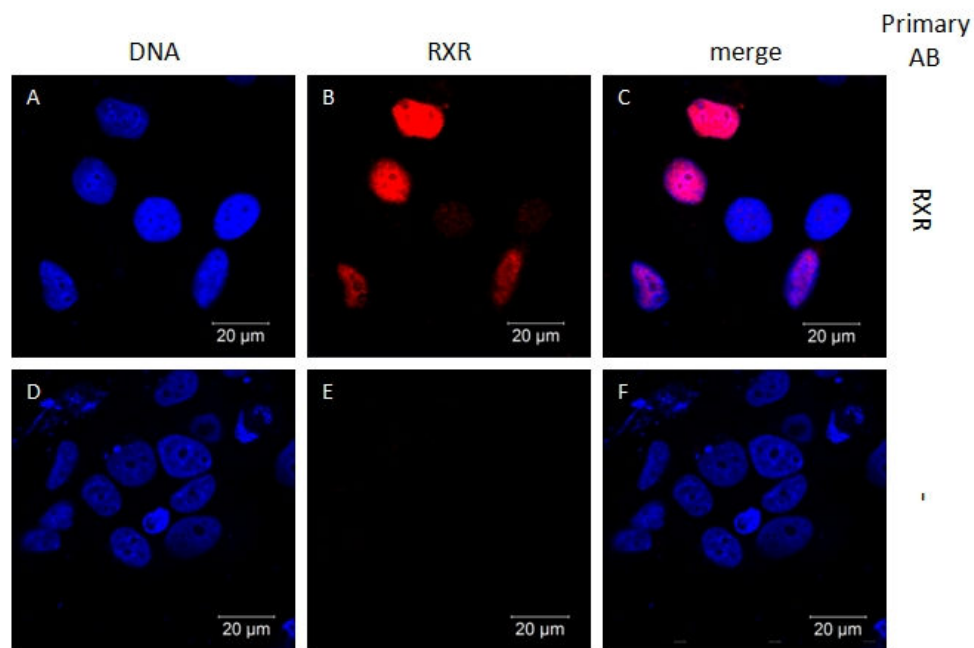


Fig. 32 Staining of RXR in transiently transfected MCF7 cells.

MCF7 cells were grown on cover slips and transfected with RXR α /pCMV. 48 h later, cells were fixed and stained with rabbit anti-RXR AB Δ N197, followed by Alexa 568 conjugated anti-rabbit AB (red). DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI; blue). **(A-C)** Cells stained with the anti-RXR AB. **(D-F)** Negative control without primary AB.

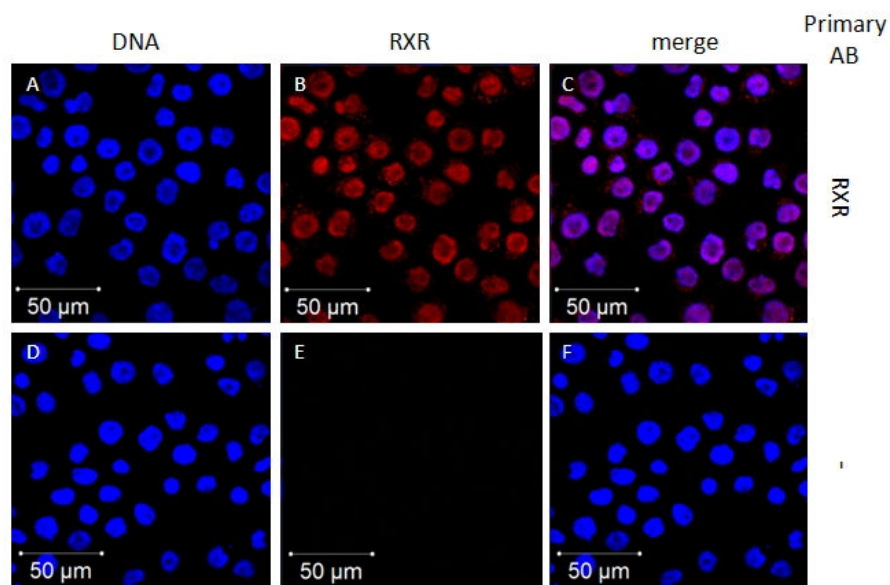


Fig. 33 Staining of endogenous RXR in U937 cells.

U937 cells were applied to slides by cytospin centrifugation, fixed, and stained with rabbit anti-RXR AB Δ N197, followed by Alexa 568 conjugate anti-rabbit AB (red). DNA was counterstained with DAPI (blue). **(A-C)** Cells stained with anti-RXR AB. **(D-F)** Negative control without primary AB.

In order to be able to confirm the known interactions between RAR and RXR as a positive control for the Duolink *in situ* assay, a mouse AB was necessary. Primary ABs RAR γ G-1, (Santa Cruz) and RXR α F-1 (Santa Cruz) raised in mouse were tested by IF in untransfected MCF7 or U937 cells but did not show any staining (data not shown). In contrast, mouse anti-RAR α AB 2C9-1F8 (Sigma-Aldrich) yielded the expected predominant nuclear staining pattern in U937 cells (Fig. 34).

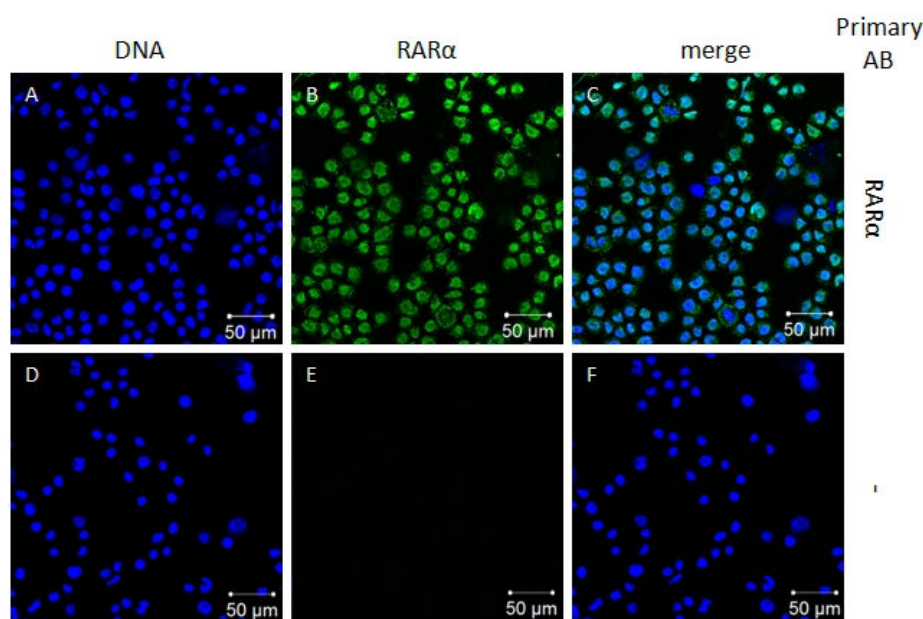


Fig. 34 Staining of endogenous RAR α in U937 cells.

U937 cells were applied to slides by cytopspin centrifugation, fixed, and stained with mouse anti-RAR α AB 2C9-1F8 and secondary FITC conjugated anti-mouse AB (green). DNA was counterstained with DAPI (blue). **(A-C)** Cells stained with anti-RAR α AB. **(D-F)** Negative control without primary AB.

As RAR and RXR are present in every cell, and no human cell line with a knock-out of one of these receptors was available in the laboratory, I decided to first probe interactions between EVI1 and retinoid receptors in cells transiently transfected with tagged proteins, in order to be able to include proper negative controls. To test the tag directed ABs, an HA-tagged version of EVI1 was transfected into MCF7 cells, and IF was performed with mouse anti-HA AB 16B12 (Covance). The HA AB yielded a clear nuclear signal in EVI1-HA/pEFzeo transfected cells but not in pEFzeo transfected cells (Fig. 35). Further experiments were done in U937 derivative cell lines which contain EVI1-HA under a tetracycline repressible promoter⁸⁹, or the corresponding empty vector (U937T_EVI1-HA or U937T_vec cells, respectively). 48 h after induction of EVI1-HA expression by tetracycline withdrawal, cells were applied to slides by cytopspin centrifugation, fixed, and stained with the anti-HA AB to detect the tagged version of EVI1, which resulted in clear nuclear staining (Fig. 36).

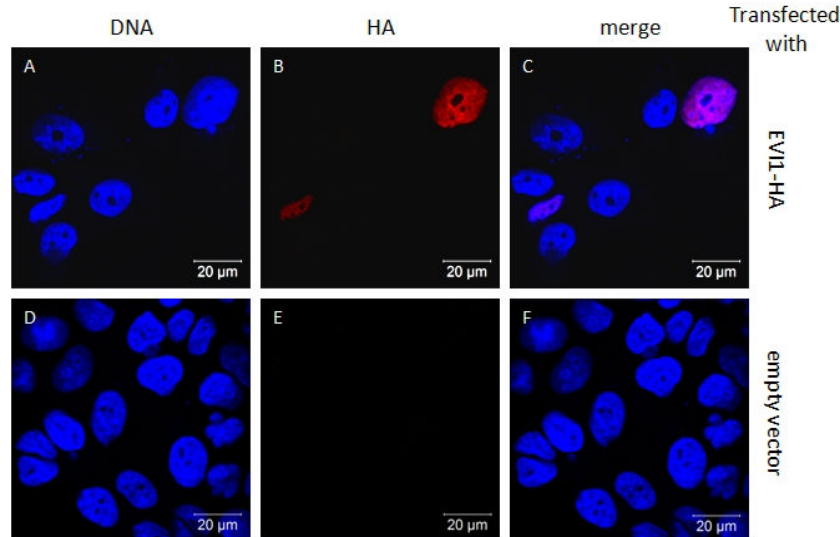


Fig. 35 Staining of EVI1-HA in transiently transfected MCF7 cells with an HA AB.
MCF7 cells were grown on coverslips and transfected with EVI-HA/pEFzeo (**A-C**) or empty vector as a control (**D-F**). 48 h later, cells were fixed and stained with mouse anti-HA.11 AB 16B12, followed by secondary TRITC conjugated anti-mouse AB (red). DNA was counterstained with DAPI (blue).

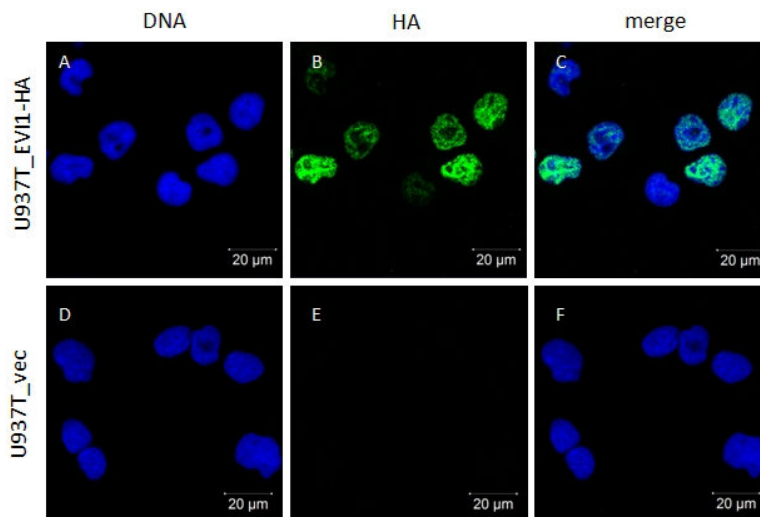


Fig. 36 Staining of EVI-HA in U937T_EVI1-HA cells with an HA AB.
U937T_EVI1-HA (**A-C**) and U937T_vec (**D-F**) cells were cultivated under withdrawal of tetracycline for 48 h to induce EVI1 expression. Cells were applied to slides by cytospin centrifugation, fixed, and were stained with mouse anti-HA.11 AB 16B12, followed by secondary FITC conjugated anti-mouse AB (green). DNA was counterstained with DAPI (blue).

Even though an AB that yielded exclusively nuclear staining in the IF experiments was available for RAR α and RXR, in order to be able to include proper negative controls a myc tag AB was used for detection of RAR. To test this AB, RAR α -mycHIS/pcDNA3.1 was transiently transfected into MCF7 cells and the protein was detected with myc AB 71D10 (cell signaling). RAR α -mycHIS/pcDNA3.1 transfected cells showed RAR α -mycHIS expression specifically only in the nucleus (Fig. 37 A-C), whereas in the empty vector transfected control cells no staining was detected (Fig. 37 D-F). An expression plasmid with an HA tagged version of RXR was constructed by

amplifying RXR α , with an engineered restriction site upstream of the stop codon, from a existing vector. This sequence was cloned into the pcDNA3 backbone and a 3xHA tag was ligated into the engineered restriction site just before the stop codon. The identity of this construct was confirmed with enzymatic digestion (see section 8.2). For detecting this tagged version of RXR, the previously described HA tag AB was used in cells transiently transfected with RXR α -HA/pcDNA3.

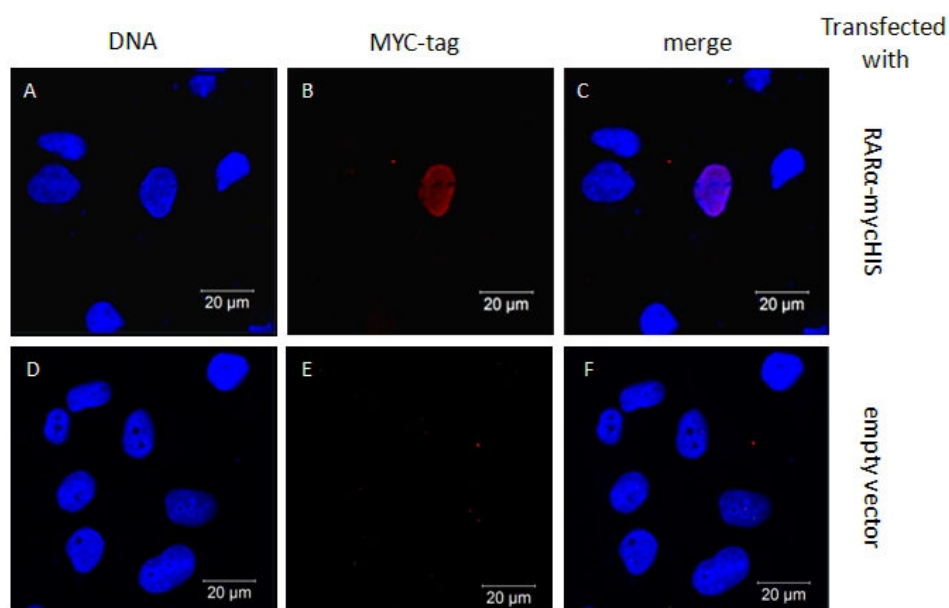


Fig. 37 Staining of RAR α -mycHis in transiently transfected MCF7 cells with a myc AB. MCF7 cells were grown on coverslips and transfected with RAR α -mycHis/pcDNA3.1 (A-C) or empty vector (D-F). 48 h later, cells were fixed and stained with rabbit anti-myc tag AB 71D10, followed by secondary Alexa 568 conjugated anti-rabbit AB (red). DNA was counterstained with DAPI (blue).

4.3.3. Duolink *in situ* assay

4.3.3.1. Establishing a positive control for the Duolink *in situ* assay

As it is a well-known fact that RAR forms heterodimers with RXR, these two proteins were chosen to establish a positive control for the Duolink *in situ* assay. As a first step, Duolink *in situ* assays for detecting interactions between RAR α and RXR α proteins were carried out in transiently transfected MCF7 cells. Cells were transfected with RAR α -mycHis/pcDNA3.1 and RXR α -HA/pcDNA3 and interactions between the two proteins were probed using the myc and HA tag ABs. In these experiments, fluorescent signals were specifically detected only in the nucleus (Fig. 38).

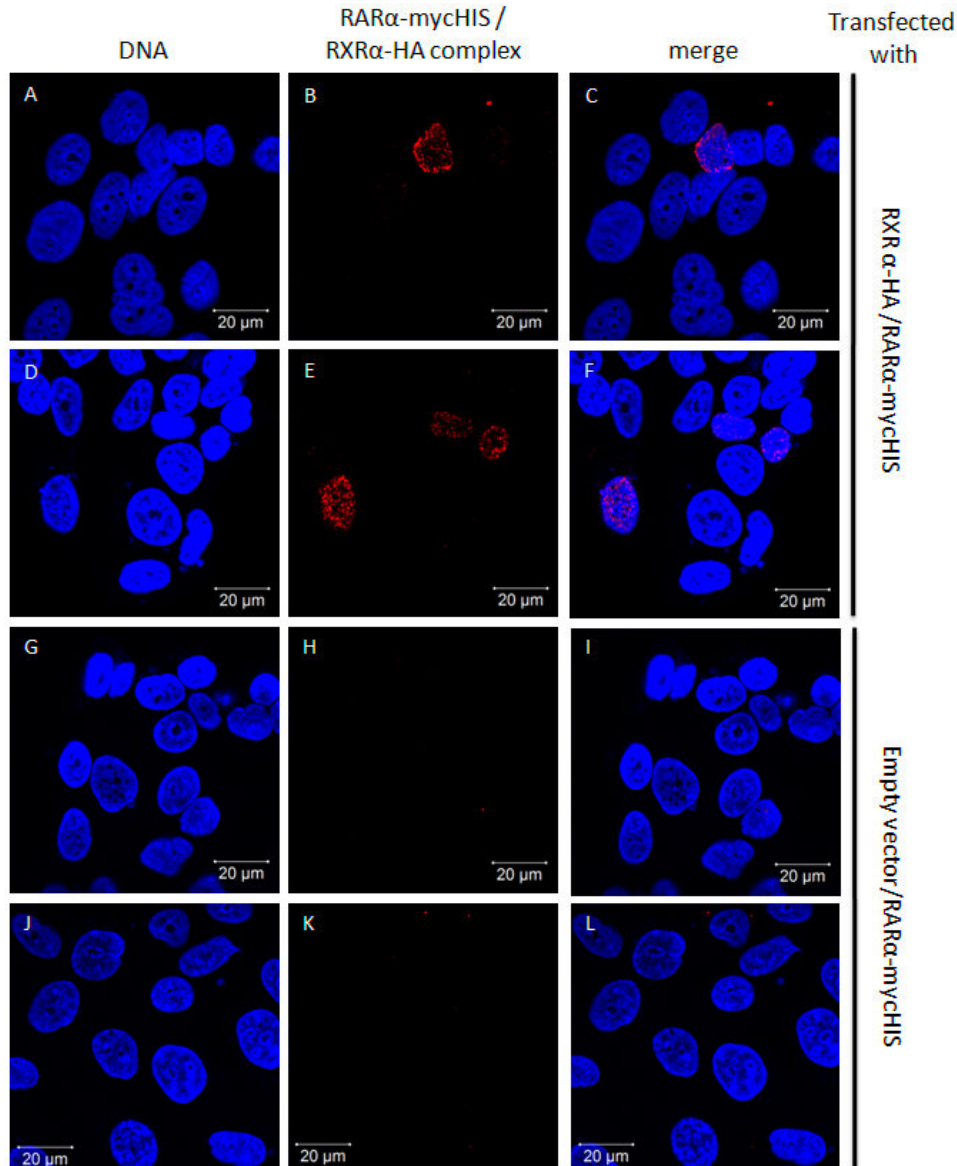


Fig. 38 Confirmation of the interaction between RXR α and RAR α in transiently transfected MCF7 cells using the Duolink *in situ* assay and tag specific ABs.

MCF7 cells seeded onto chamber slides were transfected with RAR α -mycHis/pcDNA3.1 and RXR α -HA/pcDNA3 (A-F), or RAR α -mycHis/pcDNA3.1 and pcDNA3 (G-L). Cells were treated with 1 μ M ATRA (D-F and J-L) or the respective amount of DMSO (A-C and G-I) for 24 h. Specimens were stained with primary ABs against the HA and myc tags and the Duolink *in situ* assay was carried out to detect protein-protein interactions (red). DNA was counterstained with DAPI (blue). The experiment was carried out twice, yielding comparable results.

To test whether the interaction between RAR α and RXR α could also be shown for endogenous proteins with this assay, U937 cells were immobilized on slides by cytopspin centrifugation, hybridized with primary ABs against RXR and RAR α , and subjected to the Duolink assay. Using both primary ABs, signals for interaction of RAR α and RXR in U937 cells could be detected (Fig. 39), whereas the technical negative control, where the RXR primary AB was left out, did not show signals at all. Even though the low cytoplasm/nucleus ratio of U937 cells makes this difficult to judge, signal appeared to accumulate mostly in the nucleus.

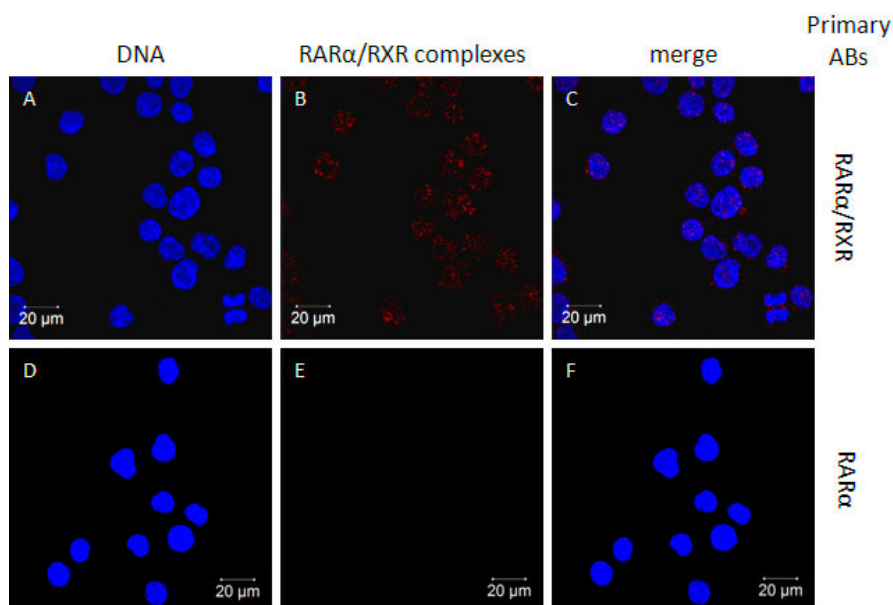


Fig. 39 Confirmation of the interaction of endogenous RXR and RAR α in U937 cells using the Duolink *in situ* assay.

U937 cells were applied to slides by cytospin centrifugation, fixed, and stained with mouse anti-RAR α AB and rabbit anti-RXR AB (**A-C**) or mouse anti-RAR α AB only (**D-F**), and the Duolink *in situ* assay was carried out to detect protein-protein interactions (red). DNA was counterstained with DAPI (blue). The experiment was carried out once.

4.3.3.2. EVI1 was in spatial proximity to RXR and RAR α in transiently transfected MCF7 cells

To investigate whether EVI1 may modulate the ATRA response via interaction with RAR or RXR, the Duolink *in situ* assay was carried out to probe the spatial proximity of EVI1-HA and untagged RXR as well as EVI1-HA and RAR α -mycHIS. The first attempts to investigate these interactions were carried out in transiently transfected MCF7 cells. After transfection with expression plasmids for both proteins of interest, cells were treated with 1 μ M ATRA or the respective amount of DMSO for 24 h. Cells transfected with an expression vector for only one of the putative interaction partners and the corresponding empty vector served as negative controls.

These experiments showed that both RXR and RAR α -mycHIS were in spatial proximity to EVI1-HA when both proteins of interest were ectopically overexpressed by transfection into MCF7 cells (Fig. 41, Fig. 40). The Duolink signal was located in the nucleus in both cases, and no signals were detected in the negative controls. Duolink signals were detected both in untreated (DMSO) as well as in ATRA treated samples. These experiments yielded clear evidence that EVI1 is in spatial proximity to RAR α as well as RXR in transfected MCF7 cells.

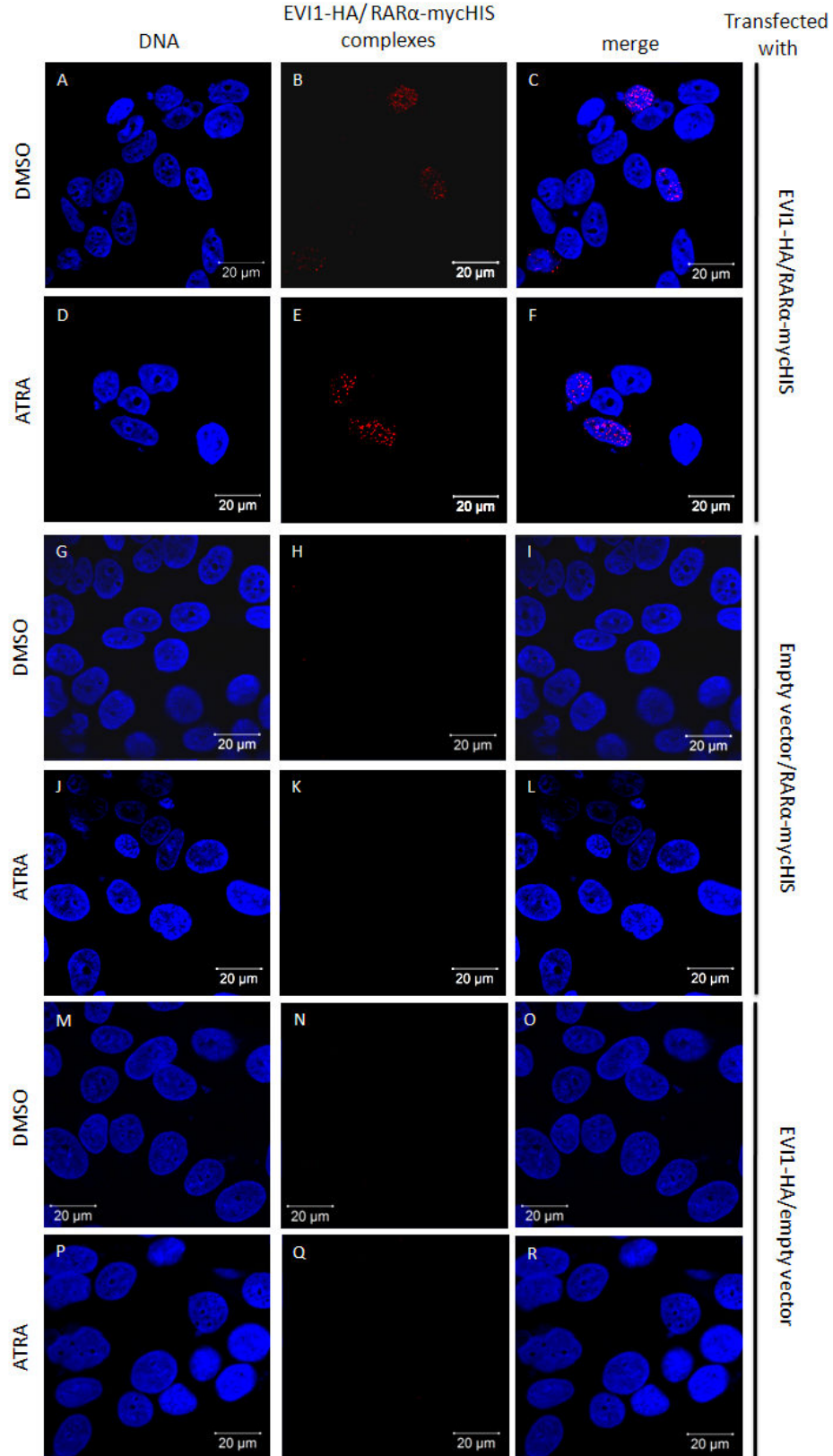


Fig. 40 Duolink *in situ* assay detected interactions between RAR α -mycHIS and EVI1-HA in transiently transfected MCF7 cells.

MCF7 cells seeded onto chamber slides were cotransfected with RAR α -mycHIS/pcDNA3.1 and EVI1-HA/pEFzeo (**A-F**), RAR α -mycHIS/pcDNA3.1 and pEFzeo (**G-L**) or EVI1-HA/pEFzeo and pcDNA3 (**M-R**). Cells were treated with 1 μ M ATRA (**D-F**, **J-L** and **P-R**) or the respective amount of DMSO (**A-C**, **G-I** and **M-O**) for 24 h. Specimens were incubated with primary ABs rabbit anti-myc and mouse anti-HA and the Duolink *in situ* assay was carried out to detect protein-protein interactions (red). DNA was counterstained with DAPI (blue). The experiment was performed twice, yielding comparable results.

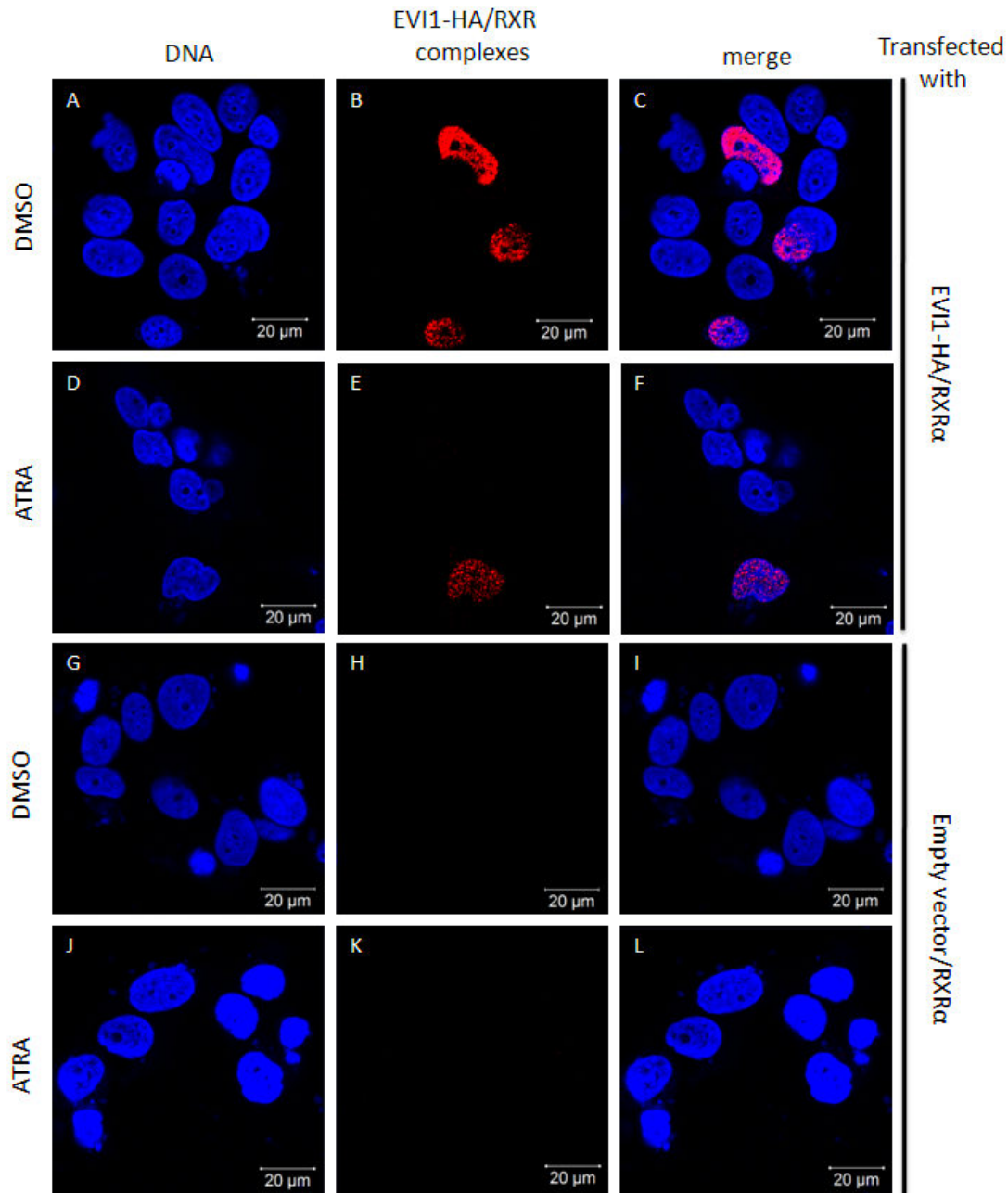


Fig. 41 Duolink *in situ* assay detected interactions between RXR and EVI1-HA in transiently transfected MCF7 cells.

MCF7 cells seeded onto coverslips were transfected with RXRα/pCMV and EVI-HA/pEFzeo (**A-F**), or RXRα/pCMV and pEFzeo (**G-L**). Cells were treated with 1 μM ATRA (**D-F** and **J-L**) or the respective amount of DMSO (**A-C** and **G-I**) for 24 h. Specimens were incubated with primary ABs rabbit anti-RXR and mouse anti-HA, and the Duolink *in situ* assay was carried out to detect protein-protein interactions (red). DNA was counterstained with DAPI (blue). The experiment was performed three times, yielding comparable results.

4.3.3.3. EVI1 protein domains involved in the interaction with RAR α

The results of the Duolink *in situ* assay showed that EVI1 is in spatial proximity to RAR α as well as RXR in transiently transfected MCF7 cells. In order to determine the domain(s) responsible for the presumed interaction, truncated versions of EVI1 were tested for interaction with RAR α in the Duolink *in situ* assay with the same ABs as used before. EVI1 consists of two ZF domains (ZF1 and ZF2), an IR, a RD and an AR¹⁷ (Fig. 42). Two deletion constructs, one containing the IR and RD domain (IR-RD), which harbors the endogenous EVI1 nuclear localization sequence (NLS), and the other containing ZF1 with an engineered NLS (ZF1-NLS) were chosen for interaction studies (Fig. 42). Both IR-RD and ZF1-NLS contained an HA tag at the N-terminus.

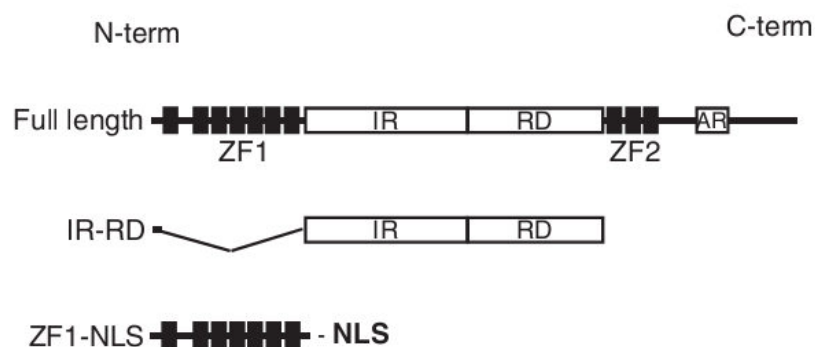


Fig. 42 Scheme of full length EVI1 protein and the deletion constructs IR-RD and ZF1-NLS.

zinc finger (ZF), intervening region (IR), repression domain (RD), acidic region (AR). Figure taken from Bingemann *et al.*⁶³, © 2009 FEBS.

These experiments revealed that both of the non-overlapping truncated versions of EVI1, IR-RD and ZF1-NLS, yielded positive signals for interaction with cotransfected RAR α -mycHis (Fig. 4Fig. 43). This may indicate that transient transfection leads to false-positive results in the Duolink *in situ* assay due to high abundance of the overexpressed proteins. Alternatively, more than one domain may be necessary for the localization of EVI1 next to the nuclear receptor RAR α .

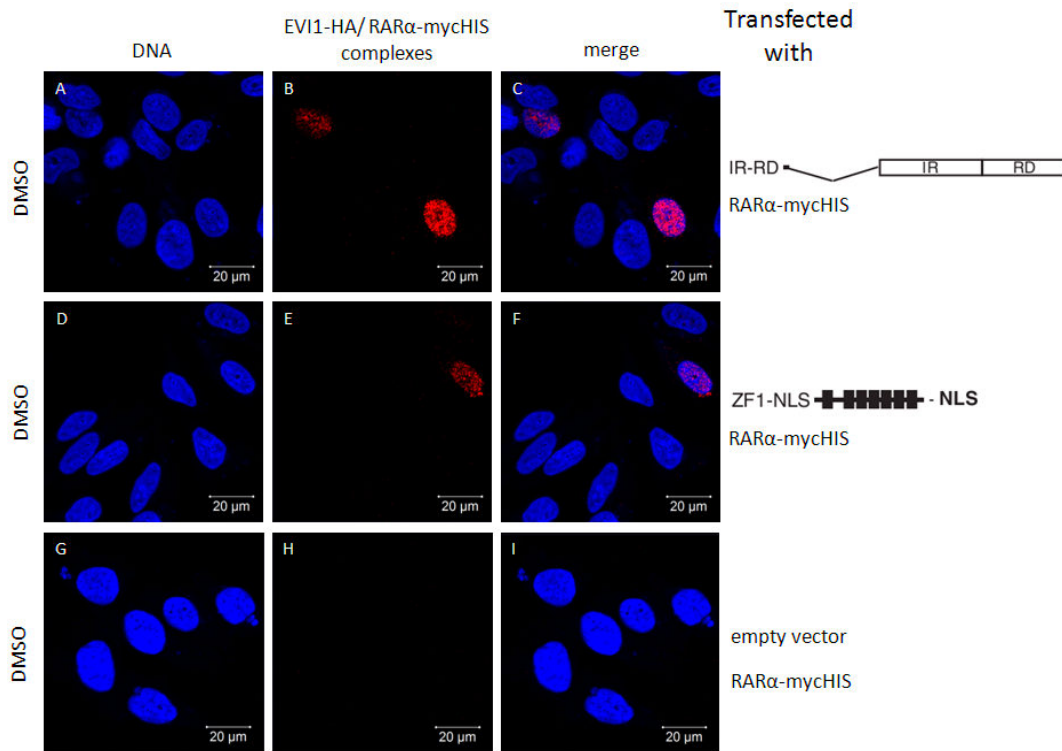


Fig. 43 Duolink *in situ* assay detected interactions between RAR α -mycHIS and IR-RD or ZF1-NLS in transiently transfected MCF7 cells.

MCF7 cells seeded onto chamber slides were cotransfected with RAR α -mycHIS/pcDNA3.1 and IR-RD-HA/pEFzeo (A-C), RAR α -mycHIS/pcDNA3.1 and ZF1-NLS-HA/pEFzeo (D-F) or RAR α -mycHIS/pcDNA3.1 and pEFzeo (G-I). Cells were treated with DMSO for 24 h in order to keep conditions similar to previous experiments. Specimens were incubated with mouse anti-HA and rabbit anti-myc ABs and the Duolink *in situ* assay was carried out to detect protein-protein interactions (red). DNA was counterstained with DAPI (blue). The experiment was performed once.

4.3.3.4. EVI1-HA was in spatial proximity to RXR in stably transfected U937 derivative cell lines

As shown above (Fig. 41, Fig. 40, Fig. 43), both RAR α and RXR are in spatial proximity with EVI1 in transiently transfected MCF7 cells. To confirm these presumed interactions for endogenous RXR and experimentally expressed EVI1, U937T_EVI1-HA and U937T_vec cells were used. U937T_EVI1 cells contain a stably integrated construct encoding a tetracycline repressible, HA tagged version of EVI1, which upon tetracycline withdrawal is expressed at levels comparable to those of endogenous EVI1 in the AML cell line HNT-34⁸⁹. Interaction of EVI1 and RXR α in U937T derivative cells was investigated with Duolink *in situ* assay. As the ABs against HA and RAR α had both been raised in mouse, the interaction between EVI1 and RAR α could not be investigated in this system, yet.

As shown in Fig. 44, U937T_EVI1-HA cells show positive signals for interaction of EVI1-HA and RXR when stained with HA and RXR ABs (A-B). In contrast, U937T_vec cells only give few signals which can be regarded as Duolink background. These results suggest that the Duolink *in situ* assay is sensitive enough to recognize spatial proximity between endogenously expressed RXR and EVI1 that is expressed experimentally, but at levels similar to endogenous ones.

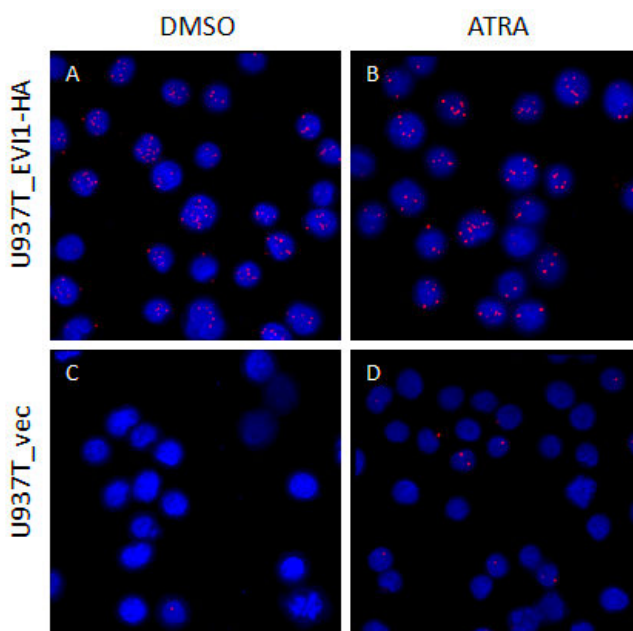


Fig. 44 Duolink *in situ* assay detected interactions between endogenous RXR and experimentally expressed EVI1-HA in U937 derivative cell lines.

U937T_EVI1-HA (A,B) and U937T_vec (C,D) cells were washed and cultivated under tetracycline withdrawal for 48 h in order to induce EVI1-HA expression in U937T_EVI1-HA cells. Cells were treated with 1 μ M ATRA (B,D) or the respective amount of DMSO (A,C) for 24 h. Cells were immobilized with Cell-Tak adhesive and incubated with primary ABs rabbit anti-RXR and mouse anti-HA and the Duolink *in situ* assay was carried out to detect protein-protein interactions (red). DNA was counterstained with DAPI (blue). The experiment was performed twice, yielding comparable results.

5. Discussion

Bingemann *et al.* showed that the *EVI1* mRNA and protein were induced after ATRA treatment in a time and dose dependent manner in the human teratocarcinoma cell line NTERA-2¹. The regulation of *EVI1* by ATRA was mediated through a DR5 RARE, which was located in exon 1a and was bound by RAR α and RXR both in the absence and in the presence of ligand¹. Furthermore, this study revealed that *EVI1* modulated the ATRA response of the *RAR β* and *EVI1* genes in NTERA-2 cells. Follow-up microarray analyses (Steinmetz *et al.*, unpublished) revealed that *EVI1* modulated the ATRA response of another 44 genes in U937 cells that had been infected with an *EVI1* expression vector or empty control vector. The expression of 34 of these genes was induced by ATRA and *EVI1* further enhanced this effect.

The purpose of this thesis was to test whether the *EVI1* enhanced ATRA response of these 34 genes is also mediated through RAREs located in their regulatory regions, as had been shown for *RAR β* and *EVI1*⁶³. To address this question, reporter gene assays had already been performed previously to this work (Steinmetz *et al.*, unpublished). For these experiments, genes had been chosen whose expression levels were enhanced by *EVI1* and ATRA but which had no previously described functional RAREs in their regulatory sequences. *In silico* predicted RAREs were tested but did not yield the expected response in reporter gene assays. Therefore, in the present thesis, genes were investigated that showed *EVI1* enhancement of the ATRA response and additionally had already been described to possess a functional RARE. Two of these genes, *ICAM1* and *HOXA1*, were chosen for further analysis.

In order to verify the microarray results showing regulation of *ICAM1* and *HOXA1* by ATRA and *EVI1*, qRT-PCR was carried out. Samples from two hematopoietic cell lines, U937 and HL60, which had been infected with an *EVI1* expression vector or empty control vector, were analyzed. For *ICAM1*, these experiments confirmed an *EVI1* enhancement (fold change between ATRA treated cells expressing or not expressing *EVI1*) of ≥ 2 - fold in both cell lines (Fig. 12 A and C). However, the *HOXA1* data obtained by microarray analysis could not be reproduced using qRT-PCR (Fig. 12 B and D). Therefore, reporter gene assays were performed mostly using the *ICAM1* RARE.

For luciferase reporter assays, cell lines were chosen according to the following criteria: Firstly, they should be transfectable with high efficiency, which is the case for several adherent but none of the commonly used suspension cell lines. Secondly, cell lines were selected in which the candidate RAREs had already been reported to be responsive to ATRA treatment. Cell lines which combine these features were MCF7 cells for *HOXA1*^{92,97} and COS1 cells for *ICAM1*^{66,68}.

Both RAREs were first cloned into the pGL3P backbone and tested in their respective cell line. For the *ICAM1* RARE neither the already described regulation by ATRA^{66,68} nor the expected *EVI1* enhancement was detected in COS1 cells. The expected regulation was also not detected when different RAR or RXR isoforms were cotransfected (Fig. 17), which had increased the ATRA response in other studies⁶⁸. As not even the positive control, the *RARβ* RARE, was responsive to ATRA in the pGL3P backbone containing the SV40 promoter (Fig. 19), I also tried a construct with a backbone and different basal promoters. However, even when the SV40 promoter was exchanged for the tk promoter, no reproducible ATRA response or *EVI1* enhancement was detected in COS1 cells, neither with the *RARβ*, nor the *ICAM1* RARE (Fig. 21). In contrast, in MCF7 cells all constructs containing the *RARβ* RARE, no matter which promoter sequence was used, showed the expected regulation (Fig. 19, Fig. 22). For the *HOXA1* RARE in the pGL3P backbone, the ATRA regulation in MCF7 cells could be reproduced but the expected *EVI1* enhancement was not observed (Fig. 27).

In summary, the responsiveness of the *ICAM1* RARE to ATRA in COS1 cells⁶⁸ could not be reproduced even though different vector backbones were used. Possible explanations might be that previous studies have been performed using yet another vector backbone⁶⁸, or that the fragment size of the *ICAM1* RARE in this thesis was different to the one that had been used in other studies⁶⁸. Furthermore, the fact that the COS1 cell line was established in 1981 favors the possibility that several mutations took place since then, leading to a different cell line version. Hence, it cannot be excluded that the COS1 cells that had been used in studies performed in 1995⁶⁸ were different to the cells used in this study. Additionally, COS1 cells contain the SV40 large T antigen⁹⁸, and the pGL3P plasmid contains an SV40 origin of replication (ori) in its promoter sequence⁹⁹. In combination, these circumstances can lead to a varying copy number of the plasmid which could have an effect on the reproducibility of the experiments. Nevertheless, several studies have reported that the pGL3P backbone, in principle, works in COS1 cells^{100,101}.

The previously demonstrated ATRA responsiveness of the *HOXA1* RARE^{92,97} could be reproduced but no *EVI1* enhancement was detected in luciferase assays using MCF7 cells. These cells, however, endogenously express *EVI1* at a moderate level. Therefore, it might be possible that the endogenous *EVI1* is already sufficient to enhance the ATRA response and no further induction can be accomplished upon ectopic *EVI1* expression. A similar phenomenon was observed by the observation that HDAC1 mediated *EVI1* repression was not further induced by ectopic expression of HDAC1¹⁰². The authors interpreted that these results were due to the endogenous high abundance of HDACs in NIH-3T3 cells¹⁰². However, this explanation does not fit the results of the positive control, where the activity of the *RARβ* RARE could indeed be enhanced upon transient transfection of *EVI1*. Anyways, a difference in affinity of *EVI1* to the RAR/RXR complex on the *HOXA1* and *RARβ* RAREs could explain why one RARE shows *EVI1* enhancement upon ectopic expression of *EVI1* and the other does not. Indeed, the ATRA response of the *HOXA1* expression in the absence of ectopically expressed *EVI1* in MCF7 cells was much higher than that of the *RARβ* expression (data not shown). Therefore it could be possible that the moderate expression of *EVI1* in MCF7 cells is high enough to enhance the ATRA response of the *HOXA1* RARE but for the *RARβ* RARE higher concentrations of *EVI1* might be needed.

Because experiments with adherent cell lines did not yield the expected results, luciferase reporter assays with the two candidate RAREs were also carried out in the hematopoietic cell line U937 (Fig. 24, Fig. 30), which had also been used for the analysis of the mRNA levels. However, also in this cell line no *EVI1* enhancement could be detected. As the reporter constructs did not even respond to ATRA treatment, and due to a lack of a positive control as well as a normalizer for transfection efficiency in this system, technical problems cannot be excluded.

Up to now, the *EVI1* enhancement was only detected in luciferase assays using the *RARβ* RARE, but not for other candidate genes like *ICAM1* or *HOXA1*. The *RARβ* RARE is present in the luciferase vector in two copies which may have an amplifying effect on the *EVI1* responsiveness. As the *HOXA1* and *ICAM1* RAREs are only present in the backbones as a single copy it would be interesting to investigate the *EVI1* enhancement with constructs containing multiple copies of the RAREs. Another explanation for the negative luciferase results may be provided by the results of Ishibashi *et al*¹⁰³. In that study, *EVI1* interacted with C/EBPβ and thereby stimulated *PPARγ2* expression in adipocytes. Furthermore, *EVI1* and C/EBPβ bound to two distant motifs (-183 and +2.6 kb from TSS) in the regulatory region of *PPARγ2*¹⁰³.

However, when the EVI1-C/EBP β binding motif was used in reporter gene assays, transient transfection of *EVI1* did not enhance the luciferase activity¹⁰³. The authors concluded that EVI1 does not directly regulate the transcription but rather attracts histone-modifying enzymes. Hence, it might be possible that necessary cofactors were not abundant enough in the tested cells to enhance the effect by ectopic EVI1 expression. Therefore, it would be interesting to identify the cofactors, necessary for EVI1 enhanced ATRA regulation.

Nevertheless, another study revealed that activation of a target gene by Evi1 does come along with responsiveness to cotransfection of *Evi1* in luciferase assays. As *Evi1* activated the *Gata-2* promoter and directly bound to an Evi1 binding site in this promoter²⁴ this site was further analyzed in luciferase assays. Fragments from the *Gata-2* promoter were cloned into the pGL3 vector and cotransfected *Evi1* enhanced the activity of these construct in a human erythroleukemia cell line, HEL, as well as in a murine stem cell factor-dependent multipotent progenitor cell line, EML C1 cells²⁴.

Further evidence of Evi1 directly regulating gene transcription was given by a study from Takahashi *et al*¹⁰⁴. Experiments showed that Evi1 activated the PLZF promoter in luciferase experiments and was bound to an Evi1-like site in the promoter of PLZF¹⁰⁴. However, Evi1 expression did not correlate with PLZF expression, as in the hematopoietic cell line K562 Evi1 was expressed, but PLZF was not. This indicates, that other tissue specific factors are necessary for PLZF expression. Studies revealed a yet unknown factor with a molecular weight of ~28kDa, to be responsible for at least 50% of the tissue specific expression of PLZF¹⁰⁴.

Since in the present study reporter gene assays did not provide further insights into the mechanism of the enhancement of the ATRA response by EVI1, I chose to investigate whether EVI1 is able to modulate transcriptional regulation of ATRA target genes via binding to retinoid receptors. Therefore, Duolink *in situ* assays were performed. In a first step, the assay was carried out in transiently transfected MCF7 cells using mostly tag-specific ABs. These experiments revealed that EVI1-HA was in spatial proximity to RAR α -mycHis (Fig. 40) as well as to RXR (Fig. 41). The interactions were detected throughout the nucleus and not in the cytoplasm.

To confirm these findings in a system where the presumed interaction partners are expressed at physiological or near-physiological levels, Duolink assays were also performed in U937T_EVI1-HA cells, a U937 cell line stably transfected with EVI1-HA (expression of EVI1 inducible to a level comparable to that in the AML cell line HNT-34). Like in the transiently transfected MCF7 cells, spatial proximity between EVI-

HA and endogenous RXR could be detected (Fig. 44). Interactions between EVI1-HA and endogenous RAR α could not be analyzed as no specific RAR α AB raised in rabbit was available in our lab at that time. For the interaction studies between EVI1-HA and RXR it has to be considered that the RXR AB may not be entirely specific for its target protein. IF experiments using the RXR AB Δ N197 (Santa Cruz) showed that the protein was located predominantly in the nucleus in U937 cells (Fig. 33) and transiently transfected MCF7 cells (Fig. 32) or COS1 cells (data not shown). However, when the AB was re-tested in U937T cells, the signal was detected throughout the whole cell and was not located predominantly in the nucleus anymore. Further tests regarding fixation and blocking agent could not restore the nuclear localization of the signal in U937T cells. It might be possible that the localization in U937T cells is different than in other cells. Several studies showed that the localization of retinoid receptors is very dependent on cell type, growth conditions, cell density and developmental state^{105–107}. However, the presumed interaction between EVI1-HA and RXR proteins should be confirmed with another AB. The interaction of RAR α -mycHIS and EVI1-HA in transiently transfected cells was carried out with tag directed ABs that were proven to be specific, at least as judged on the exclusively nuclear localization of their antigens, in all used cell lines.

To further understand the interaction between EVI1 and retinoid receptors, it was of interest to investigate the domains important for the interaction. Therefore, Duolink *in situ* assays were carried out with truncated EVI1 versions transiently cotransfected with RAR α -mycHIS into MCF7 cells. Interaction of EVI1 and RAR α -mycHIS was still detected using either of the non-overlapping deletion constructs IR-RD and ZF1-NLS, indicating that not only one domain of EVI1 is responsible for the interaction but it is rather an effect of multiple domains. This assumption is strengthened by studies of Bingemann⁹¹ which showed that the increased ATRA induction of the RAR β RARE in the presence of *EVI1* was not attributable to only one protein domain but was rather a combinatorial effect requiring all domains of EVI1.

EVI1 interacted with RAR α or RXR in Duolink *in situ* experiments in the presence as well as in the absence of ATRA, suggesting that the interaction was ligand independent. This observation corresponds with studies showing that the interaction of the EVI1 paralog PRDM16 with PPAR γ , a member of the same nuclear receptor family as RAR, was also ligand independent⁸⁶. PRDM16 was shown to be important in the switch from white to brown fat cells, as it activates genes important in brown fat cells and represses white-fat cell selective genes¹⁰⁸. PRDM16 is also a ZF protein, but for the regulation of the fat cell dependent gene expression, not direct DNA binding was

responsible¹⁰⁹. Instead binding of PRDM16 to PPAR γ is responsible for these transcriptional changes⁸⁶. Depending on the promoter, PRDM16 can be associated with PPAR coactivator 1 (PGC-1) or CtBP, a corepressor¹⁰⁸. These cofactors compete with each other and depending on which cofactor is bound to PRDM16, gene transcription is activated or repressed. Consistent with this findings, a comparable functional interplay between EVI1, RAR, and RXR can be suggested. EVI1 interacted with the coactivators CBP and P/CAF¹⁰², which were also found in complex with RAR/RXR heterodimers⁵⁴. It can be proposed that in the presence of ATRA, EVI1 might lead to a higher affinity of P/CAF and CBP to the RAR/RXR complex, thereby enhancing ATRA dependent gene transcription. Furthermore EVI1 also interacted with the corepressor CtBP^{27,110} what might explain how EVI1 mediates its own repression in the presence of ATRA⁶³.

The Duolink *in situ* assay, which was newly established in the lab in the course of this thesis, was very useful to detect protein-protein interactions *in situ*. The advantage of the system, besides the easy implementation, is that only small numbers of cells are needed. However, the bottleneck of this method is to find specific primary ABs for the target proteins, raised in two different species. Furthermore, it may be hard to find suitable negative controls for the Duolink *in situ* assay if ubiquitously expressed proteins, such as nuclear receptors, are investigated. Therefore, I chose to use transiently transfected cells and detect the proteins with tag specific ABs. As a negative control only one protein of interest was transfected. However, according to information from the company, it cannot be excluded that transient transfection leads to false positive results as the problem of high abundance due to ectopic expression has not been investigated vigorously previously. Anyways, also other groups have already published results from Duolink *in situ* experiments in transiently transfected cells^{111–113}. These groups showed negative controls either using normal IgG instead of protein specific ABs¹¹³, or two proteins known to be located in different subcellular compartments¹¹¹. However, the best negative control would be to use two proteins that are located in the same subcellular compartment but are known for certain not to be in spatial proximity to each other.

In summary, the Duolink *in situ* experiments revealed that EVI1-HA was in spatial proximity to RAR α -mycHIS and to RXR in transiently transfected MCF7 cells. In U937T_EVI1-HA cells, which express EVI1-HA experimentally and RXR endogenously, the spatial proximity between EVI1-HA and RXR was also detected. However, the specificity of the RXR AB was not fully proven. Future studies have to be carried out to

confirm the interaction of EVI1 with RAR and RXR. One suitable method would be co-immunoprecipitation.

As EVI1 is also capable of binding to DNA¹⁷ it is not yet clear whether the presumed interaction with RAR or RXR is based on direct protein-protein interactions or on protein-DNA interactions in close proximity to the DNA-bound retinoid receptors. However, ChIP analysis in NTERA-2 cells did not reveal an interaction of EVI1 with the *EVI1* RARE or the *RARβ* RARE⁹¹. As no positive control was available for these experiments, technical failure could not be excluded. The results are supported, though, by a recently published ChIP-seq study in SKOV3 cells, which did not reveal binding of EVI1 to DNA sequences in the vicinity of the *HOXA1*, *ICAM1*, *EVI1*, or *RARβ* genes²³. Because ChIP after formaldehyde crosslinking detects proteins bound to DNA in an indirect manner only very inefficiently, these studies support the results shown in the present study, that EVI1 may be bound to the protein complex RAR/RXR rather than to the RAREs themselves.

6. Abstract

The zinc finger protein ecotropic viral integration site 1 (EVI1) is important for embryonic development but also acts as an oncogene in leukemia and some solid tumors. It was identified as a transcription factor and regulates a number of target genes. Furthermore, it can also modulate gene expression by interacting with other transcription factors. In the hematopoietic system of healthy adults, EVI1 is expressed only moderately in bone marrow and not at all in peripheral blood. However, overexpression of *EVI1* occurs in approximately 10% of acute myeloid leukemia (AML) patients. Causes for its upregulation can be rearrangements of its gene locus in chromosome band 3q26 or other, still unknown mechanisms. Irrespective of the cause, the overexpression of *EVI1* is associated with a poor prognosis in AML as well as in other malignancies.

The vitamin A derivate all-*trans* retinoic acid (ATRA) is important in development, proliferation, differentiation, and apoptosis. It regulates transcription of target genes by binding to nuclear receptors, acting as transcription factors. One of the target genes of ATRA is *EVI1*. Previous studies of the host lab have shown that *EVI1* is not only regulated by ATRA, but can enhance or counteract the transcriptional regulation of certain ATRA target genes. The aim of this thesis was to unfold the mechanism behind the *EVI1* modulated ATRA regulation. *EVI1* had been shown to modulate ATRA regulation of the *RAR β* and *EVI1* gene via their retinoic acid response elements (RAREs). In this thesis, direct modulation of the ATRA response by EVI1 of two other candidate genes, *ICAM1* and *HOXA1*, via their respective RAREs, could not be demonstrated with reporter gene assays in adherent cells or hematopoietic cells.

To investigate possible interactions between EVI1 and the retinoid receptors *in situ*, a new, sensitive method, the Duolink *in situ* assay, was established during this thesis. As cells are fixed during this procedure, transient interactions can be detected *in situ*, and since detection involves a very efficient amplification step the method is sensitive enough to identify even single interactions. These experiments revealed that the proteins EVI1 and *RAR α* , as well as EVI1 and RXR, are in spatial proximity to each other in transiently transfected MCF7 cells both in the presence and absence of ATRA. An interaction between experimentally expressed EVI1 and endogenously expressed RXR could be confirmed in stably transfected U937 cells. These results suggest that EVI1 may enhance the transcriptional response to ATRA by binding to the retinoid receptors RAR and RXR.

7. Zusammenfassung

Der Transkriptionsfaktor ecotropic viral integrationsite 1 (EVI1) spielt einerseits eine wichtige Rolle in der embryonalen Entwicklung, andererseits ist er auch ein wichtiges Onkogen in manchen soliden und vor allem in myeloiden Tumoren. EVI1 kann nicht nur Gene direkt regulieren sondern nimmt auch Einfluss auf die Vorgänge in einer Zelle durch Interaktionen mit diversen Transkriptionsfaktoren und Cofaktoren. Im hämatopoetischen System eines gesunden Erwachsenen, wird *EVI1* nur noch im Knochenmark exprimiert jedoch nicht im peripheren Blut. Allerdings kommt es bei 10% der AML Patienten zu einer Überexpression von *EVI1*, ausgelöst durch eine Veränderung im Genlocus auf der Chromosomenbande 3q26 oder aber auch durch bislang unbekannte andere Mechanismen. Eine Überexpression ist in jedem Fall mit einer schlechten Prognose assoziiert, unabhängig von der Ursache.

Das Vitamin A Derivat, all-*trans* retinoic acid (ATRA), ist wichtig in der Entwicklung, Proliferation, Differenzierung und Apoptose. ATRA kann die Transkription bestimmter Gene regulieren, indem es an nukleare Rezeptoren bindet, welche als Transkriptionsfaktoren fungieren. Vorhergegangene Studien zeigten, dass *EVI1* nicht nur durch ATRA reguliert wird, sondern, dass *EVI1* auch die ATRA Regulation bestimmter Gene modulieren kann. Ziel dieser Studie war es, den Mechanismus zu untersuchen, wodurch *EVI1* die ATRA Regulation modulieren kann. *EVI1* moduliert die ATRA Regulation der beiden Gene *RAR β* und *EVI1* über deren retinoic acid response elements (RAREs). In dieser Arbeit wurde analysiert, ob dieser Mechanismus universell ist, konnte aber für die beiden Kandidatengene *ICAM1* und *HOXA1* noch nicht mittels Reporter-gen Studien gezeigt werden.

Desweiteren, wurde untersucht, ob EVI1 im Komplex mit den Rezeptoren für Retinsäure, RAR und RXR, zu finden ist. Dafür wurde eine neue Methode in unserem Labor angewendet, der Duolink *in situ* test. Mittels dieser Methode ist es möglich transiente und einzelnen Interaktionen zwischen zwei Proteinen *in situ* nachzuweisen. Diese Experimente zeigten, dass sich EVI1 in transfizierten MCF7 Zellen in räumlicher Nähe zu sowohl RAR α als auch zu RXR befindet. Diese Interaktionen wurden in Anwesenheit als auch in Abwesenheit von ATRA detektiert. Außerdem konnte die Interaktion zwischen experimentell exprimiertem EVI1 und endogen exprimiertem RXR in stabil transfizierten U937 Zellenderivaten bestätigt werden. Diese Resultate lassen vermuten, dass EVI1 die transkriptionelle Regulation durch ATRA verstärkt indem es an den Rezeptorkomplex RAR/RXR bindet.

8. Supplementary

8.1. Sequences and primers

8.1.1. *ICAM1*

Partial sequence of human *ICAM1* and its 5' region

according to the UCSC genome browser:

>hg19_dna range=chr19:10380000-10383000

```
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ICAM1-P-fwd1: catctcgag AGCTAGGTGTGGACGTGACC
 ICAM1-P-fwd2: catctcgag ACCGTGATTCAAGCTTAGCC
 ICAM1-P-rev1: catagatct TCCGGAATTTCCAAGCTAAA
 ICAM1-P-rev2: catagatct TCTGAGTAGCAGAGGAGCTCAG
 TSS: CAAGCTTAGCCTGGCCGGGA
 Translational start site: **ATG**
 RARE Sequence: **GGGTCAATCGCCCTGCCA**
 XhoI site: ctcgag
 BglII site: agatct
 small letters: repetitive sequence

8.1.2. HOXA1

Sequence of human HOXA1 and its 3' region
 according to the UCSC genome browser
 >hg19_dna range=chr7: 27127932-27132897

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 TTAATCTC**AGGTTACCGAAAGTTCAA**GAAGAGATGAATGCAGTGATGGGTCACTTTAGAATGA
GTCCCAGCAGTTTATCTGAGCTCTCAACTAAAGGCAACAATTATAGTT**CCATCCCTCTTTGGAA**
AAGGGGCAACTTTCTATTATTCTTTTGCTAAACAATGTTTACTAGAACTTAAACACTTTCC
 TCTCCCTCTTGgTtcttttcttttcttcttcttctt

Hoxa1-P-fwd1: **CAGAGTGTTCCCAAAGTAGGC**
 Hoxa1-P-fwd2: **TGGGGCAATCAGATTCAAAC**
 Hoxa1-P-rev1: **agatctCCTTTTCCAAAGAGGGATGG**
 Hoxa1-P-rev2: **agatctGCTCAGATAAACTGCTGGGACT**
 TSS: **ATTCATATCATTTTTCTTC**
 Translational start site: **ATG**
 RARE sequence: **AGGTTACCGAAAGTTCAA**
 Transcript end: **CGTATATATAAACAGAACTCCCTTCAAAGC**
 BglII site: **agatct**
 italic letters: intron

8.2. Control digests of luciferase constructs

Control digests were carried out to confirm the identity of the newly generated luciferase vectors (Fig. 45, Fig. 46, Fig. 47, Fig. 48, Fig. 49, Fig. 50).

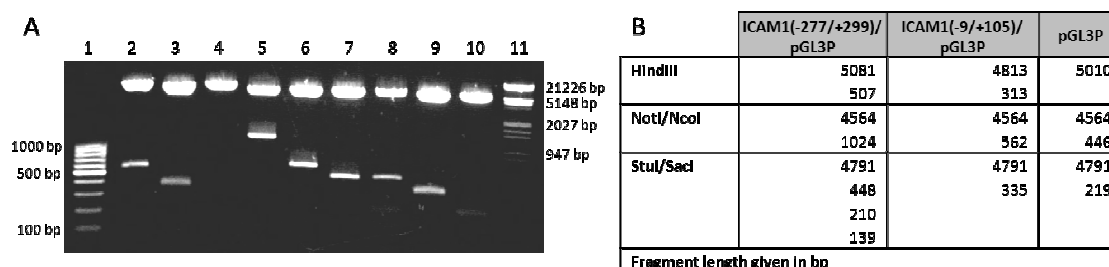


Fig. 45 Control digests of ICAM1 promoter constructs in pGL3P verifies their identity.

A, digested plasmids on a 2% agarose gel. Lane 1 and 11, GeneRuler 100 bp marker and λ marker EcoRI/HindIII, respectively. Lane 2, 5, 8, ICAM1(-277/+299)/pGL3P; lane 3, 6, 9, ICAM1(-9/+105)/pGL3P; lane 4, 7, 10, pGL3P. Lane 2-4 HindIII; lane 5-7 NotI/NcoI; lane 8-10 StuI/SacI. **B**, expected fragments according to *in silico* analysis with SerialCloner.

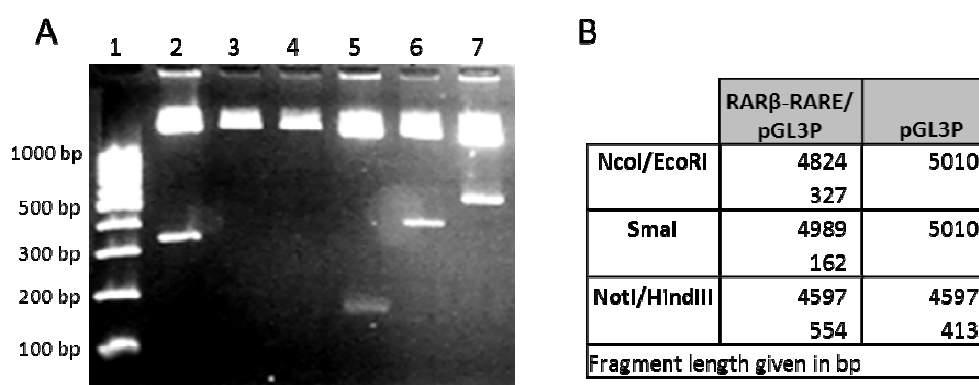


Fig. 46 Control digests of RARβ-RARE/pGL3P.

A, digested plasmids on a 2% agarose gel. Lane 1, GeneRuler 100bp marker. Lanes 2, 4, 6, RARβ-RARE/pGL3P; lanes 3, 5, 7, pGL3P. Lanes 2, 3, NcoI/EcoRI; lanes 4, 5, SmaI; lanes 6, 7, NotI/HindIII. **B**, expected fragments according to *in silico* analysis with Serial cloner.

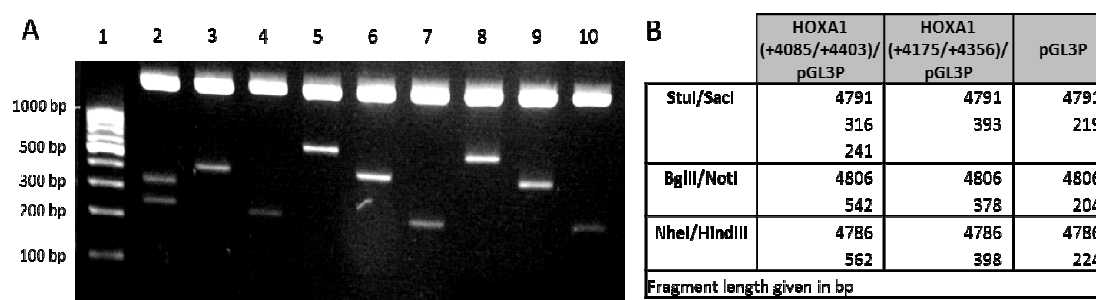


Fig. 47 Control digests of HOXA1(+4085/+4403)/pGL3P and HOXA1(+4175/+4356)/pGL3P.

A, digested plasmids on a 2% agarose gel. Lane 1, GeneRuler 100bp marker. Lanes 2, 5, 8, HOXA1(+4085/+4403)/pGL3P; lanes 3, 6, 9, HOXA1(+4175/4356)/pGL3P; lanes 4, 7, 10, pGL3P. Lanes 2-4 StuI/SacI; lanes 5-7, BglII/NotI; lanes 8-10, NheI/HindIII. **B**, expected fragments according to *in silico* analysis with Serial cloner.

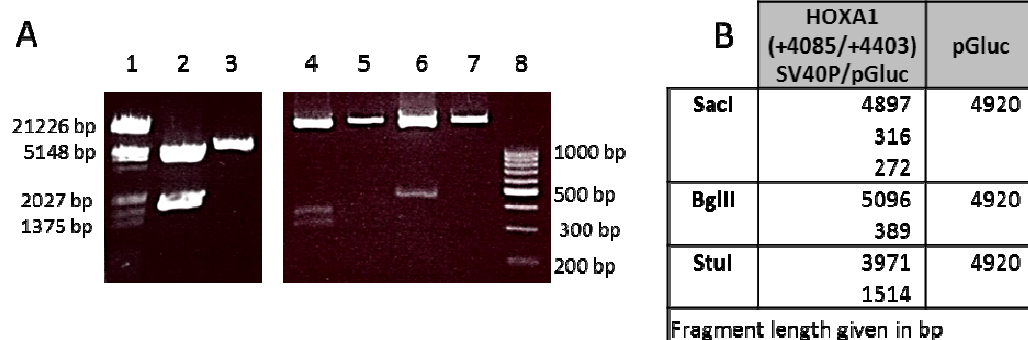


Fig. 48 Control digests of HOXA1(+4085/+4403)SV40P/pGLuc.

A, digested plasmids on a 1% (left) and a 2% (right) agarose gel. Lane 1, λ Marker EcoRI/HindIII; lane 8, GeneRuler 100bp marker. Lanes 2, 4, 6, HOXA1(+4085/+4403)SV40P/pGLuc; lanes 3, 5, 7, pGLuc. Lanes 2, 3, StuI; Lanes 4, 5, SacI; lanes 6, 7, BglII. **B**, expected fragments according to *in silico* analysis with Serial cloner.

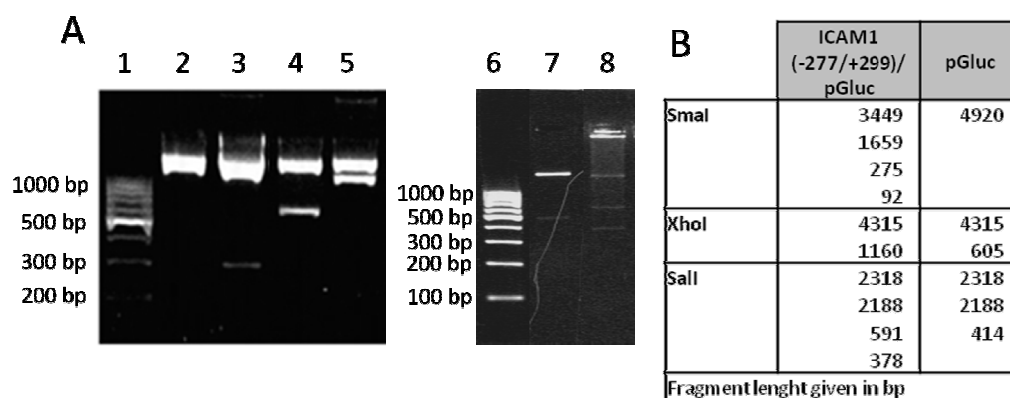


Fig. 49 Control digests of ICAM1(-277/+299)/pGLuc.

A, digested plasmids on a 2% agarose gel. Lanes 1, 6, GeneRuler 100bp marker. Lanes 3, 5, 8, ICAM1(-277/+299)/pGLuc ; lanes 2, 4, 7, pGLuc. Lanes 2, 3, SmaI; lanes 4, 5, XhoI; lanes 7, 8, Sall. **B**, expected fragments according to *in silico* analysis with Serial cloner.

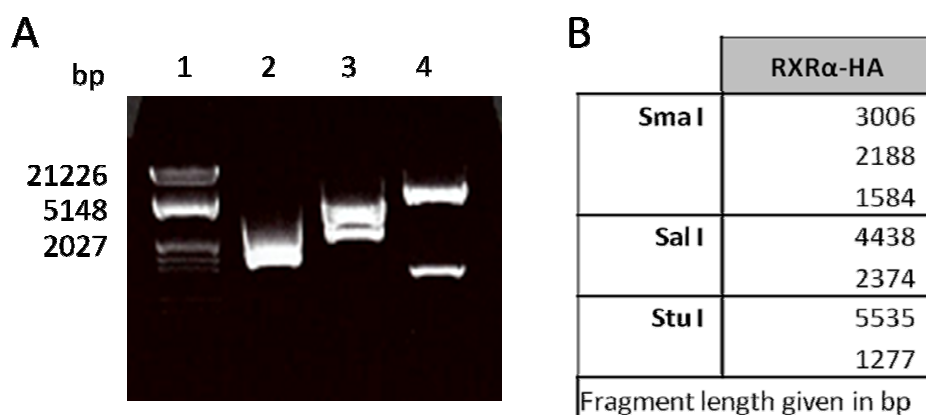


Fig. 50 Control digest RXRa-HA/pcDNA3.

A, digested plasmid on a 1% agarose gel. Lane 1, λ marker EcoRI/HindIII; Lane 2-4, RXRa-HA/pcDNA3. Lane 2, SmaI; lane 3, SalI; lane 4, StuI. **B**, expected fragments according to *in silico* analysis with SerialCloner.

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