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The oncogene, developmental regulator, and retinoic acid target gene
ecotropic viral integration site 1 as a dual modulator of the retinoic acid
response

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Abbreviations

ADH	alcohol dehydrogenase
AF-1 or AF-2	activation domain
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
AR	acidic region
AraC	Cytosine β -D Arabinofuranoside
ATRA	all- <i>trans</i> retinoic acid
bHLH	basic helix-loop-helix
CBP	co-activator cAMP-responsive element-binding (CREB) binding protein
CasBrM MuLV	CasBrM murine leukemia virus
CCR	continuous complete remission
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CRABP	cellular RA-binding protein
CRBP	cellular retinol-binding protein
CR	complete remission
C _t	cycle threshold
CtBP	C-terminal binding protein
DAPI	4',6'-diamidino-2-phenylindole dihydrochloride
DBD	DNA-binding domain
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
dNTP	deoxynucleotide triphosphate
dpc	days post coitum
DR	direct repeat
DSF	disease-free survival
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid

EFS	event-free survival
egl-43	egg laying defective family member 43
EPO	erythropoietin
ES cells	embryonic stem cells
EST e	xpressed sequence tag
EVI1 or Evi1	ecotropic viral integration site 1
FAB	French-American-British Collaborative Group system
FCS	fetal calf serum
G-CSF	granulocyte colony-stimulating factor
HA	hemagglutinin
HAT	histone acetyltransferase
HBS	HEPES bufferes saline
HDAC	histone deacetylase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HPLC	high-performance liquid chromatography
HRP	Horse radish peroxidase
HSC	hematopoietic stem cell
HS	horse serum
HSN	hermaphrodite specific neuron
IL-3	interleukin 3
IMBX	iso-methylbutylxanthine
IR	intervening region
JNK	c-jun N-terminal kinase
kD	kilo Dalton
LCoR	ligand-dependent corepressor
LBD	ligand binding domain
LTR	long terminal repeat
LXR α	liver X receptor α
MDS1 or Mds1	myelodysplasia syndrome 1
M-MLV	Moloney murine leukemia virus

NCoR	nuclear receptor corepressor
NLS	nuclear localization sequence
OD	optical density
ORF	open reading frame
OS	overall survival
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
P/CAF	p300/CBP-associated factor
PCR	polymerase chain reaction
PLZF	Promyelocytic leukemia zinc finger
PMSF	phenylmethanesulfonyl fluoride
PPAR α	peroxisome proliferator-activated receptor α
PR	PRDI-BF1-RIZ1
PRDM	PR domain containing
RA	retinoic acid
RACE	5' rapid amplification of cDNA ends
RALDH	retinaldehyde dehydrogenase
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP4	retinol binding protein
RD	repression domain
RDH	retinol dehydrogenase
RIP140	receptor interacting protein 140
RLU	relative light units
rpm	rounds per minute
RTQ-RT PCR	real time quantitative reverse transcriptase PCR
RXR	retinoid X receptor
SDS	sodium dodecylsulfate
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis

SET	Su(var)3-9, Enhancer-of-zeste, Trithorax
SHH	sonic hedgehog
Smad3	mothers against decapentaplegic homolog 3
SMCC	Srb and Mediator containing complex
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SUG-1	26S protease regulatory subunit 8
SWI/SNF	SWItch/Sucrose NonFermentable
<i>Taq</i>	<i>Thermus aquaticus</i>
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
TFIIIA	transcription factor IIIA
TGF- β	transforming growth factor β
TNF α	tumour necrosis factor α
TPA	12-O-Tetradecanoylphorbol-13-acetate
TR	thyroid hormone receptor
TSA	Trichostatin A
U	unit
UV	ultraviolet light
VDR	vitamin D receptor
WHO	World Health Organization
ZF	zinc finger

1 Introduction

1.1 EVI1

1.1.1 Identification of the murine *Evi1* gene

Evi1 is the acronym of “ecotropic viral integration site 1”. The *Evi1* locus was originally identified as a common site of ecotropic proviral integration in DNA of spontaneous occurring myeloid tumours of the susceptible inbred mouse strain AKXD. The retroviral integration occurred within a 0,6 kb region and appeared to be restricted to myeloid tumours, since it was not found in T-cell, B-cell or mixed-cell lymphomas. Based on these findings it was hypothesized that this locus contained a proto-oncogene which was ectopically activated by retroviral insertion and which played a role in the development of myeloid tumours (Mucenski ML, 1988).

From interleukin 3 (IL-3) dependent murine cell lines isolated from retrovirus CasBrM murine leukemia virus (CasBrM MuLV) induced myeloid leukemia cDNA clones were obtained which contained part of the proviral sequence fused to cellular sequences of an open reading frame (ORF) of 3126 nucleotides and coding for a protein with 1042 amino acids and a predicted molecular mass of 120 kilo Dalton (kD). The difference between the expected molecular mass of 120 kD as derived from the cDNA sequence and the observed molecular mass of 145 kD in immunoblot analysis is likely due to a combination of conformational effects on migration in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and phosphorylation (Matsugi, et al., 1990). This gene, which normally does not appear to be expressed in myeloid cells, was activated by the proviral long terminal repeat (LTR) and was termed *Evi1* (Morishita, et al., 1988).

1.1.2 General properties of the murine *Evi1* protein

First analyses of the *Evi1* protein by homology search revealed homology to the zinc finger structures of *Xenopus laevis* transcription factor IIIA (TFIIIA) which is involved in the regulation of 5S RNA expression. *Evi1* contained altogether ten repeats of zinc finger structures, clustered into an amino terminal domain with 7 repeats and a carboxy terminal domain with 3 repeats (Morishita, et al., 1988). Furthermore, *Evi1* contained a carboxy terminal acidic region. These findings suggested that *Evi1* was a transcription regulatory protein and the fact that it contained more than 3 repeats of zinc finger structures in more than one domain suggested that it was implicated in developmental regulation (Morishita, et al., 1988).

In subcellular fractioning and immunofluorescence experiments *Evi1* expression was localized to the nucleus (Matsugi, et al., 1990). Furthermore, it had zinc-dependent DNA binding capacities (Matsugi, et al., 1990).

1.1.3 Identification of the human *EVI1* gene

Human *EVI1* cDNA was first isolated and sequenced from an endometrial cell line. The ORF contained 3153 nucleotides thus coding for a protein of 1051 amino acids. It showed a high degree of homology to the murine sequence with 91% and 94% on nucleotide and amino acid level, respectively (Morishita K, 1990a). *EVI1* maps to chromosome band 3q26. The start codon ATG is located in exon 3, as in murine *Evi1*. The organization of functional domains like the two zinc finger cluster (ZF1 and ZF2) and the carboxy terminal acidic region (AR) is similar to murine *Evi1* (fig. 1.1) (Morishita K, 1990a). A transcription repression domain (RD) (Bartholomew C, 1997) is located in the central region of the *EVI1* protein (fig. 1.1). The amino terminal zinc finger domain (ZF1) and the RD are separated by an intervening region (IR) (fig. 1.1).

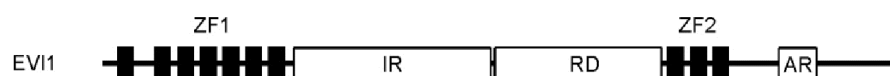


Fig. 1.1 schematic of *EVI1* functional domains modified according to Wieser (Wieser, 2007). ZF denotes zinc finger domains, IR intervening region, RD repression domain and AR acidic region. Black boxes represent zinc finger motifs. ZF1 represents the amino terminal and ZF2 the carboxy terminal zinc finger domain.

1.1.4 Identification of the *MDS1/EVI1* variant

In the course of time it was discovered that the *EVI1* transcript was not the only one in this region. cDNA clones were isolated from a human pancreas cDNA library which contained known *EVI1* sequences and novel sequences extending at the 5' end, which corresponded to the *MDS1* (*myelodysplasia syndrome 1*) gene located upstream of the *EVI1* gene. *MDS1* has been described as a unique gene (Fears, et al., 1996). In the fusion variant exon 2 of the *MDS1* transcript is fused to exon 2 of the *EVI1* transcript, thereby extending the resulting protein amino terminally by 188 amino acids (fig. 1.2). The reading frame of the *EVI1* part of this longer transcript remains unaltered. This variant was termed *MDS1/EVI1* and is conserved between humans and mice. Exon 2 of *EVI1*, which is not translated when *EVI1* is expressed as unique gene, shows high inter-species conservation, a fact that has been puzzling until the discovery of the *MDS1/EVI1* variant, in which exon 2 of the *EVI1* part is indeed translated. The discovery of the *MDS1/EVI1* variant and the requirement to translate this conserved exon provided an explanation for the high degree of homology of the respective exons 2 of murine and human *EVI1* (Fears, et al., 1996; Morishita K, 1990a). The *MDS1* part of the *MDS1/EVI1* variant exhibits homology to a PR domain. The acronym PR is derived from PRDI-BF1-RIZ1 homologous region, which has homology to the evolutionary conserved SET domain (Su(var)3-9, Enhancer-of-zeste, Trithorax). PR domains are

generally located at the amino termini of proteins, which additionally often have zinc finger domains (Buonamici, et al., 2003).



Fig. 1.2 schematic of MDS1/EVI1 functional domains modified according to Wieser (Wieser, 2007). PR denotes the PR domain, ZF zinc finger domains, IR intervening region, RD repression domain and AR acidic region. Black boxes represent zinc finger motifs. ZF1 represents the amino terminal and ZF2 the carboxy terminal zinc finger domain.

1.1.5 Conservation of *EVI1* in further species

***Xenopus laevis*:** The *Xenopus* ortholog *xEvi1* encodes a 1051 amino acid protein with a predicted molecular mass of 119 kD. The degree of identity compared to human EVI1 is 82% on the amino acid level. The organization and structure of functional domains is similar to human EVI1 (Mead, et al., 2005; Van Campenhout, et al., 2006). An Mds1/Evi1 variant has not been described so far.

***Drosophila melanogaster*:** The *Drosophila* MDS1/EVI1 ortholog *hamlet* consists of 8 exons spanning approximately 25 kb of genomic DNA. The predicted protein has 990 amino acids with a molecular mass of 109 kD (Moore, et al., 2002). An *Evi1* ortholog variant has not been described so far.

***Caenorhabditis elegans*:** The *C. elegans* ortholog *egl-43* (egg laying defective family member 43) is transcribed into two different mRNAs, denoted *egl-43L* (long) and *egl-43S* (short). *Egl-43* also shows 2 clusters of zinc finger regions but there are differences in number and arrangements of the single zinc finger units. The similarities between *egl-43* and *EVI1* are confined to the zinc finger motifs (Garriga G, 1993; Rimann and Hajnal, 2007).

Until today, databases list further *Evi1* orthologs in *Rattus norvegicus*, *Bos taurus*, *Canis lupus familiaris*, *Macaca mulatta*, *Pan troglodytes*, *Danio rerio*, and *Gallus gallus*.

In recent years, a number of genes have been characterized which contain amino terminal PR domains and one or more clusters of zinc finger, and which were united to the *PRDM* (PR domain containing) gene family. So far 17 putative *PRDM* paralogs have been described in primates which have been denoted *PRDM1-17*, with *PRDM3* as synonym for *MDS1/EVI1*. With respect to splicing pattern there is a striking conservation between paralogs *PRDM1* (*BLIMP1* or *PRDI-BF*), *PRDM2* (*RIZ*), *PRDM3* (*MDS1/EVI1*) and *PRDM16* (*MEL*, “MDS1/EVI1 like”), since these genes can code for a full length protein and an isoform without PR domain (*PRDM2*, 3) or disrupted PR domain (*PRDM1*, 16). *PRDM* genes generally code for proteins controlling critical aspects of cell functionality, such as

differentiation, growth and apoptosis (Fumasoni, et al., 2007). They also play a role in cancer and neuronal differentiation (Kinameri, et al., 2008).

1.1.6 Alternative splicing of *EVI1*

Δ324: this in-frame deletion variant lacks 324 amino acids compared to the 1051 amino acids full length protein. This leads to a loss of zinc fingers 6 and 7 of ZF1 and part of the IR. This variant is conserved between humans and mice (Bartholomew C, 1994; Bordereaux D, 1990; Morishita K, 1990a).

Δ105: this variant was first discovered in a murine cDNA and contains a deletion of 105 amino acids from the carboxy terminus with a novel predicted protein end comprising 4 amino acids. No functional domain has been described in the deleted region. This splice variant is specific for murine transcripts (Alzuherri, et al., 2006).

- **Rp9:** this variant was also first discovered in a murine cDNA and contains a 9 amino acid deletion within the RD with maintained reading frame. This variant is conserved between humans and mice (Alzuherri, et al., 2006).

Alternative promoter usage of *EVI1*: The 5' rapid amplification of cDNA ends (RACE) technique was applied to identify the transcription start site of *EVI1* with the result that there were several transcription start sites (Vinatzer U, 2003; Zoccola, et al., 2003) in addition to the already identified first exon, which was renamed *EVI1_1c* (Morishita K, 1990a). Originally identified using patient derived RNA these variants were also detected in *EVI1* expressing cell lines and in various human tissues by real time quantitative reverse transcriptase PCR (RTQ-RT PCR) and Northern blotting (Aytekin, et al., 2005). All known 5' variants and their respective transcription start sites are summarized in figure 1.1. Except for the MDS1/*EVI1* variant (see chapter 1.1.13) translation starts at the common ATG in exon 3 for variants *EVI1_1a*, *EVI1_1b*, *EVI1_1c*, *EVI1_1d* and *EVI1_3L*. Only the *EVI1_1a* and the MDS1/*EVI1* variants are conserved between humans and mice (Aytekin, et al., 2005).

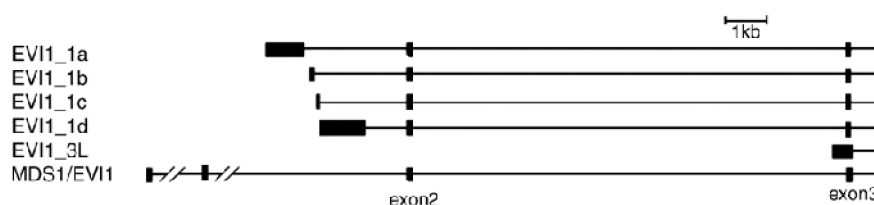


Fig. 1.3 schematic of *EVI1* alternative 5' mRNA variants including the MDS1/*EVI1* variant according to Aytekin et al. (Aytekin, et al., 2005; Wieser, 2007). Except for the MDS1/*EVI1* variant translation starts at the ATG in exon 3. Black boxes denote exons, black lines intervening sequences.

1.1.7 Tissue specific expression of *Evi1*

Due to its nature of containing zinc-finger motifs and being a DNA binding protein it was presumed that *Evi1* might be a transcription regulatory factor with roles in developmental regulation. In the developing mouse embryo *Evi1* expression followed a temporally and spatially restricted pattern. High level expression was found in the urinary system, lung, heart and developing limbs by in situ hybridisation in mouse embryos of stages 9.5 to 14.5 days post coitum (dpc) (Perkins, et al., 1991b). In adult mouse tissues *Evi1* expression was found with high levels in ovary and kidney and with lower levels in lung, uterus and heart by northern blot analyses (Morishita K, 1990b; Perkins, et al., 1991b). Since expression was particularly high in kidneys and ovaries these tissues were investigated more closely in order to determine which cell types express *Evi1*. Immunohistochemistry of murine tissue sections of kidney revealed *Evi1* staining in renal tubules. For ovaries, *Evi1* staining was found in developing oocytes, strikingly in their cytoplasm, whereas *Evi1* expression usually showed nuclear localization (Morishita K, 1990b). A similar expression pattern was found for human ovaries, namely in the oocyte cytoplasm and to a lesser extent in the stroma (Brooks DJ, 1996).

In a further study with human tissues highest expression of *EVI1* was found in adult lung, kidney and stomach by RTQ-RT-PCR (Aytekin, et al., 2005). In *Xenopus* embryos, *xEvi1* expression was found in the pronephros, parts of the brain and in head mesenchyme derived from neural crest cells (Mead, et al., 2005). In adult *Xenopus* *xEvi1* was expressed in kidney, lung, testis, spleen and stomach (Van Campenhout, et al., 2006).

1.1.8 Mouse models and further animal models

In order to study the biological functions of *Evi1* a knockout mouse was generated in which the sixth exon of *Evi1* was disrupted. This strategy did not produce a null allele since the $\Delta 324$ variant still persisted (Hoyt PR, 1997).

Matings of heterozygous animals produced no homozygous knockout offsprings, which indicated a prenatally lethal phenotype. At 9.5 dpc, homozygous knockout embryos revealed no gross phenotypic abnormalities, but at 10.5 dpc presented with obvious multiple malformations, and died in this stage due to cardiac arrest. At 11.5 dpc no viable knockout embryos were found. Heterozygous disruption of *Evi1* produced no phenotypic effects in either embryonic or adult mice (Hoyt PR, 1997).

Malformations of knockout embryos at 10.5 dpc comprised pale yolk sac, hemorrhaging, enlarged pericardial sac with an immature heart, malformed forebrain, absence of peripheral nerve bundles and spinal neurons, widespread hypocellularity, reduced body size, reduced numbers of somites, small limb buds. Heart failure seemed to be the likely cause of embryonic death. Furthermore, the development and expansion of hematopoietic stem cells was severely impaired and the vascular network was less complex compared to wild type

mice (Yuasa, et al., 2005). Apparently the $\Delta 324$ variant was unable to compensate for full length *Evi1* (Hoyt PR, 1997). Of note, the *Mds1/Evi1* variant, still undiscovered at the time of generation of this mutant, was also disrupted. Thus it could not be clearly distinguished whether the phenotypic alterations of homozygous mutant embryos were to be attributed to disrupted *Evi1* and/or *Mds1/Evi1* (Nucifora, et al., 2006).

By targeting exon 4 of *Evi1*, which is conserved in all *Evi1* isoforms, another *Evi1* knockout mouse model was generated, in which all of *Evi1* isoforms including the $\Delta 324$ variant were disrupted (Goyama, et al., 2008). Homozygous knockout mice showed a very similar phenotype to the one reported by Hoyt et al. (Hoyt PR, 1997) but survived somewhat longer, namely until 16.5 dpc. Furthermore, hematopoietic stem cells (HSCs) from these mice showed no repopulating activity when transplanted into lethally irradiated recipient mice. Heterozygous mice survived into adulthood, and their HSCs had reduced repopulating activity compared to that of wild type HSCs in transplantation experiments. The authors concluded that *Evi1* is required for HSC activity to reconstitute the hematopoietic system in recipient mice in a dose dependent manner (Goyama, et al., 2008).

To yield a conditional knockout mouse model, which allows gene deletion in a tissue specific manner, mice carrying a loxP site flanked *Evi1* allele denoted *Evi1* *f/f* were crossed with mice expressing Cre recombinase under control of the *Tie2* promoter and enhancer. *Tie2*, a protein tyrosine-kinase transmembrane receptor for angiopoietin 1, is expressed in endothelial cells of mouse embryo and in hematopoietic progenitors in the fetal liver (Li, et al., 2006). Cre recombinase mediates excision of the region within the two loxP sites in progenies, thereby disrupting *Evi1* in *Tie2* expressing cells. Most *Tie2-Cre⁺ Evi1^{f/f}* mouse embryos, i.e. both alleles of *Evi1* are inactivated in *Tie2* expressing cells, died around E13.5 to E16.5 with symptoms reproducing the phenotype of the conventional *Evi1* knockout mice (Goyama, et al., 2008).

In a transgenic mouse model *Evi1* was overexpressed in all hematopoietic lineages during early hematopoiesis under the control of the *Sca-1* (*Ly6E.1*) promoter. *Evi1* transgenic mice showed normal development, hematopoietic profile, body size and life span, however, they exhibited enhanced susceptibility for virus induced myeloid tumour formation (Louz D, 2000).

The *Drosophila* ortholog to *MDS1/EVI1*, *hamlet*, is involved in dendritic morphogenesis of neurons of the peripheral nervous system in *Drosophila*. Disruption of *hamlet* through a 1 base pair deletion, which occurs in the *ham¹* mutants, is larval lethal (Moore, et al., 2002).

In *C.elegans* mutations in the *EVI1* ortholog *egl-43* lead to a severe migration and neurotransmitter expression defect of hermaphrodite specific neurons (HSNs). Development of phasmid neurons PHA and PHB was also impaired. However, other migrating cells were not affected by the *egl-43* mutation suggesting that the gene product was not generally necessary for neuron migration in *C. elegans* (Garriga G, 1993).

The pleiotropic and severe defects of *Evi1* disruption in several organisms clearly accentuate the importance of *Evi1* and its orthologs for development.

1.1.9 Regulation of *EVI1*

1.1.9.1 Physiological regulators

To date the only known positive physiological regulator of *EVI1* is all-*trans* retinoic acid (ATRA). In acute promyelocytic leukemia (APL) patients undergoing ATRA therapy *EVI1* mRNA was detected at the beginning and during, but not before this treatment (Russell, et al., 1994; Xi ZF, 1997). Since ATRA is a well known regulator of transcription it was thus hypothesized that it might be a regulator of *EVI1*. The reported findings were further corroborated in the human APL cell line NB4, in which *EVI1* mRNA was upregulated after treatment with ATRA (Xi ZF, 1997).

Further studies extended this regulation mechanism to a different organism and other cell types. *Evi1* was upregulated during the ATRA induced neuronal differentiation of the murine embryonal carcinoma cell line P19, whereas the solvent dimethyl sulfoxide (DMSO) had no such effect on *Evi1* expression (Kazama, et al., 1999). The differentiated cells showed morphologic features reminiscent of neuronal cells such as axon-like processes, and expressed neuronal marker proteins. Similar observations were made in P19 cells which stably expressed *Evi1*. These cells exhibited a neuronally differentiated phenotype and expressed neuronal markers. This study established *Evi1* as an important regulator of neuronal differentiation in P19 cells, which is in accordance with previous findings that *Evi1* plays a role in neuronal differentiation in several species like mouse (Hoyt PR, 1997), *Xenopus* (Garriga G, 1993) and *Drosophila* (Moore, et al., 2002).

Furthermore, when addressing the question whether all known *EVI1* mRNA 5' end variants were regulated in the same way, it was observed that in the human teratocarcinoma cell line PA-1 mainly the *EVI1_1A* and *EVI1_1B* variants were induced upon ATRA treatment but not the *EVI1_1D*, *EVI1_3L* and *MDS1/EVI1* variants. In contrast, in the murine teratocarcinoma cell line F9 the only two known murine *Evi1* mRNA 5' end variants, namely *Evi1 ex1a* and the *Mds1/Evi1* variant were both upregulated, but with different kinetics (Aytekin, et al., 2005). Finally, *Xenopus* embryos treated with ATRA showed increased *xEvi1* expression (Van Campenhout, et al., 2006).

In summary, ATRA is a potent regulator of *EVI1* expression across several cell types and species. However, no RARE (retinoic acid response element) has been found in the promoter region of the *EVI1* gene so far.

Progesterone treatment of *Xenopus* oocytes, usually employed for their maturation, led to a dramatic decrease of *xEvi1* expression (Mead, et al., 2005). Furthermore, *xEvi1* expression was also negatively regulated by Wnt and Notch signalling in *Xenopus* as assessed by

mRNA injection (Van Campenhout, et al., 2006). Up to date it is unknown whether these findings can be confirmed in other organisms.

1.1.9.2 Pharmacological regulators of *EVI1*

In the human kidney renal carcinoma cell line A704 *EVI1* mRNA was induced by the adenylyl cyclase activator forskolin, and the response could be potentiated by addition of the inhibitor of cAMP phosphodiesterase iso-methylbutylxanthine (IMBX). Likewise, *EVI1* mRNA was also induced with the cAMP ortholog dibutyryl cAMP. This suggested that the induction was a direct effect of intracellular cAMP (Bartholomew C, 1994). The forskolin mediated induction was counteracted by the protein kinase C activator 12-O-Tetradecanoylphorbol-13-acetate (TPA), while TPA alone had no effect. A similar behaviour was found in the murine fibroblast cell line NIH3T3, while other kidney cell lines remained unresponsive to forskolin treatment. The presence of the protein synthesis inhibitor cycloheximide further enhanced forskolin induced *EVI1* mRNA levels in A704 cells which indicated that the induction of *EVI1* mRNA did not require *de novo* protein synthesis. Forskolin induced both the full length and the $\Delta 324$ transcript variant (Bartholomew C, 1994).

These results suggest that *EVI1* is a gene which is induced via the activation of the protein kinase A pathway and its second messenger cAMP in a highly cell specific manner (Bartholomew C, 1994).

1.1.10 *EVI1* as transcriptional regulator

1.1.10.1 Consensus DNA binding sequences

Extensive studies have been carried out to determine the consensus sequence to which the *EVI1* protein binds, with partially conflicting but also partially concordant results. In some cases it has been reported that *EVI1* acted solely as repressor (Funabiki T, 1994), in others as activator of transcription (Takahashi and Licht, 2002) (Morishita K, 1995).

In one of the earliest studies, shift-polymerase chain reaction was applied to identify a consensus sequence to which the *Evi1* protein binds. This technique uses an oligonucleotide with a random sequence flanked by defined sequences which serve as priming sites for PCR. The oligonucleotide is used as ligand in gel retardation assays with purified *Evi1* protein. The shifted band is excised and the oligonucleotide amplified by PCR. Altogether 4 cycles of alternating gel shift and PCR enrichment of the oligonucleotide to which *Evi1* protein binds are carried out before the resultant DNA, which is expected to contain the enriched consensus binding sequence for *Evi1*, is cloned and sequenced. Using this assay, it was discovered that *Evi1* binds to the sequence TGACAAGATAA (Perkins, et al., 1991a). The binding activity was attributed to the amino terminal zinc finger domain. Further studies extended and supported these findings and reported that *Evi1* binds to the sequence GA(C/T)AAGA(T/C)AAGATAA via zinc fingers 4 to 7 of the amino terminal zinc finger

domain, while zinc fingers 1-3 influenced the specificity for GACAA relative to GATAA in the first part of the consensus sequence (Delwel, et al., 1993). The binding of the amino terminal zinc finger domain of EVI1 to the consensus sequence GACAAGATAA was revealed to be very specific since single base pair removals and methylation changes interfered with EVI1 binding (Perkins and Kim, 1996). Another study reported that the amino terminal zinc finger domain of Evi1 binds to sequences containing repeats of (GATA)_n sequences (Matsugi T, 1995). Of note, this (GATA)_n motif is contained in the other consensus sequences determined by Perkins (Perkins, et al., 1991a; Perkins and Kim, 1996) and Delwel (Delwel, et al., 1993).

The consensus sequence to which the carboxy terminal zinc finger domain of the Evi protein binds, was determined to be GAAGATGAG, with zinc finger number 9, which is the middle zinc finger of this domain, being crucial for DNA binding (Funabiki T, 1994).

Yet another study addressed the question of how the amino- and carboxy terminal zinc finger domains interacted to influence transcription of target genes (Morishita K, 1995). The results suggested that full length *EVI1* can indeed act both as transcriptional activator as well as suppressor. In reporter gene assays EVI1 acts a transcriptional repressor when the reporting constructs contain the consensus binding sequence for the amino terminal zinc finger domain, but acts as transcriptional activator when the reporting constructs contain the consensus binding sequence for both zinc finger regions or only for the carboxy terminal zinc finger region.

This established *EVI1* as a bifunctional transcriptional regulator of transcription and gave an explanation for opposing results from previous studies (Morishita K, 1995). However, none of these studies presented *bona fide* target genes for EVI1.

In a screen for putative EVI1 target genes using an EVI1 amino terminal zinc finger-VP16 chimera which has the ability to activate EVI1 reporter genes, 16 genes were identified which were upregulated after EVI1-VP16 overexpression. For 9 of these genes, namely *Dcn*, *Drd1ip*, *Gadd45g*, *Gata2*, *Map3K14*, *Nabp*, *Skil*, *Tnnt2*, and *Zfpm2*, the upregulation after EVI1-VP16 overexpression was confirmed by RTQ-RT-PCR. Promoter occupancy by Evi1-VP16 and native Evi1 was verified for these 9 genes by chromatin immunoprecipitation (ChIP) in mouse cell lines (Yatsula, et al., 2005). These genes are probable *bona fide* target genes of Evi1, however, the results have up to date not been confirmed in human cell lines.

1.1.10.2 EVI1 as a negative regulator of transcription

A well investigated example for the transcriptional repressive activity of Evi1 is its interaction with GATA-1, a transcription factor essential for erythroid differentiation. The determined consensus binding sequences for the amino terminal zinc finger domain of EVI1 showed a partial overlap with the consensus binding sequence (A/T)GATA(A/G) for GATA-1 (Kreider, et al., 1993) (Perkins and Kim, 1996). Cotransfection experiments in murine myeloid

32DEpol cells with Evi1- and/or GATA1 expression constructs together with a reporting construct containing Evi1 consensus binding sequence revealed in the subsequent reporter gene assay that Evi1 interfered with GATA1 mediated transcriptional activation. Evi1 did not interfere with Adenovirus E4 promoter driven reporter gene activation which suggested that Evi1 was not a general inhibitor of transcription (Kreider, et al., 1993). The finding that Evi1 repressed GATA1 induced transcriptional activation, while not interfering with basal transcription factors, was reproduced by reporter gene cotransfection experiments in murine fibroblast NIH3T3 cells (Perkins and Kim, 1996). A further study elucidated the mechanism of EVI1 mediated repression of GATA1 transcriptional induction: protein-protein interactions between the amino terminal zinc finger domain of EVI1 and the carboxy terminal zinc finger domain of GATA1 prevented GATA1 from binding to DNA *in vivo* as well as *in vitro*. Thus GATA1 was sequestered to the nucleoplasm by EVI1. EVI1 did not bind to the GATA1 consensus binding site by itself. Disruption of the first and sixth zinc finger of the amino terminal zinc finger domain of EVI1 eliminated binding of EVI1 to GATA1 and abolished all repressive effects of EVI1 on GATA1 mediated transcription activation (Laricchia-Robbio, et al., 2006). These findings gave a very feasible explanation for the erythroid differentiation impairment observed in *Evi1* overexpressing 32DEpol cells (Kreider, et al., 1993) and in *Evi1* overexpressing murine bone marrow cells (Laricchia-Robbio, et al., 2006).

Another mechanism for Evi1 mediated transcriptional repression is competition for DNA binding with GATA6 at the *Calreticulin* promoter. GATA6 is an important factor for cardiac development and activates the promoter of the *Calreticulin* gene in reporter gene assays, which is counteracted by cotransfection of an Evi1 expression plasmid. Both Evi1 and GATA6 bind in living cells and *in vitro* to the GATA consensus binding sequence present in the *Calreticulin* promoter which suggested a competition mechanism by which Evi1 abrogated GATA6 dependent *Calreticulin* transcriptional activation (Qiu, et al., 2008). These findings were in accordance with the phenotype of an immature heart in *Evi1* knockout mice (Hoyt PR, 1997).

A further example for competition with a transcription factor as a mechanism by which EVI1 exerts transcriptional repression has been reported for the PU.1 mediated activation of the *M-CSFR* promoter. PU.1 is a member of the Ets family of transcription factor. It is a master myeloid transcription factor and it is important for hematopoietic stem cell differentiation into different lineages, thereby relying on the recruitment of other transcription factors such as c-Jun. Interaction between the amino terminal zinc finger region of EVI1 and the carboxy terminal region of PU.1 disturbed the binding of c-fos to PU.1. This subsequently interfered with PU.1 mediated transcriptional activation of the *M-CSFR* promoter. EVI1 did not affect the binding of PU.1 to DNA (Laricchia-Robbio, et al., 2009).

Interaction with factors that modify chromatin structure is a common theme for transcriptional regulators, and this has also been found for EVI1. EVI1 directly interacts with histone deacetylase (HDAC1) and shows repression of a reporter gene construct containing the EVI1

consensus binding sequence TGACAAGATAAGATAA as reported by Perkins and Kim (Perkins and Kim, 1996). The repression is presumably HDAC1 dependent since it was attenuated by the HDAC1 inhibitor Trichostatin A (Vinatzer U, 2001). The regions necessary for the interaction between EVI1 and HDAC1 were mapped to the amino terminal zinc finger region of EVI1 and the amino terminal region of HDAC1 (Vinatzer U, 2001) (Chakraborty, et al., 2001). Further evidence for the involvement of Evi1 in epigenetic processes was provided with the finding that Evi1 interacts with histone methyltransferase SUV39H1 via the amino terminal zinc finger domain of Evi1 and the SET domain of SUV39H1, in which the histone methyltransferase activity resides. However, the interaction did not interfere with the enzymatic activity. SUV39H1 catalyzes the methylation of histone H3 at lysine residue 9 (H3K9), which according to the histone code influences the chromatin and results in silenced heterochromatin. The Evi1-SUV39H1 complex enhanced transcriptional repression in a reporter gene assay in a dose dependent manner (Spensberger and Delwel, 2008).

1.1.10.3 EVI1 as a positive regulator of transcription

Promyelocytic leukemia zinc finger (PLZF) is a transcriptional repressor important for the development of hematopoietic progenitor cells. The *PLZF* promoter contains a putative EVI1 binding site. In reporter gene assays, EVI1 was found to activate the *PLZF* promoter in a cell type specific manner, and it was shown that EVI1 binds *in vitro* to the sequence TTCTCTTGTA in the *PLZF* promoter (Takahashi and Licht, 2002). A similar mechanism was described for the *GATA-2* gene, to whose promoter Evi1 bound *in vivo* and thus transactivated transcription, resulting in increased proliferation of hematopoietic stem cells (Yuasa, et al., 2005).

Other promoters who are reported to be activated by Evi1 are *c-fos* (Tanaka, et al., 1994), and – in cooperation with BRG1 – *E2F* (Chi, et al., 2003). For the activation of the *c-fos* promoter the amino terminal zinc-finger region of Evi1 was important (Tanaka, et al., 1994) and for the activation of the *E2F* promoter the interaction with BRG1 via the carboxy terminal zinc finger region of Evi1 (Chi, et al., 2003). It remained unclear whether Evi1 bound to these promoters directly or not.

EVI1 interacts with the transcriptional co-activator cAMP-responsive element-binding (CREB) binding protein (CBP), which has histone acetyltransferase (HAT) activity. For this interaction the IR of EVI1 was necessary. In reporter gene assays CBP was able to alleviate EVI1 mediated transcriptional repression (Chakraborty, et al., 2001). This is another example for the ability of EVI1 to influence epigenetic processes, in this context towards transcriptional activation. Furthermore, EVI1 interacts with the coactivator p300/CBP-associated factor (P/CAF) but a precise effect this interaction might have on a potential target gene was not investigated (Chakraborty, et al., 2001). However, EVI1 was acetylated by both CBP and P/CAF (Chakraborty, et al., 2001).

1.1.11 *EV11* and its role in leukemia

Retroviral activation of murine *Evi1* is a transforming event in murine myeloid leukemia, and therefore the question emerges, whether *EV11* is involved in human myeloid leukemia as well (Morishita K, 1992). In acute myeloid leukemia a number of recurring chromosomal aberrations are known, and some of them involve the 3q26 chromosome band where the *EV11* gene is located.

1.1.11.1 Myelodysplastic syndromes (MDS)

MDS are defined as stem cell disorders characterized by ineffective haematopoiesis in one or more lineages of the bone marrow. MDS can arise *de novo*, as a consequence of chemo- or radiotherapy, or due to exposure to other genotoxic substances, and it can progress to myeloid leukemia. The syndrome belongs to the clonal hemopathies, i.e. one or more aberration(s) of a hematopoietic progenitor cell give(s) rise to an aberrant clone and finally to the entire disease. Patients present with peripheral cytopenia and contrasting to this cellular or hypercellular bone marrow with dysplastic changes of differentiated cells or their precursors. Clinical symptoms are anemia, bleeding, infection, fever and fatigue (Corey, et al., 2007).

There are two classification systems: the French-American-British (FAB) Collaborative Group system (established in 1982) and the World Health Organization (WHO) system (established in 1997). Both systems refer to bone marrow morphology, and the WHO classification system also takes chromosomal aberrations into account. Characteristic features of MDS are differentiation failure, increased apoptosis, nuclear and cytoplasmic dysplasia of bone marrow cells. Deletions of chromosomes 5q and/or 7 are frequently found (Corey, et al., 2007).

MDS is considered as a disease of the ageing with a mean age of 70 at diagnosis. With the exception of bone marrow transplantation for high risk patients as the only curative treatment, all other treatments with chemotherapeutic substances or erythropoietin are palliative rather than curative. This stresses the need for the identification of more effective therapies for myelodysplastic syndromes (Corey, et al., 2007).

1.1.11.2 Acute myeloid leukemia (AML)

AML may arise *de novo*, through progression of MDS or it may be therapy-induced (t-AML). According to the FAB classification, a blast count, i.e. an immature blood cell, of 30% or more in the peripheral blood is considered as AML, while in the WHO classification the threshold of 20% blast count in the peripheral blood is used in order to distinguish AML from MDS. Features of MDS and AML partially overlap, in both conditions there is a reduced or absent production of normal hematopoietic cells, however, the two diseases diverge in

aetiopathology, therapy and prognosis (Corey, et al., 2007). In AML bone marrow is often hypercellular due to increased proliferation and block in differentiation at early stages and the peripheral blood shows elevated blast count. In most cases of MDS bone marrow is also hypercellular but due to failure of terminal differentiation or increased cell death or apoptosis there is a lack of terminally differentiated cells, and pancytopenia can be found in the peripheral blood (Corey, et al., 2007).

Like MDS, AML is also considered as a disease of the ageing, with the incidence rate rising from 3,5 per 100 000 in adults of 50 years of age to 35 per 100 000 in adults of 90 years of age. However, AML also occurs in children and young adults. Generally, with increased age of AML onset survival decreases. In children and young adults with AML the survival rate is approximately 50%, in patients older than 60 years only approximately 10% - 15% (Giles, et al., 2002). Apart from patient age another important prognostic factor is the presence of specific chromosomal aberrations which are categorized as (i) favourable, (ii) intermediate, (iii) unfavourable. In accordance with this, chromosomal aberrations of the category "favourable" are found more frequently in younger patients, while unfavourable aberrations occur more often in older patients. These chromosomal aberrations may lead to either fusion genes and hence fusion proteins with altered functions, or to the inappropriate activation of genes due to juxtaposition to e.g. enhancer elements. Aberrations involving the *EV11* locus may lead to inappropriate expression of *EV11* and are categorized to the unfavourable group of cytogenetic aberrations (Giles, et al., 2002), and are often accompanied by additional unfavourable aberrations (Barjesteh van Waalwijk van Doorn-Khosrovani, et al., 2003).

Depending on patient age, leukemia karyotype and other factors, the complete remission (CR) rates range from 40 % to 90% and the cure rates range from less than 10% to 70% (Giles, et al., 2002).

1.1.12 Transforming potential of *EV11*

Overexpression of *Evi1* in the IL-3 dependent myeloid leukemia cell line 32Dcl3 abrogated the ability of the cell to terminally differentiate into granulocytes upon granulocyte colony-stimulating factor (G-CSF) treatment, and finally to loss of viability. Yet requirement for IL-3 and expression of G-CSF receptor remained unchanged (Morishita, et al., 1992). A similar example of transformation by disturbing the ability to respond to a differentiation factor was observed in murine myeloid 32DEpol cells, which do not express *Evi1* and whose growth depends on IL-3 or erythropoietin (EPO). These cells lost their ability to proliferate, survive and differentiate into erythroid cells in the presence of EPO when *Evi1* was overexpressed (Kreider, et al., 1993).

Apart from blocking differentiation, *Evi1* overexpression was found to increase the self-renewing capacity of hematopoietic stem cells, accompanied with a distinct delay in the progress of differentiation in colony formation assays (Laricchia-Robbio and Nucifora, 2008).

Infection of murine bone marrow cells with an *Evi1* expressing retroviral vector and subsequent transplantation into lethally irradiated syngenic recipient mice lead to the development of a myelodysplastic syndrome. The blood profiles showed peripheral pancytopenia and paradoxically hypercellular bone marrow, a contradictory condition typically observed in MDS. An explanation for this was that Evi1 interfered with the differentiation of hematopoietic stem cells and simultaneously upregulated the rate of proliferation (Buonamici S, 2004).

In summary, Evi1 interferes with the normal development of hematopoietic stem cells by affecting cell cycle and differentiation.

In reporter gene and gel shift assays in HepG2 and the mink lung endothelial cell line Mv1Lu it was shown that Evi1 was capable of interfering with transforming growth factor β (TGF- β) signalling by binding and thus quenching the intracellular mediator Smad3 (mothers against decapentaplegic homolog 3). This counteracted the growth repressive effects of TGF- β signalling. The functional region of Evi1 necessary for this effect was the RD located between the two zinc finger regions (Kurokawa, et al., 1998). Evi1 contains two possible binding motifs for the transcriptional corepressor C-terminal binding protein (CtBP). One of them, the more C-terminal PLDLS motif, interacts with CtBP in intact cells. This interaction was important for TGF- β mediated growth inhibition in Mv1Lu cells (Izutsu, et al., 2001). Furthermore, this interaction was also shown to be essential for the transforming activity in the Rat1 cell transformation assay since mutation of this site abrogated colony formation (Palmer, et al., 2001). Altogether, interference of EVI1 with TGF- β signalling and an interaction between EVI1 and CtBP have been confirmed independently by several investigators.

Another example, in which Evi1 interferes with mechanisms controlling cell growth, is the c-jun N-terminal kinase (JNK) pathway. This pathway is implied in apoptotic cell death by e.g. ultraviolet light (UV), Fas ligand or tumour necrosis factor α (TNF α). The direct binding of Evi1 to JNK inhibits JNK dependent phosphorylation of its substrates. While Evi1 itself is not phosphorylated by JNK it disturbs the interaction with its substrates. Evi1 thus protects cells from stress-induced apoptosis which is dependent on the JNK pathway which is very likely to contribute to the oncogenic potential of Evi1 (Kurokawa M, 2000).

The Rat1 cell transformation assay is a well established assay to examine the transforming capability of presumptive oncogenes by conferring anchorage independent growth when overexpressed in these cells. Rat1 cells infected with *Evi1* cDNA in a retroviral vector formed macroscopic colonies in soft agar, while cells infected with the empty vector produced fewer and smaller colonies, thus indicating transforming potential of Evi1 (Bartholomew C, 1997). This biological activity was attributed to the amino terminal zinc finger domain, the intervening region and the repressor domain, since deletion of these regions impaired colony

formation in the Rat1 transformation assay. Notably, the $\Delta 324$ variant also showed impaired colony formation (Kilbey A, 1998).

These findings clearly demonstrate the transforming properties of Evi1 in myeloid precursor cells as well as in adherent cells, which is very well in line with the presumption that *Evi1* is an oncogene.

Taken together, *EV11* is very versatile in influencing transcriptional activities which corroborate its transforming capabilities. The different modes of action are summarized in table 1.1, and the functional domains of EVI1 together with binding sites of interaction partners are depicted in figure 1.4. Though EVI1 has DNA binding capabilities, interaction with a consensus binding sequence or even the presence of a consensus binding sequence is not always necessary for EVI1 to exert or participate in transcriptional regulation.

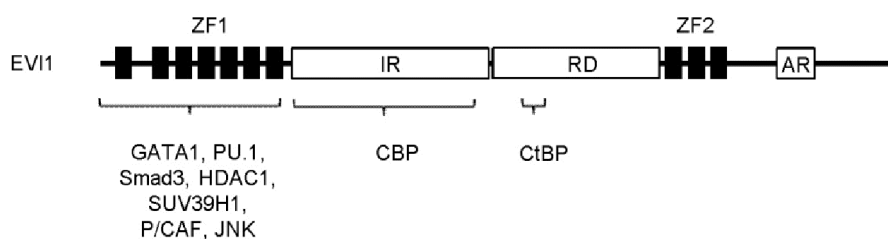


Fig. 1.4 schematic of EVI1 functional domains modified according to Wieser (Wieser, 2007) and respective interaction partners. PR denotes the PR domain, ZF zinc finger domains, IR intervening region, RD repression domain and AR acidic region. Black boxes represent zinc finger motifs. ZF1 represents the amino terminal and ZF2 the carboxy terminal zinc finger domain.

EVI1 interaction partner	Mechanism	Effect
GATA1	Direct binding between EVI1 and GATA1 prevents GATA1 from binding to <i>EpoR</i> promoter (Laricchia-Robbio, et al., 2006)	Repression of <i>EpoR</i> and inhibition of EpoR induced erythroid differentiation (Laricchia-Robbio, et al., 2006)
BRG1	Direct binding between EVI1 and BRG1 (Chi, et al., 2003)	Activation of reporter gene construct under control of <i>E2F</i> promoter and promotes proliferation (Chi, et al., 2003)
GATA6	Evi1 and GATA6 compete for DNA binding at the <i>Calreticulin</i> promoter (Qiu, et al., 2008)	Abrogation of GATA6 dependent transcriptional activation of reporter gene construct und control of the <i>calreticulin</i> promoter (Qiu, et al., 2008)
PU.1	Direct binding of EVI1 to PU.1 disturbs binding of c-fos to PU.1 (Laricchia-Robbio, et al., 2009)	Interference with PU.1 mediated transcriptional activation of a reporter gene construct under control of the <i>M-CSFR</i> promoter (Laricchia-Robbio, et al., 2009).
Smad3	Direct binding between Evi1 and Smad3 disturbs binding of Smad3 to DNA (Kurokawa, et al., 1998).	Repression of TGF β signalling in reporter gene assays (Kurokawa, et al., 1998)
HDAC	Direct binding between EVI1 and HDAC1 (Vinatzer U, 2001)	Transcriptional repression of reporter gene construct (Vinatzer U, 2001).
SUV39H1	Direct binding between Evi1 and SUV39H1 (Spensberger and Delwel, 2008)	Transcriptional repression of reporter gene construct (Spensberger and Delwel, 2008)
CtBP	Direct binding between Evi1 and CtBP leads to recruitment of further corepressors like HDACs to Smad3 target genes (Izutsu, et al., 2001).	Interference with TGF β signalling in reporter gene assays (Izutsu, et al., 2001) and oncogenic transformation in Rat1 cell transformation assays (Palmer, et al., 2001).
JNK	Direct binding between Evi1 and JNK disturbs interaction between JNK and its substrate c-Jun (Kurokawa M, 2000).	Protection from stress-induced cell death in UV exposure assays (Kurokawa M, 2000).

EVI1 interaction partner	Mechanism	Effect
CBP	Direct binding between EVI1 and CBP (Chakraborty, et al., 2001)	Acetylation of EVI1 and alleviation of EVI1 mediated transcriptional repression of reporter gene construct (Chakraborty, et al., 2001)
P/CAF	Direct binding between EVI1 and P/CAF (Chakraborty, et al., 2001)	Acetylation of EVI1 (Chakraborty, et al., 2001)

Table 1.1 overview of EVI1 interaction partner and effects of interaction.

1.1.13 *EVI1* and its presumptive antagonist *MDS1/EVI1*

Regarding the function of *MDS1/EVI1* (described in chapter 1.1.6) reports have been conflicting. In some assays *MDS1/EVI1* acted in an opposite manner compared to *EVI1* (Sitailo S, 1999; Soderholm J, 1997; Sood R, 1999), in others it acted in similar or in partially similar ways (Cuenco and Ren, 2004; Nanjundan, et al., 2007; Vinatzer U, 2001). In a reporter gene assay using the the *EVI1* consensus binding sequence TGACAAGATAAGATAA (Perkins and Kim, 1996) both *EVI1* and *MDS1/EVI1* acted as transcriptional repressors (Vinatzer U, 2001), while in a reporter gene assay using a reporting plasmid containing a genomic DNA fragment that has shown high-affinity to *Evi1* (Kreider, et al., 1993) *EVI1* acted as transcriptional repressor and *MDS1/EVI1* acted as transcriptional activator (Soderholm J, 1997; Sood R, 1999). This might be due to different reporting plasmids and/or different cell types used in these assays.

Overexpression of *MDS1/EVI1* did not interfere with granulocytic differentiation of 32Dcl3 cells (Sood R, 1999), in contrast to overexpression of *Evi1* in 32Dcl3 cells, which caused failure to terminally differentiate and loss of viability (Morishita, et al., 1992). Furthermore, while *MDS1/EVI1* inhibited the replicative ability of embryonic stem (ES) cells and their potential to grow in a semisolid medium, *EVI1* enhanced it significantly (Sitailo S, 1999). In bone marrow retroviral transduction and transplantation experiments it was shown that overexpression of both *EVI1* and *MDS1/EVI1* enhanced the growth of myeloid and immature progenitor cells and suppressed erythropoiesis in the bone marrow (Cuenco and Ren, 2004). A subset of mice which had received a transplantation of *EVI1* overexpressing bone marrow developed leukemia related diseases but no acute myeloid leukemia. However, none of the mice which had received a transplantation of *MDS1/EVI1* overexpressing bone marrow developed leukemia related diseases (Cuenco and Ren, 2004). These findings imply that *EVI1* might have a more aggressive tumour promoting potential than *MDS1/EVI1*.

Further evidence for this notion was brought with a correlation of patient prognosis and *EVI1* and *MDS1/EVI1* mRNA levels in ovarian carcinoma patients. Intriguingly, patients with elevated levels of c*EVI1*, a region common to all known *EVI1* mRNA 5' end variants, showed significantly longer survival than those with low levels of c*EVI1*. However, when assayed specifically for *EVI1*_1d mRNA levels, survival for patients with high expression of this variant was significantly shorter than for those with low levels of *EVI1*_1d mRNA (Nanjundan, et al., 2007). The effects of short term overexpression of *EVI1* and *MDS1/EVI1* in cell motility and proliferation assays were similar for both proteins, both increased proliferation and cell motility to an equal extent (Nanjundan, et al., 2007).

Further evidence for *EVI1* as a contributor to more aggressive forms of cancer while *MDS1/EVI1* acted contrarily was brought with cases for AML with high *EVI1* but low *MDS1/EVI1* expression. These cases showed a significantly worse prognosis with respect to

overall survival (OS), event-free survival (EFS) and disease-free survival (DSF), than those with both *EVI1* and *MDS1/EVI1* overexpression (Lugthart, et al., 2008).

Differences between *EVI1* and *MDS1/EVI1* in response to TGF- β signalling have been observed by independent groups, namely that in contrast to *EVI1*, *MDS1/EVI1* did not influence or even enhanced the growth inhibitory effect of TGF- β in 32Dcl3 cells (Sood R, 1999). In accordance with this, *MDS1/EVI1* showed only very weak interaction with CtBP (Nitta, et al., 2005). Of note, the interaction between *Evi1* and CtBP has been determined to be essential for repressing TGF- β signalling (Izutsu, et al., 2001). However, these differences seem to be cell type and/or assay specific, since in assays using the TGF- β responsive plasminogen activator inhibitor-1 (PAI-1) promoter both *EVI1* and *MDS1/EVI1* repressed TGF- β induced induction of the native PAI-1 promoter, but when using the PAI-1 derived so called CAGA promoter, which contains multiple TGF- β response conferring CAGA boxes but which otherwise lacks elements of the native promoter, opposing effects of *EVI1* and *MDS1/EVI1* were observed. *EVI1* repressed the CAGA promoter while *MDS1/EVI1* even increased TGF- β induced CAGA promoter activity (Nanjundan, et al., 2007).

In summary, differences between the functions and biological properties of *EVI1* and *MDS1/EVI1* cannot be ruled out, though detailed understanding of how these come about, still lacks.

1.1.14 Chromosomal rearrangements affecting the *EVI1* locus

A number of chromosomal rearrangements have been described which have chromosomal breakpoints either 5' or 3' of *EVI1*, e.g. inv(3)(q21q26) or t(3;3)(q21;q26) (Suzukawa, et al., 1994). However, other rearrangements with one breakpoint in 3q21 or 3q26 and the second breakpoint involving other chromosomes have been described (Wieser, 2002).

The consequences of these rearrangement events may be the generation of fusion genes, e.g. *AML1-EVI1* in t(3;21) (Mitani K, 1994) or *ETV6-MDS1/EVI1* in t(3;12) (Peeters, et al., 1997), or juxtaposition of *EVI1* to enhancer elements, e.g. the *Ribophorin1* enhancer in inv(3)(q21q26) (Suzukawa, et al., 1994), the *MSI2* locus in t(3;17) (De Weer, et al., 2008) or the *TCR β* enhancer element with a complex translocation der(3)t(3;7;8) (Suzukawa K, 1999). In other cases of AML with rearrangements of the 3q21 or 3q26 chromosome band and *EVI1* overexpression, neither fusion transcripts nor juxtaposition to a distinct enhancer element could be elucidated, and the overexpression was merely explained by a positional effect (Storlazzi, et al., 2004). Of note, one contrary case has also been observed: no *EVI1* overexpression was detected in an AML patient with a rearrangement involving the 3q26 locus, albeit somewhat further distant than most 3q26 breakpoints (Poppe B, 2006). Another interesting exception was that a rearrangement involving the 3q26 was also sporadically observed in two cases of lymphoblastic leukemia (Poppe B, 2006), but in a subsequent

screening of acute lymphoblastic leukemia patients and cell lines no *EVI1* overexpression could be detected (De Weer, et al., 2006).

However, *EVI1* overexpression was also observed in AML patients without detectable cytogenetic abnormalities of the *EVI1* locus suggesting that rearrangements are not the only cause for *EVI1* overexpression (Barjesteh van Waalwijk van Doorn-Khosrovani, et al., 2003; Russell, et al., 1994).

1.1.14.1 Inappropriate expression of *EVI1* and its prognostic significance in myeloid leukemia

Overexpression of *EVI1* has been observed in approximately 10% of AML and other forms of myeloid malignancies (Langabeer SE, 2001; Morishita K, 1992; Poppe B, 2006; Vinatzer U, 2003), and several studies attempted to correlate *EVI1* expression with prognostic factors. In primary studies, which used semiquantitative RT-PCR to assay *EVI1* expression and which did not discriminate between *EVI1* and its 5' end mRNA variants including the MDS1/*EVI1* variant, no evidence was found for a correlation between *EVI1* expression and a poorer prognosis (Langabeer SE, 2001). In another study, in which the more precise method of RTQ-RT PCR was used to assay *EVI1*_1d and MDS1/*EVI1* expression in a larger cohort of patient samples, overexpression of *EVI1*_1d, but not of MDS1/*EVI1*, was associated with shortened overall and event free survival of AML patients. (Barjesteh van Waalwijk van Doorn-Khosrovani, et al., 2003). However, in a further study which took all known *EVI1* 5' splice variants - except for the hardly detectable variant *EVI1*_1c - and additional prognostic factors such as the duration of continuous complete remission (CCR) and CR, it was shown that high expression of all *EVI1* mRNA 5' end variants, including the MDS1/*EVI1* variant, were associated with a shorter duration of CCR, though none of the variants were a prognostic parameter for the achievement of CR or the duration of OS (Haas K, 2008).

Altogether there is plenty of evidence that *EVI1* is the oncogene for the 3q26 chromosome band, whose inappropriate expression contributes to an aggressive course of myeloid leukemia. However, precise mechanisms of how the overexpression of *EVI1* is induced still remain unclear for a variety of cases.

1.1.15 *EVI1* and its role in solid tumours

The first human *EVI1* cDNA was cloned from the endometrial carcinoma cell line HEC1B (Morishita K, 1990a), and further investigations brought evidence that *Evi1* was expressed in the developing urogenital system of embryonic mice and in kidney and ovaries of adult mice (Morishita K, 1990b; Perkins, et al., 1991b). Because of this expression pattern it was presumed that *EVI1* might play a role in the development of ovarian tumours. High *EVI1* expression was found in a variety of ovarian tumour cell lines. Furthermore,

immunohistochemistry of ovary tumours revealed a change in the cellular localisation of EVI1. While EVI1 is found in the cytoplasm of oocytes of healthy ovaries, it is present in the cytoplasm as well as in the nucleus in primary ovarian cancer (Brooks DJ, 1996). In whole genome expression profiling *EVI1* mRNA was upregulated in 93% of early and in 100% of advanced stage ovarian cancer, which was confirmed by quantitative PCR. Furthermore, comparative genomic hybridisation revealed an increased gene copy number of *EVI1* in microdissected ovarian cancer, which also correlated very well with levels of *EVI1* mRNA expression (Nanjundan, et al., 2007; Sunde, et al., 2006). Increases in mRNA levels of *EVI1* were greater than the increases in DNA copy number which suggested that there must be additional factors which influenced the increased mRNA expression levels. Altogether these data suggest that *EVI1* contributes to the development of ovarian cancer.

1.2 Retinoic acid signalling

1.2.1 Retinoic acid properties and metabolism

Retinoic acid (RA) is the biologically active form of vitamin A (retinol). It belongs to the family of retinoids, which are essential nutrients obtained from the diet in the form of retinyl esters from meat or as β -carotene from plants. RA exists in several isoforms: all-*trans* RA (ATRA) and, 9-*cis* RA as the active isoforms, 5,8-epoxy-RA, 4-OH-RA and 4-oxo RA as the inactive degradation products (Chambon, 1996).

The lipophilic retinol is directly absorbed by the intestinal epithelium. In the circulating blood it is bound by retinol binding protein (RBP4) present in serum and then transported to tissues. In order to be utilized retinol must be converted to the biologically active form ATRA, and cells that respond to retinoic acid are usually equipped with the biochemical properties to carry out this conversion, while in non-RA-responding cells this pathway is inactive.

Retinol enters cells via the specific receptor STRA6, and is then bound by cellular retinol-binding protein (CRBP), which also facilitates conversion of retinol to retinyl esters for storage, e.g. in the liver. In RA-target cells retinol is oxidized to retinaldehyde by either alcohol dehydrogenase (ADH) or retinol dehydrogenase (RDH), and in the final and irreversible step retinaldehyde is oxidized to RA by retinaldehyde dehydrogenase (RALDH). RA is bound by cellular RA-binding protein (CRABP) that facilitates transport to the nucleus. CRBP and CRABP differ in their ability to bind different metabolites of vitamin A, i.e. CRBP binds retinol whereas CRABP binds RA. Once RA enters the nucleus it binds nuclear RA receptors (RARs) or retinoid X receptors (RXRs) which are ligand activated transcription factors. Non-RA target cells usually express CYP26A1 or CYP26B1, which initiate the further oxidation of RA for degradation and excretion (Chawla, et al., 2001; Duester, 2008; Maden, 2002).

Lack of vitamin A leads to characteristic changes in adult animals, namely keratinization of epithelia, a decrease of the immune function, anaemia, xerophthalmia and nerve degeneration. In the opposite case, an excess of vitamin A, or more precisely, of the biologically active form retinoic acid, was found to be highly teratogenic and caused severe malformations in animal and human embryos to whose mothers high doses of retinoic acid was administered. Malformations were found mainly in the central nervous system (CNS), and comprised spina bifida, encephalocele and microcephaly (Maden, 2002; P. J. McCaffery, 2003). In adult organisms, hypervitaminosis A causes swelling of the brain by an unknown mechanism. This condition is described as pseudotumour cerebri since it mimics the symptoms of a brain tumour.

Therapeutically, retinoic acid is used in the treatment of severe acne (P. J. McCaffery, 2003).

1.2.2 Nuclear retinoic acid receptors and their response elements

Once RA has entered the nucleus it serves as a ligand for two families of nuclear receptors that directly bind DNA and regulate transcription: (1) the RA receptors (RAR α , RAR β , and RAR γ) which bind ATRA and 9-*cis*-RA, and (2) the retinoid X receptors (RXR α , RXR β , and RXR γ) which bind only 9-*cis*-RA. Each RAR and RXR isoform is encoded by a separate gene (Chambon, 1996), and RAR and RXR receptors usually form RAR/RXR heterodimers (Kastner, et al., 1997) (Mark, et al., 1999).

Retinoid receptors are composed of conserved domains: (1) the DNA-binding domain (DBD) mediates DNA recognition via a zinc-finger structure, (2) the ligand binding domain (LBD), which is the most conserved region and contains phosphorylation sites, (3) two transcriptional activation domains (AF-1 and AF-2), which also contain phosphorylation sites (Rochette-Egly, 2003), and (4) a hinge region between the DBD and the LBD, which allows a rotation of the DBD to adopt different conformations (Bastien and Rochette-Egly, 2004). The AF-1 domain functions as constitutive ligand-independent transcriptional activation domain, whereas the AF-2 is ligand-dependent; the synergism between AF-1 and AF-2 at a given promoter appears to be dependent on the promoter context, namely on other transactivators binding to the promoter region (Chambon, 1996). Given the prerequisite of an open chromatin conformation RAR/RXR heterodimers usually bind to their DNA recognition sequence independently of binding of the retinoic acid ligand. Transcription of the target gene is initiated in the presence of the RA ligand, while in the absence of ligand transcription is repressed (Dilworth FJ, 2001).

Retinoic acid response elements (RARE) have been identified in many promoters of retinoic acid target genes. Canonical response elements are made up of the two core hexamers motifs PuG(G/T)TCA, which are separated by several spacer nucleotides and are usually oriented in like polarity, except for atypical RAREs which may be oriented in “head-to-head” or “tail-to-tail” polarity. Hexamers are usually separated by 1, 2, or 5 nucleotides, and these sites are therefore referred to as DR1, DR2 or DR5 elements, respectively, where DR stands for direct repeat. RAREs are as likely to be located on the coding as on the non-coding strand. Many of the sites show interspecies conservation (Balmer and Blomhoff, 2005). In DR2 and DR5 elements the 5' hexameric motif is occupied by RXR and the 3' hexameric motif by RAR. This configuration is reversed in DR1 elements (Chambon, 1996). One of the most intensively studied RARE is that of the RAR β 2 promoter, a classical DR5 element (Bastien and Rochette-Egly, 2004).

1.2.3 Transcription of retinoic acid target genes

Retinoic acid receptors rely on coregulator proteins to increase or decrease transcription of target genes, i.e. to implement the signalling pathway given by the ligand retinoic acid. These coregulators can be broadly subdivided into 3 categories:

- (1) coregulators which silence transcription in the absence of ligand, such as nuclear receptor corepressor (NcoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (Bastien and Rochette-Egly, 2004),
- (2) coregulators which potentiate transcription in the presence of ligand, such as members of the p160 family, the SRC group, p300/CBP and P/CAF (Bastien and Rochette-Egly, 2004), and
- (3) a recently discovered group of coregulators which, unlike the first category, repress transcription in the presence of a ligand, such as receptor interacting protein 140 (RIP140) and ligand dependent nuclear receptor corepressor (LcoR) (Gurevich, et al., 2007). More precisely, these coregulators counteract the effect of the ligand, whether this may be an activation or repression of the target gene. In the case that ligand binding leads to increased transcription of the target gene, as is the case for most nuclear receptor target genes, then this response may be repressed (Augereau P, 2006; Subramaniam, et al., 1999).

The discovery and characterization of the 3rd group of coregulators has challenged the previously rather simplistic and generally accepted view that coactivators are recruited to nuclear receptors in a ligand-dependent manner while corepressors are recruited in a ligand-independent manner (Chen, et al., 2004).

Retinoic acid receptors usually bind to their cognate recognition sequences independent of the presence of the ligand. In the absence of retinoic acid, unliganded and DNA bound retinoid receptors recruit corepressors NcoR and SMRT and thus repress transcription (Bastien and Rochette-Egly, 2004).

Upon ligand binding the receptors undergo a conformational change, which leads to dissociation of corepressors and recruitment of coactivators (Glass and Rosenfeld, 2000). Both RAR and RXR within the heterodimer can bind their cognate ligands, but only the RAR ligand - when added alone - activates transcription, thus RXR is considered to be subordinated to the RAR partner. This is due to the inability of RXR to dissociate corepressors after ligand binding, and consequently, coactivators cannot be recruited (Bastien and Rochette-Egly, 2004).

However, two ligands synergize transcription and the RXR ligand enhances the stability of promoter occupancy (Minucci, et al., 1997). This is in line with the finding that RXR is an obligate heterodimer partner for other nuclear receptors, e.g. vitamin D receptor (VDR) or thyroid hormone receptor (TR), by serving as a scaffolding protein (Chawla, et al., 2001).

In order to facilitate binding of the basal transcription machinery to the promoter of RA target genes, condensed chromatin needs to adopt an open state, thus the first activities after ligand binding to the retinoid receptors is the sequential binding of those coactivators, which possess chromatin remodeling activities. Members of the p160 family directly bind to the AF2 domain of RAR. This, in turn, leads to recruitment of p300/CBP to the RAR/RXR complex via p160. P300/CBP then increases the number of productive preinitiation complexes within the promoter and modify histones through their acetyltransferase activity. P160 dissociates from RAR and is replaced by Srb and Mediator containing complex (SMCC), which recruits the RNA polymerase II holoenzyme. The last step of the chromatin remodeling procedure is the recruitment of SWItch/Sucrose NonFermentable (SWI/SNF), which then, by using its ATP-dependent chromatin remodeling activity, displaces nucleosomes and thus establishes optimal conditions for the tight binding of the basal transcription machinery to the promoter of the retinoid target gene, so that transcription can proceed (Dilworth FJ, 2001; Glass, et al., 1997; Glass and Rosenfeld, 2000; Lee JW, 2001; Wei, 2003).

The transcription of retinoid target genes can be modulated through the integration of diverse phosphorylation events which target the RAR/RXR heterodimer and/or the involved coregulators. These events usually cooperate to achieve maximal transcriptional activity, but can also lead to termination or attenuation of the ligand response. The RAR or RXR isoforms have individual phosphorylation patterns and are substrates for a number of kinases, which together orchestrate a rather complex interplay (Rochette-Egly, 2003) (Bastien and Rochette-Egly, 2004).

Termination of the retinoid response is achieved through degradation of the RAR/RXR heterodimer by the ubiquitin-proteasome system. The RAR partner is polyubiquitinated, which leads to recruitment of the proteasome to the heterodimer via 26S protease regulatory subunit 8 (SUG-1) and then to its degradation. SUG-1 is one of the 6 ATPases of the 19S regulatory unit of the 26S proteasome (Bastien and Rochette-Egly, 2004).

Another mechanism for the termination of the retinoid response is a negative feedback loop via retinoic acid mediated upregulation of CYP26A1, which degrades retinoic acid (Chawla, et al., 2001). These mechanisms control the magnitude and duration of retinoid-mediated transcription. At least 532 genes have been described to be regulated directly or indirectly by retinoic acid (Balmer and Blomhoff, 2002).

1.2.4 Retinoic acid in development

Vitamin A is not metabolised by all cells of the body at all stages of development but is produced in a unique spatiotemporal pattern. RA which is generated in a cell can enter adjacent cells, where it is either degraded - if the cell expresses CYP26 - or induces gene expression. During development the concentration of RA must be within a very narrow range

to avoid both deficiency and teratogenicity, which would result in severe developmental defects. In many cellular systems retinoid signalling has a pronounced antiproliferative potential, which is linked to its ability to induce differentiation (Rochette-Egly, 2003). RA is essential for the development of the spinal cord, hindbrain, heart, eye, skeleton, forelimb bud, lung, pancreas and the genitourinary tract. It provides an instructive signal for the posterior neuroectoderm, and at later stages contributes to the development of the eye and many other organs, mainly by inducing various homeobox genes (Duester, 2008).

Especially in neuronal differentiation RA has an important and crucial role (Maden, 2002). It induces neuronal differentiation of embryonic and teratocarcinoma cell lines, and, when added to cells that already have neuronal properties, increases the number and length of neurites (Maden, 2002; P. J. McCaffery, 2003). The highest concentration of endogenous RA measured by high-performance liquid chromatography (HPLC) in the mouse embryo at 10.5 and 13 dpc was found in the spinal cord, an intermediate concentration was found in the hindbrain, while the lowest concentration was found in the fore- and midbrain. In all other regions there was an intermediate concentration of retinoic acid. This demonstrates an anteroposterior difference in the spatial distribution of RA along the neural tube (Horton C, 1995).

Of all *Raldh* isoforms, only the homozygous deletion of *Raldh2*, but not of *Raldh1* or *Raldh3*, is embryonic lethal in mice, *Raldh2* is the most important RA generating enzyme during embryonic development (Duester, 2008). *Raldh2* expression pattern during mouse embryonic development coincides very well with the retinoic acid gradient, with the exception that it is not directly expressed in the neural tube but in the adjacent mesoderm. The current theory is that RA is provided by mesodermal *Raldh2*, and then diffuses into the neural tube and into the hindbrain (P. J. McCaffery, 2003). One of the differentiation processes RA induces is - together with sonic hedgehog (SHH) – establishment of motor neuron fate at the ventral side of the spinal cord. RA necessary for this process is synthesized by *RALDH2* in the somitic mesoderm adjacent to the spinal cord, which then travels to the spinal cord neuroectoderm, which is in line with the theory of RA as a diffusable morphogen (Duester, 2008).

Of note, mice with a homozygous disruption of *Raldh2* show a partially overlapping phenotype like *Evi1*^{-/-} mice, e.g. embryos are smaller, lack an organized network of extraembryonic vessels in their yolk-sac reminiscent of the pale yolk-sac observed in *Evi1*^{-/-} mice, show heart dilation, spinal cord and hindbrain defects and small somites. Embryos usually die at 9.5 dpc, which is the same day of embryonic development that *Evi1*^{-/-} mice die (Hoyt PR, 1997; Niederreither K, 1999). These observations suggest that *Evi1* may be involved in mediating some of the biological effects of ATRA during embryonic development.

1.3 Goals of this thesis

When the work on this thesis was begun, the mechanisms by which ATRA regulates *EVI1* expression were not yet understood and the role of *EVI1* in differentiation processes were only characterized to a very limited extent. Therefore, the main goal of this thesis was to elucidate the regulation of *EVI1* by ATRA.

The influence of ATRA on *EVI1* expression was to be investigated using semiquantitative RT-PCR, and then, if results indicated that *EVI1* is indeed positively regulated by ATRA in NTERA-2 cells, a more precise analysis is to be carried out using RT-RTQ PCR to determine whether the known *EVI1* 5' mRNA variants are differentially regulated by ATRA. Furthermore, the expression of *EVI1* and *MDS/EVI1* was to be monitored during ATRA induced neuronal differentiation of NTERA-2 cells.

In order to locate a presumptive RARE within the *EVI1* and *MDS1/EVI1* promoter, reporter gene assays were to be carried out in NTERA-cells, and physical binding of retinoid receptors to this RARE *in vivo* was to be confirmed by ChIP assay.

By coincidence it was found during this work that the ATRA mediated regulation of the *EVI1* gene is modulated by *EVI1* itself. Therefore, a characterization of this regulatory mechanism was added to the goals of this thesis.

2 Materials and Methods

2.1 Cell culture

2.1.1 Maintenance of NTERA-2 cells

NTERA-2 cells were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). This cell line is derived from a 22 year old male caucasian and has the complex karyotype 48-58<2n>XXXY, +1, +16, +20, +22, +3-4mar, add(X)(p11), add(X)(q275), der(X;1)(p10;q10), add(1)(p35-36), add(11)(p15), add(13)(q371) - sideline with i(3q). The cells have epitheloid morphology, grow in monolayer and contain prominent granula. NTERA-2 cells respond very well to ATRA and express *RAR* and *RXR* (Moasser MM, 1994; Moasser MM, 1995; Spinella MJ, 1998). Another important property is that this cell line can undergo neuronal differentiation and thus can serve as a model system to elucidate the role of *EVI1* during this developmental process (Leypoldt, et al., 2002; Megiorni, et al., 2005).

NTERA-2 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5% horse serum (HS) as growth medium in a humidified incubator with 5% CO₂. In the first 2 weeks after receipt the cells were kept in collagen coated flasks.

For passaging, confluent cells were washed with 1x phosphate buffered saline (PBS) and then treated with trypsin/EDTA solution at 37°C until the cells became completely detached. The cells were resuspended in growth medium, diluted 1:5 and left to reattach. The cells were usually maintained in the same dish for up to 2 weeks. Splitting was carried out twice a week, usually when cells had reached a confluence of 100%. Only cells between passage numbers 6 and 20 were used for experiments. For long term storage, NTERA-2 cells were detached as described and resuspended in growth medium, then centrifuged at 1000 rounds per minute (rpm) for 5 min at room temperature. The cell pellet resuspended in growth medium supplemented with 10% dimethyl sulfoxide (DMSO). Aliquots were immediately frozen in a 2-propanol-filled freezing jar for the gradual freezing of -1°C/min until the final temperature of -70°C.

After approximately 24 h at -70°C, the samples were finally transferred to liquid nitrogen.

10x PBS

1,37 M NaCl

27 mM KCl

1 M Na₂HPO₄

20 mM KH₂PO₄

pH 6,8 with NaOH.

Upon dilution to 1x PBS the pH changes to 7,4

Both 10x PBS and 1x PBS were autoclaved

NaCl, ACS reagent, ≥99.0%	Sigma-Aldrich
KCl, analysis grade	Merck
Na ₂ HPO ₄ x 2 H ₂ O, analysis grade	Merck
KH ₂ PO ₄ , analysis grade	Merck
DMEM, high glucose	Invitrogen
FCS	Invitrogen
HS	Invitrogen
0,25% Trypsin/EDTA	Invitrogen
Cell culture grade plastic ware	Iwaki
Collagen coated cell culture flasks	BD Bioscience
Humidified incubator	Heraeus Instruments
Lamina "LaminAir"	Holten

2.1.2 All-*trans* retinoic acid (ATRA) treatment of NTERA-2 cells

ATRA was dissolved in DMSO to a concentration of 10 mM, and kept at -20°C. Aliquots were thawed maximal 3 times. All experiments involving ATRA were carried out under subdued light conditions because of sensitivity of ATRA towards light. ATRA was directly added to the growth medium to a final concentration of 10 µM. For smaller volumes of growth medium, when necessary ATRA volumes failed to exceed 5 µl, ATRA was diluted 1:10 in growth medium prior to adding the cells. For negative control cells, the solvent DMSO was added in the same manner as ATRA.

ATRA, ≥98% (HPLC), powder	Sigma-Aldrich
DMSO, cell culture grade	AppliChem

2.1.3 Transfection procedures

At the beginning of this work, transfections for reporter gene assays were carried out using the DAC-30 reagent. However, production of this reagent was discontinued, therefore the transfection procedure for reporter gene assays had to be changed. Instead of DAC-30, FuGENE®6 was used in later experiments. Other transfection reagents unsuccessfully tested were Metafectene Pro (Biontex), GenePorter (Genlantis) and FuGene HD (Roche). Effectene (Qiagen) showed very low transfection efficiency.

2.1.3.1 Transfection of NTERA-2 cells using DAC-30 for reporter gene assays

18 – 20 h before transfection, NTERA-2 cells grown in a 100% confluent layer were trypsinized and 80 000 cells were seeded per well of a 24-well tissue culture grade dish in 500 µl growth medium. Prior to transfection cells were microscopically examined for viability and confluence of about 70-85%. Unviable or misshaped cells were not used for transfection experiments.

The growth medium was removed and replaced with 400 µl serum free DMEM. 2 µl DAC-30 were added to 200 µl serum free DMEM, and 600 ng plasmid DNA were added to another 200 µl serum free DMEM. The proportion of renilla plasmid with respect to the total amount of plasmid DNA was 60 ng (1/10) per well for the pRL-TK plasmid. The two mixtures were combined and incubated at room temperature for 30 min. The transfection mix was added dropwise to the cells and plates were shaken very gently. After 6 hours, 800 µl of DMEM containing the double amount of serum used for growth medium, i.e. 20% FCS and 10% HS, were added to the 800 µl of serum free transfection mix, thus yielding growth medium with the usual percentage of serum. 23-25 h after transfection start 10 mM ATRA stock solution or the solvent DMSO were diluted 1:5 in growth medium and 8 µl were added per well to give a final concentration of 10 µM ATRA or an equal volume of DMSO. Incubation was continued for another 23-25 h. Usually, luciferase assays were carried out 46-50 h after transfection start, with the exception for reporter gene assays using *MDS1* promoter constructs, for which the ATRA incubation time was extended to 46-50 h. All transfections were carried out in duplicates.

DAC-30 transfection reagent Eurogentec

2.1.3.2 Transfection of NTERA-2 cells using FuGENE®6 for reporter gene assays

18 – 20 h before transfection, NTERA-2 cells grown in a 100% confluent layer were trypsinized and 80 000 cells were seeded per well of a 24-well tissue culture grade dish in 500 µl growth medium. Prior to transfection cells were microscopically examined for viability and confluence of about 70-85%. Unviable or misshaped cells were not used for transfection experiments.

A transfection mix was set up consisting of 20 µl DMEM without serum plus 3 µl FuGENE®6 per well. After a 5 min incubation at room temperature a total of 600 ng plasmid DNA in a volume of 0,5 – 2 µl was added and incubated for 15 min at room temperature. The proportion of renilla plasmids with respect to the total amount of plasmid DNA was 6 ng (1/100) per well for the pRL-SV40 plasmid and 60 ng (1/10) per well for the pRL-TK plasmid. *EV11* expression construct and reporting luciferase construct were added to equal parts. The transfection mix was added directly to the growth medium and plates were shaken very

gently. 23-25 h after the transfection start 10 mM ATRA stock solution or the solvent DMSO were diluted 1:10 in growth medium and 5,2 µl were added per well to give a final concentration of 10 µM ATRA or an equal volume of its solvent DMSO. Incubation was continued for another 23-25 h. Usually 46-50 h after transfection start luciferase assay was carried out, with the exception of reporter gene assays using the *MDS1* promoter constructs, for which the ATRA incubation time was extended to 46-50 h. All transfections were carried out in duplicates.

FuGENE®6 transfection reagent Roche

2.1.3.3 Transfection of NTERA-2 cells using Calcium Phosphate

NTERA-2 cells were transiently transfected using the calcium phosphate transfection method for subsequent immunofluorescence, western blot or chromatin immunoprecipitation (ChIP) experiments. For immunoblot or immunofluorescence, transfection was carried out in 6-well plates, for ChIP experiments, for which large numbers of NTERA-2 cells were necessary, transfection was carried out in 150 mm dishes.

6-well plates:

18 – 20 h before transfection 500 000 NTERA-2 cells, trypsinized from a 100% confluent plate, were seeded per well of a 6-well tissue culture grade dish in 2 ml growth medium. If cells were going to be used for immunofluorescence experiments, an autoclaved coverslip was placed into the well. Prior to transfection cells were microscopically examined for viability and confluence of about 70-85%. Unviable or misshaped cells were not used for transfection experiments.

3,5 µg plasmid DNA and 37 µl 2M CaCl₂ were mixed and adjusted to a volume of 300 µl with sterile H₂O. 300 µl 2x HEPES buffered saline (HBS) were added, the mixture was vortexed immediately and incubated for 10 min at room temperature. The mixture was added dropwise to the growth medium, the plate was shaken gently and the cells were returned to the humidified incubator. After 6 h to 8 h cells were washed twice with 2 ml 1x PBS to remove the dust-like precipitate of calcium phosphate, fresh growth medium was added, and cells were left to grow for another approximately 40 h.

150 mm dishes:

18 – 20 h before transfection 8x 10⁶ cells NTERA-2 cells trypsinized from a 100% confluent plate were seeded on a 150 mm plate in 20 ml growth medium. Prior to transfection cells were microscopically examined for viability and confluence of about 70-85%. Unviable or misshaped cells were not used for transfection experiments.

35 µg plasmid DNA and 125 µl 2M CaCl₂ were mixed and adjusted to a volume of 1 ml with sterile H₂O. 1 ml 2x HBS was added, the mixture was vortexed immediately and incubated

for 10 min at room temperature. The mixture was added dropwise to the growth medium, the plate was shaken gently and the cells were returned to the humidified incubator. After 6 h to 8 h the cells were washed twice with 20 ml 1x PBS to remove the dust-like precipitate of calcium phosphate, fresh growth medium was added, and the cells were left to grow for another approximately 40 h.

2x HBS

280 mM NaCl

10 mM KCl

1,5 mM Na₂HPO₄ x 2 H₂O

12 mM Dextrose

50 mM HEPES, Na-salt

pH 7,0 with NaOH, readjustment of pH after filling up to the final volume, since correct pH within limits of 0,05 units is essential for the procedure.

sterilized by filtration

2 M CaCl₂

Sterilized by filtration

CaCl ₂ x 2 H ₂ O, ACS, Reag. Ph Eur	Merck
NaCl, ACS reagent, ≥99.0%	Sigma-Aldrich
KCl, analysis grade	Merck
Na ₂ HPO ₄ x 2 H ₂ O, analysis grade	Merck
Dextrose, ≥99.5%	Sigma-Aldrich
HEPES, Na-salt, cell culture tested, ≥99.5%	Sigma-Aldrich

2.1.4 Neuronal differentiation of NTERA-2 cells

Upon exposure to ATRA and the use of differential adhesion matrices, NTERA-2 cells are able to develop morphological characteristics of postmitotic neurons. The procedure described by Megiorni et al (Megiorni, et al., 2005) and Leypoldt et al (Leypoldt, et al., 2002) consists of an initial aggregation phase in which NTERA-2 cells are plated on bacterial grade dishes. This augments formation of aggregates and impedes proliferation in order to facilitate differentiation in the second phase, in which the morphologically visible differentiation takes place after plating NTERA-2 aggregates on a poly-D-lysine and laminine adhesion matrix in the presence of mitosis inhibitors.

10-12 150 mm dishes of NTERA-2 cells at 100% confluence were harvested by trypsination. Cells were adjusted to a density of 1-1,5x10⁶ cells/ml in growth medium and then plated onto

bacterial grade dishes to avoid attachment and to allow formation of aggregates. Per required sample for RNA isolation 1 compartment of a 100 mm septate dish containing 5 ml of cell suspension, was sufficient. After 1 d, when first aggregates had formed, ATRA was added to a final concentration of 10 μ M (5 μ l of 10 mM ATRA stock solution to 5 ml of cell suspension). During the next 19 days cells were maintained on bacterial grade petri dishes in growth medium containing 10 μ M ATRA. Cell aggregates of varying sizes formed, some of which became slightly attached to the bottom of the bacterial grade dishes, while some remained in suspension. Cells did not appear to proliferate. The medium containing ATRA was changed every second to third day. Cell suspension was carefully resuspended and attached aggregates were gently rinsed off the dish, with the least possible disturbance of their integrity, and transferred to a 14 ml tube. Cell aggregates were pelleted by centrifugation for 5 min at 1000 rpm and room temperature. Supernatant was aspirated and replaced by the same volume of fresh growth medium containing 10 μ M ATRA. Cell aggregates were transferred to fresh bacterial grade dishes. During the course of time, cell aggregates appeared to become more stable and more resistant to resuspension. Single cells were not observed, all of them had aggregated, even though some of them to very small aggregates. Generally, the integrity of the cell aggregates was preserved as much as possible throughout the aggregation period.

Cell culture grade 6-well plates were coated with 1 ml of 10 μ g/ml poly-D-lysine in 1x PBS for 1 h at room temperature, then washed twice with 3 ml 1x PBS per well. Following this, plates were coated with 1 ml of 10 μ g/ml mouse laminin per well for 2 h at 37°C in a humidified incubator with 5% CO₂. Plates were washed 3 times with 3 ml 1x PBS per well. An autoclaved coverslip was placed in 6-well plates prior to the coating procedure for those cells designated for immunofluorescence experiments. Coated plates were stored at 4°C in 3 ml 1x PBS until use and were stable for up to one month.

On day 19, NTERA-2 aggregates were gently resuspended with the least possible disruption of aggregates, transferred to 14 ml tubes and centrifuged for 5 min at 1000 rpm at room temperature. Growth medium containing 10 μ M ATRA was aspirated and the cell pellet from 1 compartment resuspended in 8 ml growth medium containing no ATRA but the mitosis inhibitors at 10 μ g/ml Cytosine β -D Arabinofuranoside (AraC) and 1 μ g/ml uridine. Per well of a poly-D-lysine and laminine coated 6-well plate 2 ml of resuspended cell aggregates were plated, i.e. 1 compartment was sufficient for 4 wells of a 6-well plate. In contrast to cultivation on bacterial grade dishes, cell aggregates adhered tightly to the bottom of the coated dishes. Thus, in order to change growth medium containing 10 μ g/ml AraC and 1 μ g/ml uridine every second day, it could be aspirated and replaced. After 2-3 days neurite like structures started growing out of the attached cell aggregates, which formed more complex networks in the course of time. On day 29, massive cell death set in, and by day 30 the cells were dead. On

days 0, 1, 2, 4, 7, 10, 13, 16, 19, 20, 22, 24, 26 and 28 cells were harvested, those in aggregation phase by lysing and resuspending cells from 1 compartment of a bacterial grade dish, pelleted by centrifugation at 1000 rpm and 5 min, in 1 ml of Trizol reagent, and those in differentiation phase by adding 0,5 ml Trizol reagent directly to 1 well of a 6-well plate of adherent cells and resuspending them. Samples were transferred to 1,5 ml Eppendorf cups and stored at -70 °C until RNA isolation for gene expression analysis.

Stock solutions:

Uridine: 10 mg/ml in H₂O, sterile filtered, stored at -20°C, freshly prepared before each neuronal differentiation experiment

AraC: 10 mg/ml in H₂O, sterile filtered, stored at -20°C, freshly prepared before each neuronal differentiation experiment

Poly-D-lysine, molecular weight 70,000-150,000 g/mol: 50 mg/ml in sterile H₂O, stored at 4°C

Laminine from Engelbreth-Holm-Swarm murine sarcoma: 1 mg/ml in 50 mM Tris-HCl pH 7,5, 150 mM NaCl, sterile filtered, stored at -20°C.

Bacterial grade petri dishes	Greiner Bio-One
Poly-D-lysine, molecular weight 70,000-150,000 g/mol	Sigma-Aldrich
Laminine from Engelbreth-Holm-Swarm murine sarcoma	Sigma-Aldrich
AraC	Sigma-Aldrich
Uridine	Sigma-Aldrich
Centrifuge Labofuge 400	Heraeus
Microscope	Nikon eclipse

2.2 Reporter gene assays

2.2.1 Luciferase assay

Transfected cells were washed with 500 µl of 1x PBS per well at room temperature and then lysed with 100 µl 1x passive lysis buffer (supplied with the Dual Luciferase Reporter Assay System) per well of a 24-well plate for 15 min at room temperature with gentle agitation on a rocking platform. Lysates were resuspended and 30 µl transferred to a white 96-well plate. Firefly and renilla luciferase activity were measured according to the parameters suggested in the Dual Luciferase Reporter Assay System manual. First, 50 µl of the substrate for firefly luciferase were injected using the automatic injection device of the luminometer, followed by a 2 sec premeasurement delay and a 10 sec measurement with signal integration. Then 50 µl of substrate for renilla luciferase were injected followed by a measurement at the same conditions. Relative light units (RLU) values obtained for firefly luciferase were normalized against values for renilla luciferase. RLU values for cells which had been treated with ATRA

were generally increased by a factor of approximately 1,5 to 2,0. After measurements, white 96-well plates were washed thoroughly with H₂O and could be reused when dry.

Dual Luciferase Reporter Assay System	Promega
Aureon PhL Luminometer	Aureon Biosystems
White 96-well plates	Wallac

2.3 Agarose gel electrophoresis

Agarose gels were fabricated by boiling 1-2% agarose in 0,5x Tris/Borate/EDTA (TBE) buffer to yield a homogenous solution. After cooling to approximately 50°C, ethidium bromide (10 mg/ml aqueous solution) was added to a final concentration 0,5 µg/mL to enable visualization of DNA by UV-transillumination. The gel was then cast into trays in which combs were placed to leave loading slots after gelation. The gel was left to cool down and placed into the gel chamber filled with 0,5x TBE buffer.

The DNA samples whose fragments were to be separated according to their molecular weight were diluted 1:6 with 6x loading mix and the mixture loaded into slots. For every run 100 ng of molecular weight marker were included. A constant voltage of 135 V was applied so that negatively charged DNA travelled through the gel matrix towards the anode. Retention through the molecular sieve is dependent on the molecular weight and hence on the length of the DNA fragments, therefore longer DNA fragments migrate slower than shorter DNA fragments. While traveling the DNA fragments are separated by their size.

DNA fragments were visualized by UV-transillumination at 312 nm and an image of the gel was recorded using a digital camera and printed using a thermoprinter

10x TBE buffer

890 mM Trizma-base
890 mM boric acid
20 mM EDTA
pH 8,3 by self-regulation

6x loading mix

30% Glycerol
50 mM EDTA
0,2% Bromphenol Blue

peqGold Universal Agarose	Peqlab
Boric acid, for molecular biology, ~99%	Sigma-Aldrich
Bromphenol Blue	Sigma-Aldrich
EDTA for molecular biology, 99.0-101.0%	Sigma-Aldrich
Glycerol, for molecular biology, ≥99%	Sigma-Aldrich
Trizma® base Biotechnology Performance Certified, ≥99.9%	Sigma-Aldrich
Ethidium bromide 10 mg/mL, for molecular biology	Sigma-Aldrich

DNA molecular weight standards:

GeneRuler™ 100 bp marker	Fermentas
λ-EcoRI/HindIII marker	Promega
Mupid-exu advance agarose gel electrophoresis chamber	i-mupid
digital camera Canon PowerShotG9	Canon
UV-illuminator TFX-20M	MWG Biotech
Thermoprinter Mitsubishi 91	Mitsubishi

2.4 RNA extraction, cDNA synthesis, reverse transcriptase PCR (RT–PCR) and real time quantitative reverse transcriptase PCR (RTQ-RT-PCR)

2.4.1 RNA extraction using Trizol reagent

Adherent cells were harvested by directly adding Trizol reagent to the tissue culture dish after the removal of media, and resuspending cells several times with pipet until all cells became detached and lysed. 500 µl of Trizol were used per well of a 6-well plate and 1000 µl for a 10 cm diameter dish. Homogenized samples were transferred to a 1,5 ml reaction tube. At this stage the procedure could be interrupted and the samples stored at -70°C until further processing.

Samples were incubated for 5 min at room temperature for complete dissociation of nucleoprotein complexes. 1/5th volume of chloroform was added and thoroughly vortexed. After an incubation of 2 min at room temperature samples were centrifuged at 12000 rpm and 4°C for 10 min. The upper aqueous phase was transferred to a fresh tube. RNA was precipitated by addition of 1 volume of isopropanol relative to the volume of the transferred supernatant and pelleted by a 10 min centrifugation at 12 000 rpm and 4°C. The pellet was washed with 75% ethanol and then air-dried without letting it overdry, as this would decrease its solubility. It was then dissolved in RNase free H₂O for 10 min at 56°C.

To determine the concentration of RNA in the sample the optical density (OD) at 260 nm was measured using a spectral photometer. The RNA concentration was calculated using the formula

$$c [\mu\text{g}/\mu\text{l}] = \text{OD}_{260} \times \text{dilution factor} \times \text{multiplication factor}$$

multiplication factor for RNA: 0,04

Generally, RNA was diluted 1:100 prior to measurement. RNA was stored at -70°C.

Trizol	Invitrogen
Chloroform, analysis grade, ACS,ISO,Reag. Ph Eur	Merck
2-Propanol, ACS reagent, ≥99.5%	Sigma-Aldrich

96% Ethanol, Ph Eur,BP

Merck

RNase free H₂O

Invitrogen

Hitachi U-2000 spectral photometer

Hitachi

2.4.2 DNase I digestion of RNA

In order to remove contaminant genomic DNA prior to cDNA synthesis a DNase I digestion was carried out with the isolated RNA. This step was only necessary and carried out when subsequent applications were disturbed by the presence of genomic DNA, e.g. PCR with primers which would not only amplify cDNA but also genomic DNA.

500 ng RNA were incubated with 1 unit (U) DNase I in DNase I reaction buffer for 30 min at 37°C in a final volume of 10 µl. For inactivation of DNase I, EDTA was added to a final concentration of 2,5 mM followed by an incubation of 10 min at 70°C. Until further processing, RNA was stored on ice.

DNase I, amplification grade Invitrogen

25 mM EDTA Invitrogen

2.4.3 cDNA synthesis

500 ng DNase I treated RNA were incubated with 0,9 mM each deoxynucleotide triphosphate dATP, dCTP, dGTP, dTTP, 10 mM dithiothreitol (DTT), 62,5 ng random hexamer primer and 150 U Moloney murine leukemia virus (M-MLV) reverse transcriptase in M-MLV reverse transcriptase buffer for 1 h at 37°C in a total volume of 20 µl. The reaction was subsequently heat inactivated for 10 min at 95°C. cDNAs were stored at -20°C. In order to verify successful cDNA synthesis, a control PCR with primers specific for the housekeeping gene *cyclophilinD* was carried out after cDNA generation (chapter 2.4.4).

Reagent	Concentration stock solution	Volume (µl)	Final concentration in 20 µl reaction
M-MLV reverse transcriptase buffer	5x	4	1x
DTT	100 mM	2	10 mM
dNTPs	10 mM each	1,8	0,9 mM each
Random hexamer primer	50 ng/µl	1,25	3,125 ng/µl
M-MLV reverse transcriptase	200 U/µl	0,75	7,5 U/µl

M-MLV reverse transcriptase Invitrogen

100 mM DTT Invitrogen

dNTPs, molecular biology grade	Fermentas
random hexamer primer	Invitrogen

2.4.4 Conventional RT-PCR

A reaction mix was set up containing 1 µl of cDNA, 500 nM of each primer, 1 U of *Thermus aquaticus* (*Taq*) DNA polymerase, 125 nM of each dATP, dCTP, dGTP and dTTP, a final concentration of 1.5 mM MgCl₂ in *Taq* DNA polymerase buffer in a total volume of 20 µl. The PCR programme consisted of an initial denaturation step of 2 min at 94°C, followed by 25-35 cycles of 94°C denaturation for 15 sec, annealing at the primer specific annealing temperature for 20 sec, and elongation at 72°C for 1 min per kb. Since most products of conventional RT-PCR were shorter than 500 bp, elongation time was usually 30 sec. The PCR programme was finished with a final elongation step at 72°C for 5 min. Primers for conventional RT-PCR with sequence, annealing temperatures, along with the accession numbers of their reference sequence and the location therein are listed in the appendix in table 6.1. After amplification, PCR products were analysed by horizontal agarose gel electrophoresis (chapter 2.3).

Reagent	Concentration stock solution	Volume (µl)	Final concentration in 20 µl reaction
<i>Taq</i> DNA polymerase buffer	10x	2	1x
MgCl ₂	50 mM	0,6	1,5 mM
dNTPs	2,5 mM each	1	125 nM each
Primer forward	10 µM	1	500 nM
Primer reverse	10 µM	1	500 nM
<i>Taq</i> DNA polymerase	5 U/µl	0,2	0,05 U/µl
cDNA		1	

dNTPs, molecular biology grade	Fermentas
<i>Taq</i> DNA polymerase	Invitrogen
PCR plastic disposables	Greiner Bio-One
Thermocycler:	
PTC-200	MJ Research
Primus 96plus	MWG biotech

2.4.5 RTQ-RT-PCR

A master mix was set up containing 10 µl of 2x *Power* SYBR® Green PCR Master Mix, 400 nM of each primer and H₂O to a volume of 15 µl per well. 15 µl of the master mix were pipetted into each well. cDNA was diluted 1:5 and 5 µl were added per well, so that the final reaction volume was 20 µl. RTQ-RT-PCR was carried out in an ABI PRISM 7700® Sequence Detection System. Following the amplification programme a melting curve programme was performed to analyse the specific dissociation profile of the generated PCR products.

Assays for the housekeeping gene *cyclophilinD* were carried out in duplicates, all other assays were carried out in triplicates. Samples whose melting curve profile grossly deviated from the profile of that of the other samples were excluded from analysis, as were samples whose cycle threshold (C_t) value differed more than 0,5 from the other two replicate C_t values. Primers for RTQ-RT-PCR are listed in the appendix in table 6.2.

Reagent	Concentration stock solution	Volume (µl)	Final concentration in 20 µl reaction
<i>Power</i> SYBR® Green PCR Master Mix	2x	10	1x
Primer forward	10 µM	0,8	400 nM
Primer reverse	10 µM	0,8	400 nM
cDNA, diluted 1:5		5	

Amplification programme:

Hold 95°C, 2 min
Cycle (x45) 95°C, 15 sec
 60°C, 1 min

Melting curve programme:

95°C 15 sec
60°C 20 sec
ramp to 95°C in 20 min
95°C 20 sec

Relative expression values for *EV11* or its 5'-end variants relative to *cyclophilinD* and to a reference value were calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). In contrast to absolute quantification, in which the input copy number of the transcript of interest is determined, relative quantification describes the change in expression of the target gene relative to a reference such as a sample at time zero or in an untreated state.

Prerequisites are:

internal control: usually a housekeeping gene whose expression remains unaffected by the experimental treatment, for normalisation.

Calibrator: untreated control or sample at time zero, for which the $\Delta\Delta C_t$ value equals zero and the $2^{-\Delta\Delta C_t}$ value equals one, serves as reference for the treated samples.

Amplification efficiencies of the target and reference gene must be very close to optimal.

$$\Delta\Delta C_t = (C_t \text{ target} - C_t \text{ cyclo})_{\text{time } x} - (C_t \text{ target} - C_t \text{ cyclo})_{\text{time } 0}$$

To calculate the value of relative expression, the $\Delta\Delta C_t$ value is set as negative exponent to the basis 2:

$$\text{Relative expression} = 2^{-\Delta\Delta C_t}$$

The C_t values of replicates of the housekeeping gene *cyclophilinD* and the target gene at time zero were averaged before calculating the $\Delta C_{t \text{ time zero}}$ value. This part of the equation constitutes a constant value for each assay (Livak and Schmittgen, 2001).

The $\Delta C_{t \text{ time } x}$ value was calculated by subtracting the average $C_t \text{ cyclo}_{\text{time } x}$ value from each replicate $C_t \text{ target}_{\text{time } x}$ value. Standard deviation and average value was calculated from replicate $2^{-\Delta\Delta C_t}$ values obtained for each sample (Livak and Schmittgen, 2001).

Power SYBR® Green PCR Master Mix	Applied Biosystems
Primer synthesis	Operon, Ingenetix
Optical PCR plastic disposables	Applied Biosystems
ABI PRISM 7700® Sequence Detection System	Applied Biosystems
Sequence Detection Systems software	Applied Biosystems

2.5 Cloning procedures and strategies, preparation of plasmid DNA

2.5.1 Cloning strategies

2.5.1.1 *EV11* promoter constructs

EV11 promoter fragments -2629/-72, +15/+1106, and +1258/+3903 (fig. 2.1) were amplified from BAC clone RP11-33A1 using DyNAzyme™ EXT DNA Polymerase with proofreading activity. Position of the constructs are relative to the transcription start site of *EV11_1a* according to Genbank accession number BX640908. Primers used to generate the PCR products introduced restriction sites for cloning. The obtained PCR-products were cloned into pCR®2.1-TOPO®. One clone was chosen, respectively, and the insert was transferred into

the luciferase reporter plasmids pGL3basic and pGL3 promoter using the engineered restriction sites.

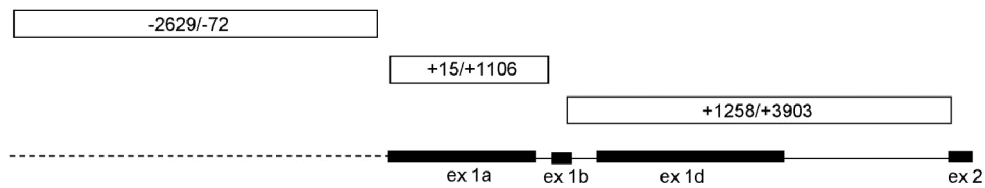


Fig. 2.1 schematic of *EVI1* promoter constructs in relation to the putative *EVI1* promoter region. Dashed line: *EVI1* putative promoter region, continuous line: *EVI1* intronic regions, black boxes: *EVI1* exons.

construct	Forward primer	Reverse primer
-2629/-72	EVI1prom-2629 Bgl II fwd	EVI1prom-72 KpnI rev
+15/+1106	EVI1prom+15 KpnI fwd	EVI1prom+1106 Bgl II rev
1258/+3903	EVI1prom+1258 KpnI fwd	EVI1prom+3903 XhoI rev

Table 2.1 Primers used to generate PCR products for *EVI1* promoter constructs. Primer sequences, positions, and restriction sites are listed in the appendix in table 6.3.

Only promoter fragment +15/+1106 was subjected to sequence analysis, and the obtained sequence corresponded exactly to the amplified part of BAC clone RP11-33A1.

Deletion subclone +86/+1106 (fig. 2.3) was derived from EVI1(+15/+1106)/pGL3basic by restriction digestion with Bgl II and ligating the +86/+1106 *EVI1* promoter fragment into pGL3basic. Deletion subclone +653/+1106 (fig. 2.3) was derived from EVI1(+15/+1106)/pGL3basic by restriction digestion with SacI, and religation of the remaining plasmid.

Deletion subclones +255/+1106, +325/+1106, +587/+1106, +86/+274 and +140/+227 (fig. 2.3) of the *EVI1* promoter were amplified from BAC clone RP11-33A1 using DyNAzyme™ EXT DNA Polymerase with proofreading activity. Position of the constructs are relative to the transcription start site of EVI1_1a according to Genbank accession number BX640908. Primer used to generate the PCR products introduced restriction sites for cloning and are listed in table 6.3. The obtained PCR products were cloned into pCR®2.1-TOPO®. One clone was chosen, respectively, and the insert transferred into the luciferase reporter plasmid pGL3basic using the engineered restriction sites. Only deletion subclone +140/+227 was subjected to sequence analysis, and the obtained sequence corresponded exactly to the amplified part of BAC clone RP11-33A1.

The +86/+1106 mut construct carries a mutation in the second half site of the *EVI1* DR5 retinoic acid response element (RARE) with the nucleotide sequence changed from

TGACCT to CTTTAG according to Jiang et al. (Jiang, et al., 2005). Two PCR products were generated, the first with a regular forward primer also used to generate the +15/+1106 construct and a reverse primer carrying the desired mutation of the *EV11* DR5 RARE, the second product was generated with a forward primer carrying the desired mutation of the *EV11* DR5 RARE and a regular reverse primer also used to generate the +15/+1106 construct. These two PCR products were used as templates in a fusion PCR with the two most outward flanking primer, and were thus joined to yield the +86/+1106 mut construct as depicted in fig. 2.2.

The resulting fragment was cloned into pCR®2.1-TOPO® and successful mutagenesis but otherwise unaltered sequence was verified by sequencing analysis. One clone with the correct sequence was chosen and transferred into pGL3basic using the engineered Bgl II site of the *EV11*prom+1106 Bgl II rev primer and a naturally occurring Bgl II site at position +68.

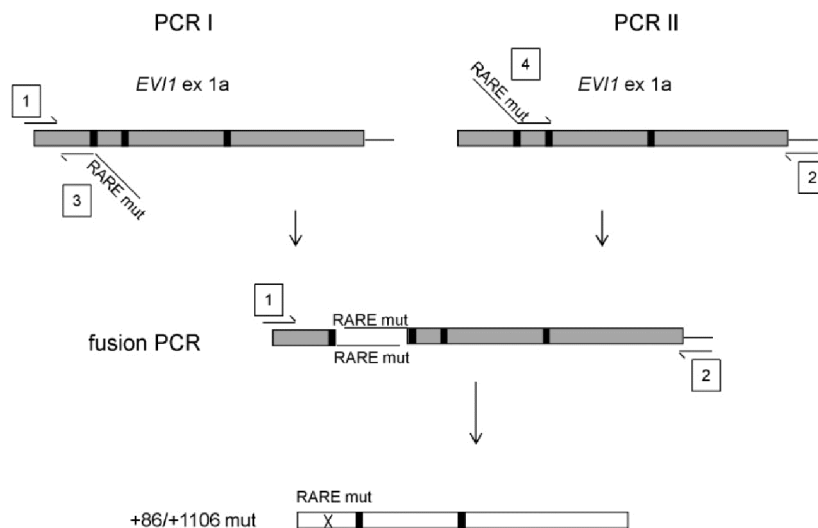


Fig. 2.2 schematic of cloning strategy for the +86/+1106 mut construct. Primers and oligonucleotides used to generate the +86/+1106 construct in square boxes are 1) *EV11*prom+15 KpnI fwd 2) *EV11*prom+1106 Bgl II rev, 3) *EV1*prom RAREmut rev, 4) *EV1*prom RAREmut fwd. Length of primer not drawn to scale. Black boxes: predicted RAREs; X: mutated RARE.

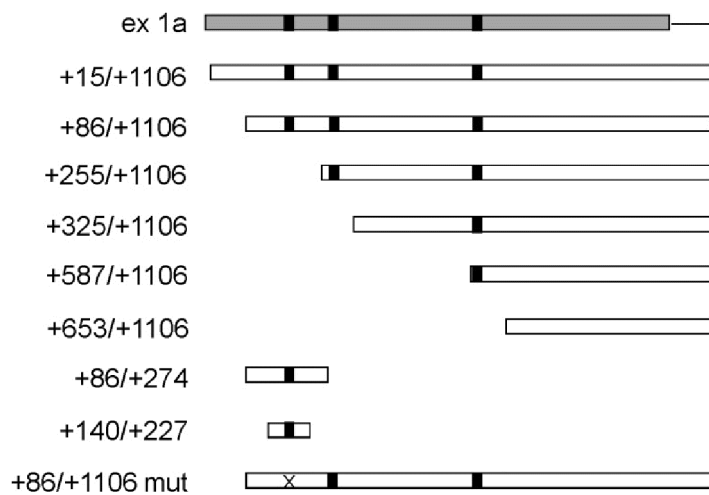


Fig. 2.3 schematic of EVI1 promoter deletion constructs in relation to EVI1 exon 1a (in grey). Continuous line: intronic region, black boxes: predicted RAREs; X: mutated RARE.

construct	Forward primer	Reverse primer
+255/+1106	EVI1prom+255 NheI fwd	EVI1prom+1106 Bgl II rev
+325/+1106	EVI1prom+325 NheI fwd	EVI1prom+1106 Bgl II rev
+587/+1106	EVI1prom+587 NheI fwd	EVI1prom+1106 Bgl II rev
+86/+274	EVI1prom+15 KpnI fwd	EVI1prom+274 HindIII rev
+140/+227	EVI1prom+140 NheI fwd	EVI1prom+227 Bgl II rev
+86/+1106 mut – PCR I	EVI1prom+15 KpnI fwd	EVI1prom RAREmut rev
+86/+1106 mut – PCR II	EVI1prom RAREmut fwd	EVI1prom+1106 Bgl II rev
+86/+1106 mut-fusion PCR	EVI1prom+15 KpnI fwd	EVI1prom+1106 Bgl II rev

Table 2.2 Primers used to generate PCR products for *EVI1* promoter deletion constructs. Primers used to generate the +86/+1106 mut construct are listed in fig. 2.2.

Primer sequences, positions, and restriction sites are listed in the appendix in table 6.3.

2.5.1.2 *MDS1/EVI1* promoter constructs

MDS1/EVI1 promoter fragments -2968/-1975, -2027/-915, -920/+357, and +310/+2456 (fig. 2.4) were amplified from BAC clone RP11-816J6 using DyNAzyme™ EXT DNA Polymerase with proofreading activity. Position of the constructs are relative to the transcription start site of *MDS1* according to Genbank accession number NM_004991. Primers used to generate the PCR products introduced restriction sites for cloning, or naturally occurring sites within the amplified region were used. PCR products were cloned into pCR®2.1-TOPO®. One clone was chosen and the insert transferred into the luciferase reporter plasmid pGL3basic using either the engineered restriction sites or the naturally occurring restriction sites.

For the construct -2027/-915, a naturally occurring NheI restriction site towards the 3' end of the PCR product was used for cloning. This restriction site was also used for the construct -920/+357, only then it was towards the 5' end of the PCR product. For the construct +310/+2456, a naturally occurring HindIII restriction site towards the 3' end of the PCR product was used for cloning.

MDS1/EVI1 promoter fragments were not subjected to sequence analysis.

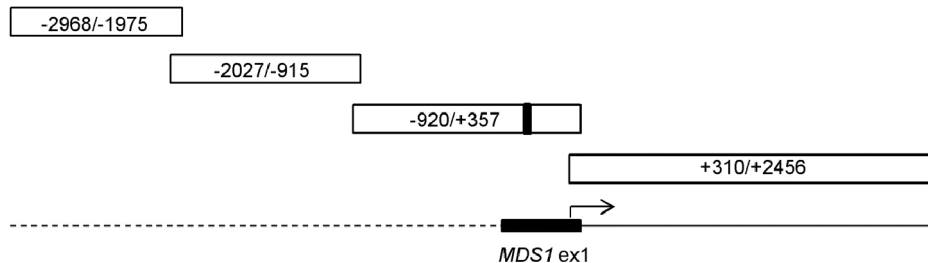


Fig. 2.4 schematic of *MDS1/EVI1* promoter constructs in relation to the putative *MDS1* promoter region. Dashed line: *MDS1/EVI1* putative promoter region, continuous line: *MDS1* intron 1, arrow: transcription start site, black bar within -920/+357 construct: predicted DR2 RARE.

construct	Forward primer	Reverse primer
-2968/-1975	MDS1prom-2968 NheI fwd	MDS1prom-1975 Bgl II rev
-2027/-915	MDS1prom-2027 KpnI fwd	MDS1prom-915 rev
-920/+357	MDS1prom-920 fwd	MDS1prom+357 XhoI rev
+310/+2456	MDS1prom+310 Bgl II fwd	MDS1prom+2456 rev

Table 2.3 Primers used to generate PCR products for *MDS1/EVI1* promoter constructs.

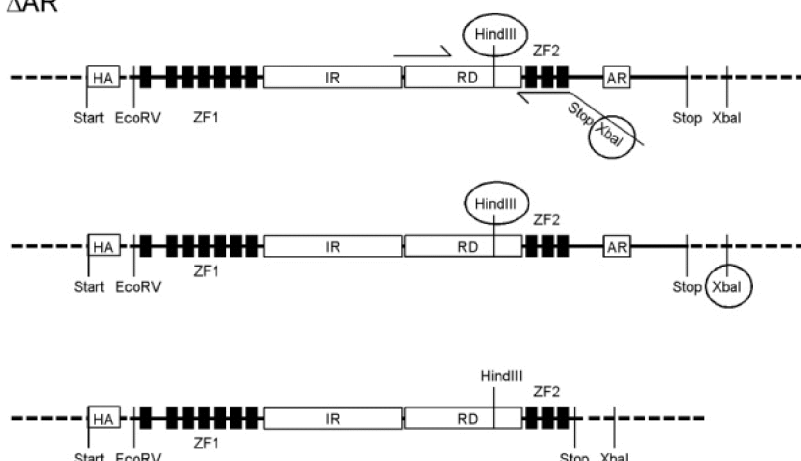
Primer sequences, positions, and restriction sites are listed in the appendix in table 6.3.

2.5.1.3 *EVI1* deletion constructs

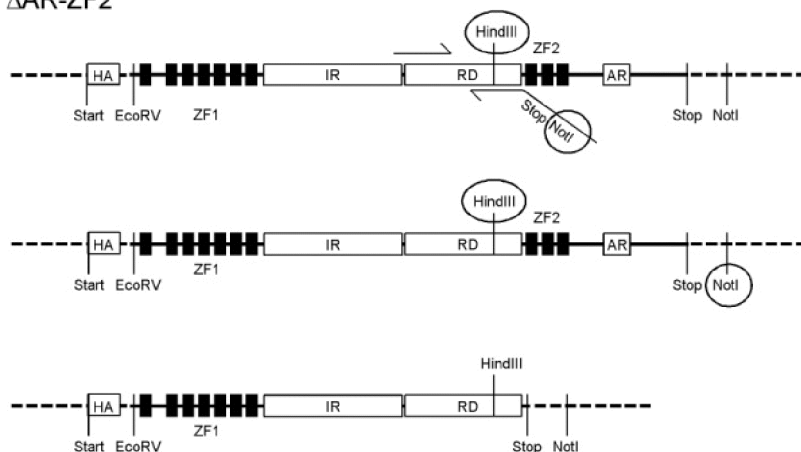
The plasmid HA-*EVI1*/pEFzeo contains the human *EVI1* cDNA, an engineered Kozak consensus for translation initiation and an amino terminal hemagglutinin (HA) epitope tag, under the control of the *eIF1 α* promoter (Vinatzer U, 2001). This construct served as a basis for *EVI1* deletion constructs. The Kozak sequence and the HA tag remained unaltered. Using the internal restriction sites EcoRV and HindIII, or the vector restriction sites NotI or XbaI, internal fragments were replaced by PCR products with engineered restriction sites and/or – if required - engineered stop codons and carrying the desired deletions, and thus creating *EVI1* deletion constructs (fig. 2.5). For the *EVI1* ZF1-NLS construct, two complementary oligonucleotides containing a tandem repeat of the SV40 large T antigen nuclear localization sequence (NLS) (Fischer-Fantuzzi and Vesco, 1988) followed by a stop codon, were annealed. A PCR product covering the *EVI1* ZF1 region was generated with a reverse primer carrying part of the NLS sequence. The products of these two reactions were used as

templates in a fusion PCR, which yielded a ZF1-NLS PCR-product (fig. 2.5). All PCR products were generated with DyNAzyme™ EXT DNA Polymerase with proofreading activity. Prior to replacing internal *EVI1* fragments of the HA-EVI1/pEFzeo with their suitable PCR products with engineered restriction sites and/or – if required - engineered stop codons and carrying the desired deletions, these PCR products were cloned into pCR®2.1-TOPO®, sequence verified using vector specific M13 fwd and M13 rev primer, and if necessary *EVI1* specific primers. One clone with the correct sequence was chosen, and the insert transferred into HA-EVI1/pEFzeo.

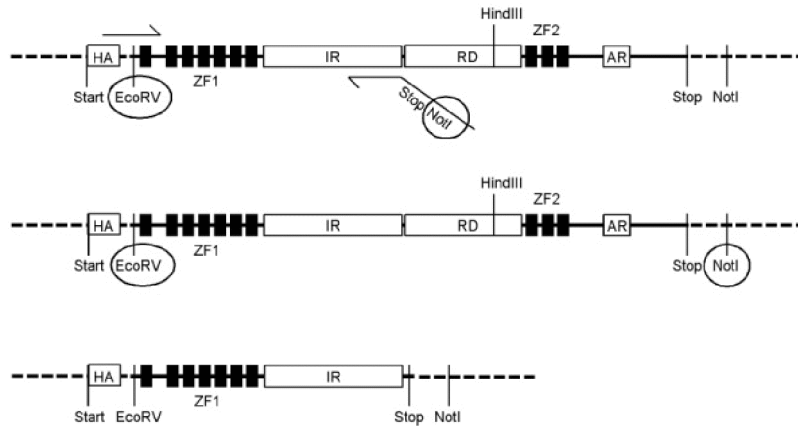
ΔAR



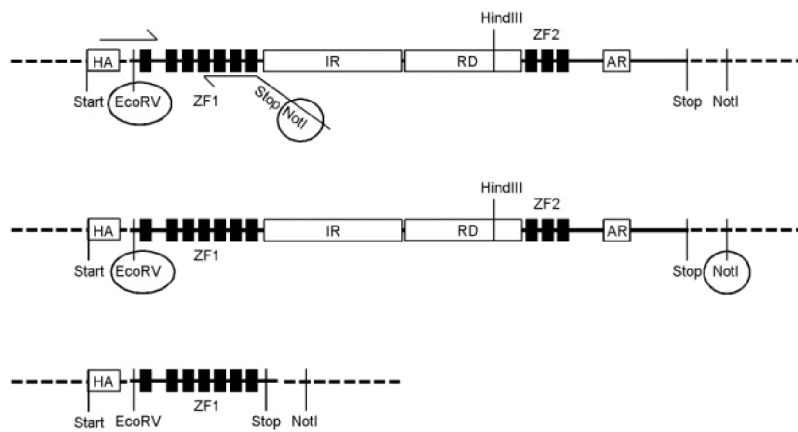
ΔAR-ZF2



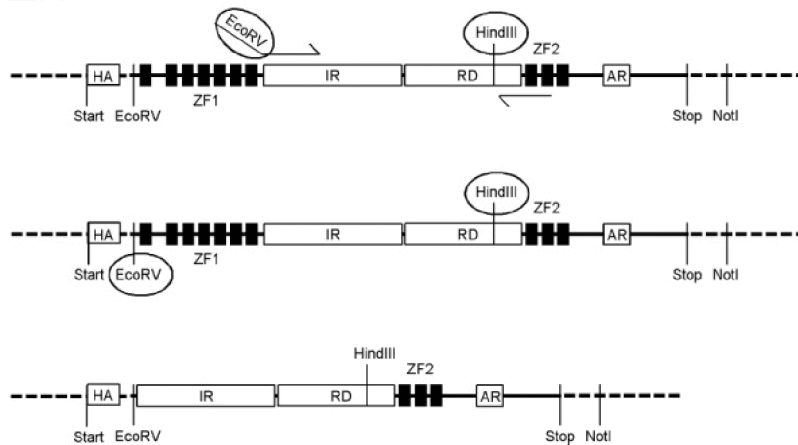
ZF1-IR



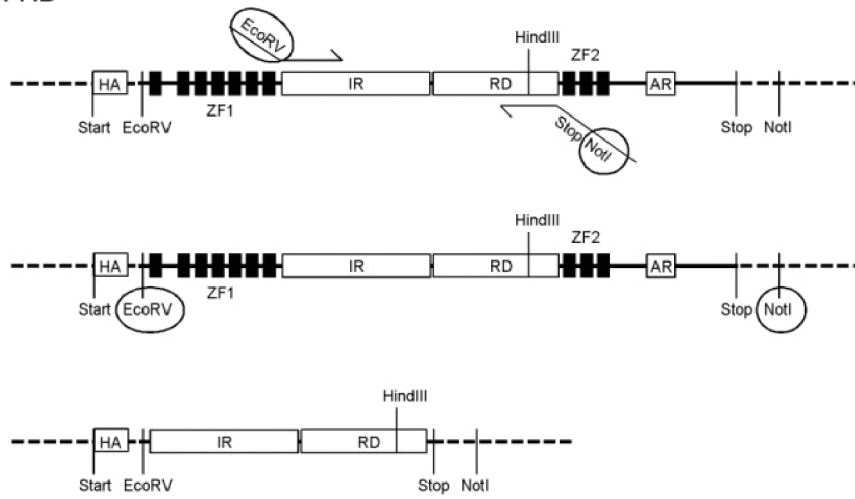
ZF1



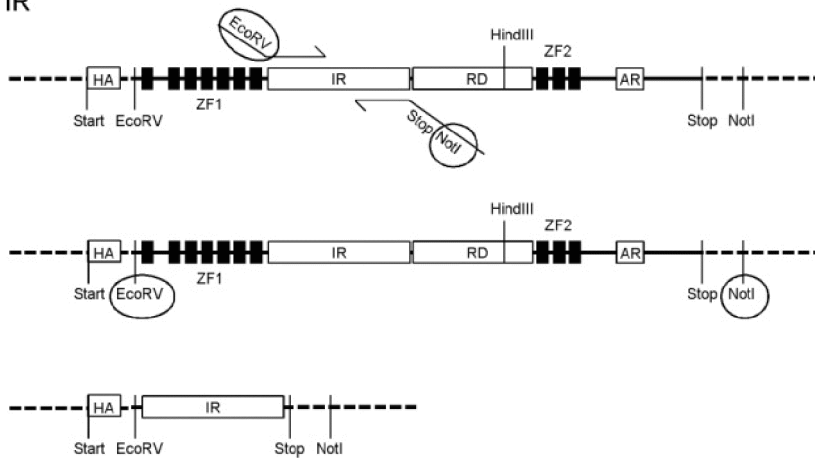
Δ ZF1



IR-RD



IR



ZF1-NLS

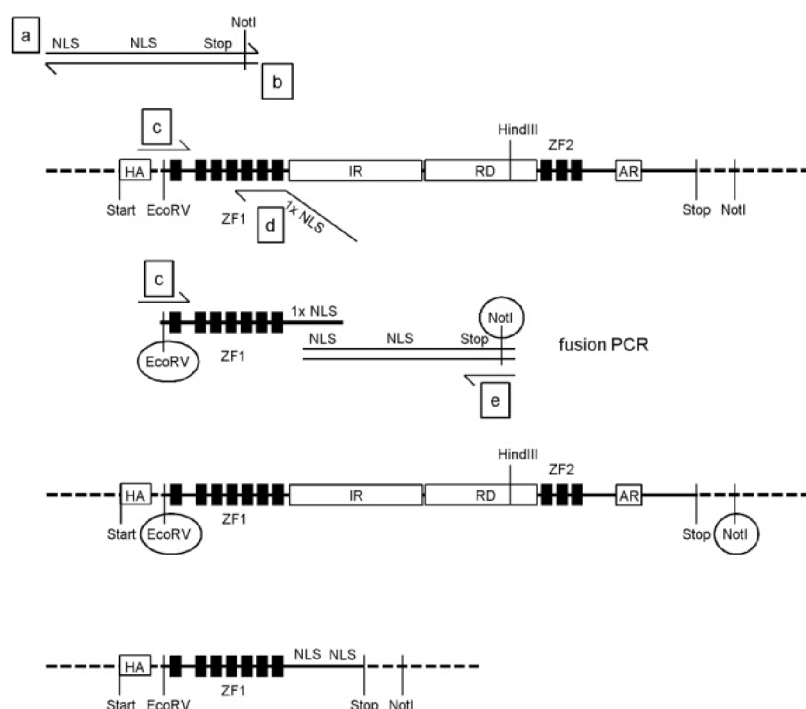


Fig. 2.5 schematic of cloning strategies for EVI1 deletion constructs.

Upper scheme, respectively: original plasmid, serving as a template for PCR with indicated primer pairs, some of them introducing restriction sites and/or stop codons as indicated.

Middle scheme, respectively: original plasmid from which internal element was removed using indicated restriction sites, in order to be replaced with the PCR-product. Restriction sites used for cloning are circled.

Lower scheme, respectively: obtained *EVI1* deletion construct

dashed line: vector sequence, continuous line: *EVI1* cDNA sequence. Functional domains of EVI1 are ZF1 = amino terminal zinc finger region, IR = intervening region, RD = repression domain, ZF2 = carboxy terminal zinc finger region, AR = acidic region

primers and oligonucleotides used to generate the ZF1-NLS construct in square boxes are a) NLSfwd, b) NLS Stop NotI rev, c) EVI1 270 fwd, d) ZF1fus NLS rev, e) NLS Stop NotI rev. Length of primer not drawn to scale.

construct	Forward primer	Reverse primer
Δ AR	EVI1 2147 fwd	EVI1 2698 Stop Xbal rev
Δ AR-ZF2	EVI1 2147 fwd	EVI1 2427 Stop NotI rev
ZF1-IR	EVI1 270 fwd	EVI1 1767 Stop NotI rev
ZF1	EVI1 270 fwd	EVI1 1039 Stop NotI rev
Δ ZF1	EVI1 1118 EcoRV fwd	EVI1 2441 rev
IR-RD	EVI1 1118 EcoRV fwd	EVI1 2427 Stop NotI rev
IR	EVI1 1118 EcoRV fwd	EVI1 1767 Stop NotI rev

Table 2.4 Primers used to generate PCR products with which internal fragments of the plasmid HA-EVI1/pEFzeo were replaced in order to generate *EVI1* deletion constructs. Primer and oligonucleotides used to generate the EVI1-NLS construct are listed in fig. 2.5.

2.5.1.4 Further observations and strategies

It was observed in reporter gene assays that some constructs changed their behaviour in the course of time and that the activity, e.g. an ATRA response, eventually faded and was lost. In DNA sequencing analyses alterations of the plasmid sequence were not found. However, recloning has been helpful in reconstituting the original activity of that construct. The plasmid was restriction digested, insert and vector DNA were separated by gel electrophoresis, and subsequently both insert and vector DNA were isolated from the gel. Vector and insert were religated to yield the same construct again. To avoid the necessity of another sequencing analysis, the very construct, which has been used in experiments, was used for recloning. The freshly prepared and recloned construct then showed the original activity in all observed cases of reporter gene assays. If necessary, this procedure was repeated.

2.5.2 Preparation of electrocompetent cells (DH5 α , XL1-blue)

200 ml LB medium were inoculated with 3 ml of a preparatory over night culture of DH5 α or XL1-blue *E. coli* bacteria and grown at 37°C and 200 rpm in a 500 ml Erlenmeyer flask. Growth was monitored by determining OD₆₀₀ and cells were cooled on ice when OD₆₀₀ had reached a value between 0,4 and 0,5.

Bacteria were centrifuged for 10 min at 4000 rpm at 4°C and the pellet was washed three times with ice cold sterile H₂O using the same volume of H₂O as the bacteria culture volume, with gentle resuspension after centrifugation. After the last wash bacteria were resuspended in 720 μ l 10% DMSO in H₂O. 40 μ l aliquots were snap frozen in liquid nitrogen and stored at -70°C.

To determine transformation efficiency, 10 pg of pUC vector were transformed into one aliquot of electrocompetent cells. Different amounts of bacteria were plated and the following

day the number of colonies were counted. The number of transformants per μg DNA was calculated. Only cells with transformation efficiency of more than $10^6/\mu\text{g}$ were used. Usually values between 10^8 and $10^9/\mu\text{g}$ were achieved.

LB medium

1,0% Peptone

0,5% Yeast Extract

0,5% NaCl

pH 7,2 with NaOH

Peptone, for microbiology

Merck

Yeast Extract, for microbiology

Merck

NaCl, ACS reagent, $\geq 99.0\%$

Sigma-Aldrich

Incubator Certomat®U, Certomat®H

B. Braun Biotech International

2.5.3 Electroporation of competent *E. Coli* strains

In order to transform competent bacteria (DH5 α or XL-1 blue strain), 40 μl electrocompetent cells were mixed with 1 μl of the respective plasmid or ligation reaction and transferred to an electroporation cuvette with a 2 mm distance between the electrodes. The mix was kept on ice. Cells were subjected to an electric pulse of a voltage of 2,5 kV, a capacity of 25 μF and a resistance of 200 Ω , and then rinsed out of the electroporation cuvette with 460 μl SOC medium, in which they were left to recover at 37°C in a thermomixer with agitation at 700 rpm for 30 min.

Cells were spread on agar plates containing the appropriate antibiotic for selection of transformed bacteria and then grown at 37°C over night until visible colonies appeared.

SOC medium

0.5% Yeast Extract

2% Tryptone

10 mM NaCl

2.5 mM KCl

10 mM MgCl_2

10 mM MgSO_4

20 mM Glucose

pH 7,0 with NaOH

LB medium

1,0% Peptone

0,5% Yeast Extract

0,5% NaCl

pH 7,2 with NaOH

LB Agar

LB medium + 1,5% Agar-Agar

Kanamycin stock solution 50 mg/ml in H_2O , concentration in medium 50 $\mu\text{g}/\text{ml}$

Ampicillin stock solution 50 mg/ml in H₂O, concentration in medium 50 µg/ml

Yeast Extract, for microbiology	Merck
Peptone, for microbiology	Merck
Tryptone, for microbiology	Merck
Agar-Agar, for microbiology	Merck
NaCl, ACS reagent, ≥99.0%	Sigma-Aldrich
KCl, analysis grade	Merck
MgCl ₂ x 6 H ₂ O, analysis grade, ACS,ISO,Reag. Ph Eur	Merck
MgSO ₄ x 7 H ₂ O, analysis grade, ACS,Reag. Ph Eur	Merck
Glucose, mouse embryo tested	Sigma-Aldrich
Ampicillin, Na-salt	Sigma-Aldrich
Kanamycin, disulfate salt	Gibco BRL
Petri dishes	Greiner Bio-One
Electroporation cuvettes	BioRad
Electroporator, consisting of the three units	
• Gene Pulser II	
• Pulse Controller Plus	
• Capacitance Extender Plus	BioRad
Thermomixer comfort	Eppendorf

2.5.4 Restriction digest and isolation of DNA fragments from agarose gels

In order to analyse plasmid preparation for the presence of an insert or to verify plasmid identity, restriction control digests were carried out.

Approximately 0,5 – 1 µg of plasmid DNA or 1-2 µl plasmid from small scale preparation of plasmid DNA (chapter 2.5.8) with 5 U of each restriction enzyme, or altogether 5 U if only one enzyme was used, in appropriate buffer supplied by the manufacturer in a total volume of 20 µl for 1 hour. The volume of enzymes was not to exceed 10% of the total reaction volume. 10 µl of digested plasmid DNA were separated by horizontal agarose gel electrophoresis (see chapter 2.3).

If plasmids were digested to obtain vector or insert fragments for cloning, 3 µg of plasmid DNA were digested with 20 U of each restriction enzyme, or altogether 20 U if only one enzyme was used, in appropriate buffer supplied by the manufacturer in a total volume of 70 µl for 3-5 hours. The restriction digest reaction was mixed with 6x loading mix and loaded onto 1-2% agarose gels with wide slots which have the capacity for large volumes. The DNA fragments were then separated by electrophoresis (see chapter 2.3).

The DNA bands required for subsequent cloning steps were excised from the gel and the DNA extracted using the Illustra™ GFX Gel Band Purification kit according to the manufacturer's instructions. Gel slices were melted in capture buffer, the mixture was transferred to a column to whose glass fibre matrix the DNA bound. Contaminants were removed by wash steps and purified DNA was eluted with elution buffer supplied in the Illustra™ GFX Gel Band Purification kit.

In order to estimate the approximate DNA concentration of the isolated fragment, a small portion of the eluate was subjected to agarose gel electrophoresis (see chapter 2.3) together with 100 ng of the λ -EcoRI/HindIII marker, whose band intensity corresponded to specific amounts of DNA. By comparing the band intensity of the isolated DNA fragments to a band of similar intensity of the λ -EcoRI/HindIII marker the DNA concentration of the isolated fragment could be estimated.

Restriction enzymes	Roche
Illustra™ GFX Gel Band Purification kit	GE Healthcare
λ -EcoRI/HindIII marker	Promega

2.5.5 Ligation

In order to generate plasmid constructs compatible ends of vector and insert were joined in a ligation reaction.

20 ng of vector DNA were mixed with a stoichiometric triple oversupply of insert DNA and 1 U of T4 DNA Ligase in T4 DNA Ligase buffer in a total volume of 10 μ l. The ligation reaction mix was incubated for 3 hours at room temperature or at 16°C overnight. 0,5-1 μ l were transformed into electrocompetent bacteria (see chapter 2.5.2).

T4 DNA Ligase	Roche
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2.5.6 T/A cloning of PCR products

PCR products designated to be cloned into a vector were generated using DyNAzyme™ EXT DNA Polymerase with proofreading activity to minimize the eventuality of point mutations. Furthermore, this DNA polymerase has the capacity of adding unspecific adenine residues to the 3' ends of its reaction products, thereby facilitating ligation into a vector with complementary thymidine residues. A reaction mix was set up containing approximately 50 ng of template BAC DNA, 500 nM of each primer, 0,8 U of DyNAzyme™ EXT DNA Polymerase, 125 nM of each dATP, dCTP, dGTP and dTTP, in DyNAzyme™ buffer in a total volume of 20 μ l. The PCR programme consisted of an initial denaturation step of 2 min at 94°C, followed by 25 cycles of 94°C denaturation for 15 sec, annealing at the primer specific

annealing temperature for 20 sec, and elongation at 68°C for 1 min per kb. Primers used to generate PCR products for cloning are listed in the appendix in table 6.3.

Reagent	Concentration stock solution	Volume (µl)	Final concentration in 20 µl reaction
DyNAzyme™ buffer	10x	2	1x
dNTPs	2,5 mM each	1	125 nM each
Primer forward	10 µM	1	500 nM
Primer reverse	10 µM	1	500 nM
DyNAzyme™ EXT DNA Polymerase	2 U/µl	0,4	0,04 U/µl
DNA template		1	

After amplification, an aliquot of each PCR reaction was analysed by horizontal agarose gel electrophoresis (see chapter 2.3). If agarose gel electrophoresis revealed a single band of the expected size, the product was cloned into pCR®2.1-TOPO®, which has single overhanging thymidine residues on both ends. This residue binds to the complementary adenine overhang of the PCR-product unspecifically added by DNA polymerase during amplification. Ligation of the vector and insert is carried out by vector-bound Topoisomerase. During gel electrophoresis the rest of the PCR product was stored on ice, and after verification of successful amplification, the product was ligated immediately into the pCR®2.1-TOPO® vector. 4 µl PCR product were mixed with 1 µl pCR®2.1-TOPO® vector and 1 µl salt solution supplied in the pCR®2.1-TOPO® kit and incubated at room temperature for 15 min. Chemically competent One Shot® TOP10 cells were thawed on ice, mixed with 2 µl of the ligation reaction and incubated on ice for 5 min. The cells were heat-shocked for 30 sec at 42°C in a waterbath and shortly chilled on ice. Transformed Cells were resuspended in 250 µl SOC medium supplied in the pCR®2.1-TOPO® vector kit and grown at 37°C with agitation at 700 rpm in a thermomixer for 30 min and then plated on agar plates containing the appropriate antibiotic overnight until visible colonies had appeared.

DyNAzyme™ EXT DNA Polymerase	Finnzyme
pCR®2.1-TOPO® vector kit	Invitrogen
One Shot®TOP10 competent cells	Invitrogen
Waterbath	Haake D8
Thermomixer comfort	Eppendorf
Thermocycler:	
PTC-200	MJ Research
Primus 96plus	MWG biotech

2.5.7 Annealing of oligonucleotides

Complementary oligonucleotides were annealed at a final concentration of 2 μ M of each oligonucleotide in PCR buffer, which was supplied with DyNAzyme™ EXT DNA Polymerase by the manufacturer, in a total volume of 50 μ l. The mixture was incubated in a boiling water bath for 5 min, and then left to cool down to room temperature for approximately 20 min.

DyNAzyme™ EXT DNA Polymerase buffer Finnzyme

2.5.8 Small scale preparation of plasmid DNA

4 ml LB medium containing appropriate antibiotic were inoculated with a single colony of transformed bacteria and grown overnight at 37°C with agitation at 200 rpm. 2 x 1,5 ml of bacteria culture were sequentially pelleted by centrifugation at 5000 rpm at room temperature for 5 min in a table top centrifuge and supernatant LB medium was discarded. The bacterial pellet was resuspended in 200 μ l resuspension buffer. Cells were lysed by addition of 400 μ l alkaline lysis buffer, gentle mixing and incubation for 5 min at room temperature. Lysis was stopped by addition of 300 μ l of neutralisation solution and an incubation for 5 min on ice. Precipitated cellular debris was pelleted by centrifugation at 13000 rpm and 4°C and 10 min in a table top centrifuge. The supernatant was transferred to a new tube, and plasmid DNA was precipitated by the addition of 700 μ l ethanol and subsequent centrifugation in a table top centrifuge for 20 min at 13000 rpm and 4°C. Residual salts were removed by washing the plasmid DNA pellet with 70% ethanol. The pellet was air-dried and dissolved in 40 μ l H₂O containing 2 μ l RNase A to degrade bacterial RNA. The DNA yield was approximately 20 μ g. For analysis of isolated plasmid DNA a restriction digest was carried out.

Resuspension buffer	alkaline lysis solution	Neutralisation solution
50 mM Glucose	1% SDS	3M Potassium-Acetate
25 mM Tris base	0,2 M NaOH	pH 5,5 with acetic acid
10 mM EDTA		
pH 8,0 with HCl		

RNase A

10 mg/ml in H₂O, 95°C 10 min before use

Glucose, mouse embryo tested	Sigma-Aldrich
Trizma® base Biotechnology Performance Certified, $\geq$ 99.9%	Sigma-Aldrich
EDTA for molecular biology, 99.0-101.0%	Sigma-Aldrich
SDS, molecular Biology Grade	Calbiochem

NaOH, reagent grade, ≥98%	Sigma-Aldrich
Potassium-Acetate, Ph Eur, BP, E 261	Merck
96% Ethanol, Ph Eur, BP	Merck
RNaseA, for molecular biology	Sigma-Aldrich
Table top centrifuge 5415R	Eppendorf
Incubator Certomat®U, Certomat®H	B. Braun Biotech International

2.5.9 Large scale preparation of plasmid DNA

In order to prepare 100 – 200 µg of plasmid DNA of high purity suitable for transfection, 50 ml of LB medium containing the appropriate antibiotic were inoculated with a single colony of transformed bacteria and grown overnight at 37°C with agitation at 200 rpm. Bacterial suspension was pelleted for 10 min at 4000 rpm and 4°C and supernatant LB medium was discarded. Plasmid DNA was extracted according to the manufacturer's instructions of plasmid DNA isolation kits "Illustra™ plasmidPrep Midi flow kit" (GE Healthcare) or "Plasmid Midi kit" (Qiagen), which contained all necessary buffers and solutions and which worked according to the same principles.

Bacterial cells were resuspended, followed by alkaline lysis and neutralisation. Cell debris was removed by centrifugation and the plasmids were purified by anion exchange chromatography. Impurities were removed by washing steps and plasmid DNA was eluted with 200 µl 1x TE buffer. OD₂₆₀ was determined with a spectrophotometer and DNA concentration was calculated according to the formula

$$c [\mu\text{g}/\mu\text{l}] = \text{OD}_{260} \times \text{dilution factor} \times \text{multiplication factor}$$

multiplication factor for DNA: 0,05

Generally, plasmid DNA was diluted 1:100 prior to measurement. For analysis of isolated plasmid DNA 4-5 restriction analyses were carried out with 0,5 – 1,0 µg plasmid DNA. When very small restriction fragments were expected, up to 2 µg of plasmid DNA were analyzed.

Illustra™ plasmidPrep Midi flow kit	GE Healthcare
Plasmid Midi kit	Qiagen
Incubator Certomat®U, Certomat®H	B. Braun Biotech International
Centrifuge	Megafuge 2.0R Heraeus
Hitachi U-2000 Spectrophotometer	Hitachi

2.5.10 DNA sequencing of plasmids

Sequencing of plasmids was carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit, with 200 ng of plasmid DNA, 500 nM primer, BigDye® Terminator v3.1 and Sequencing buffer in a total volume of 10 µl.

Reagent	Concentration stock solution	Volume (µl)	Final concentration in 10 µl reaction
BigDye® Terminator v3.1	5x	1	0,5x
Sequencing Buffer	5x	1	0,5x
Primer	10 µM	0,5	500 nM
DNA		1-2	

The reaction conditions were:

93°C	4 min	x 25 cycles
92°C	12 sec	
55°C	5 sec	
60°C	2 min 15 sec	

The reaction products were stored at -20°C until further processing. Purification of the sequencing reaction and loading onto the 3130x/ Genetic Analyzer capillary sequencer was carried out by personnel entitled to operate this device.

BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems
Sequencing Buffer, 5X	Applied Biosystems
Thermocycler:	
PTC-200	MJ Research
Primus 96plus	MWG biotech
3130x/ Genetic Analyzer	Applied Biosystems

2.5.10.1 Other plasmids used

The following plasmids were used but not constructed during the work on this thesis:

- HA-MDS1/EVI1/pEFzeo: The human cDNA of *MDS1/EVI1*, an engineered Kozak consensus for translation initiation and an amino terminal HA epitope tag were cloned into the expression vector pEFZeo under the control of the *eIF1α* promoter (Kovarik, et al., 2001; Vinatzer U, 2001).
- EVI1 wt/pME18S and EVI1 DL/AS/pME18S: These plasmids contain the human wild type (wt) EVI1 cDNA, or a mutated version which changed the amino acids DL at

positions 586 and 587 to AS, which abrogates binding of CtBP to the EVI1 protein. Both plasmids were kindly provided by Drs. Goyama and Kurokawa, Tokyo University, Japan (Izutsu, et al., 2001).

- pERE/luc: this luciferase reporter plasmid contains two copies of the recognition sequence for the amino terminal zinc finger domain of EVI1 according to Perkins et al (Perkins and Kim, 1996) and the *tk* minimal promoter in the pGL3basic vector backbone (Vinatzer U, 2001).
- pRARE-tk-luc and pGL2-tk-luc: the former contains two copies of the RARE of the human *RAR β* gene promoter (de Thé H, 1990) and a *tk* minimal promoter in pGL2; the latter is the corresponding empty control vector lacking the RAREs. Both plasmids were kindly provided by Dr. Hanna Harant, Ingenetix, Vienna, Austria.

2.6 Protein assays

2.6.1 Subcellular localization of proteins

To determine the subcellular location of the protein products derived from *EVI1* deletion constructs and full length exogenously expressed *EVI1*, NTERA-2 cells grown on coverslips in 6-well plates were transiently transfected with the respective plasmids using the calcium phosphate method (chapter 2.1.3.3). These constructs carried an amino terminal HA-tag, therefore the protein products could be detected with a mouse-anti-HA antibody.

For staining of endogenous EVI1, native NTERA-2 cells were used. 300 000 cells were seeded per well of a 6-well plate on autoclaved coverslips, 18h later 10 μ M ATRA or an equal volume of DMSO was added and incubation continued for 72 h.

Staining of the neuronal marker protein β III tubulin was carried out with neuronally differentiated NTERA-2 cells on day 24, which had been plated on coverslips in 6-well plates 5 days earlier on day 19.

2.6.2 Immunofluorescence

All steps were carried out at room temperature and, if not indicated otherwise, with 3 ml of the indicated solution or buffer per well of a 6-well-plate.

Cells grown on coverslips were washed with 1x PBS. For staining with the mouse-anti-HA 16B12 antibody cells were fixed with ice cold methanol for 3 min. Methanol was removed with 3 washes with 1x PBS. For staining with the rabbit-anti-EVI1- or mouse-anti- β III tubulin-antibody cells were fixed with 3% paraformaldehyde in 1x PBS followed by 3 washes with 1x PBS. In either case, after fixation cells were permeabilized with 1x PBS containing 1% Triton X-100 for 1 min and washed 3 times with 1x PBS. Preblocking of unspecific binding sites was carried out with 1 ml 5% goat serum in 1x PBS for 45 min per well.

Primary antibodies were freshly diluted in blocking solution:

- mouse anti-HA.11 clone 16B12 at 1:200
- rabbit anti-EVI1 C50E12 at 1:200
- mouse anti- β III tubulin clone TU-20 at 1:100

100 μ l of diluted antibody per coverslip were pipetted onto a piece of parafilm placed into a 150 mm petri dish. Coverslips were placed on the parafilm with the cell side facing the diluted antibody solution in a manner that bubbles did not get trapped. Wetted tissues were placed at the sides of the petri dish to create a humidified chamber. The petri dish was closed and incubated for 1 h. Following that, the coverslips were returned to the 6-well plate - the cell side facing upwards - and unbound antibodies were removed by 3 washes with 1x PBS.

Secondary antibodies were freshly diluted in blocking solution:

- FITC-conjugated goat-anti-mouse at 1:200 and
- Alexa 568 conjugated goat-anti-rabbit at 1:750
- Alexa 568 conjugated goat-anti-mouse at 1:750

100 μ l of diluted antibody per coverslip were pipetted onto a piece of parafilm placed into a 150 mm petri dish. Coverslips were placed on the parafilm with the cell side facing the diluted antibody solution in a manner that bubbles did not get trapped. Wetted tissues were placed at the sides of the petri dish to create a humidified chamber. The petri dish was closed and incubated for 1 h under subdued light conditions. Following that, the coverslips were returned to the 6-well plate - the cell side facing upwards - and unbound antibodies were removed by 3 washes with 1x PBS.

Nuclei were counterstained with 1 ml of 2 μ g/ml 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) in 1x PBS for 5 min, followed by a final wash with 1x PBS. Coverslips were mounted with Vectashield onto glas slides. Slides were examined under the fluorescence microscope using filtersets 01 to visualize DAPI staining, filterset 13 to visualize fluorescein staining and filterset 43 to visualize Alexa 568 staining. Images were recorded with a magnification of 400x.

10x PBS: see chapter 2.1.1

Paraformaldehyde, 95%

Sigma-Aldrich

Triton X-100, for molecular biology

Sigma-Aldrich

Methanol, Ph Eur,BP,NF

Merck

Primary antibodies:

mouse anti-HA.11 clone 16B12

Covance

rabbit anti-EVI1 C50E12

Cell Signaling Technology

mouse anti β III tubulin clone TU-20

Chemicon

Secondary antibodies

Fluorescein-conjugated Goat-anti-Mouse IgG (H+L)	Jackson ImmunoResearch
Alexa 568-conjugated goat-anti-rabbit IgG	Invitrogen
Alexa 568-conjugated goat-anti-mouse IgG	Invitrogen
Goat serum	Jackson ImmunoResearch
DAPI	Roche
VectaShield mounting medium	Vecta Laboratories
Glass slides, coverslips	Marienfeld
Microscope	Axioplan 2 (Zeiss)
Filtersets 01, 13, 43	Zeiss

2.6.2.1 Immunoblotting

2.6.3 Preparation of cell lysates

For expression analysis of engineered *EVI1* deletion constructs and full length exogenously expressed *EVI1*, NTERA-2 cells were transiently transfected with the respective constructs using the calcium phosphate method in 6-well plate format as described in chapter 2.1.3.3.

For analysis of endogenously expressed *EVI1*, native NTERA-2 cells grown in 100 mm dishes were used. 1×10^6 cells were seeded per dish and left to adhere for 18-20 h. ATRA was added to a final concentration of 10 μ M, an equal volume of DMSO was added to the cells serving as negative control.

NTERA-2 cells were detached by trypsin treatment as described (2.1.1) and resuspended in growth medium containing serum to neutralize trypsin. All subsequent steps were carried out on ice and with ice cold reagents. Cells were pelleted by centrifugation and washed with 1x PBS. Cells were then lysed by adding 20 μ l Hunt buffer for cells harvested from one well of a 6-well plate and 100 μ l Hunt buffer for cells harvested from a 100 mm dish. Resuspended cells were subjected to 2 freeze-thawing cycles in liquid nitrogen and 37°C for 2 min each. Insoluble cell debris was pelleted by a centrifugation for 10 min at 13000 rpm and 4°C in a table top centrifuge, and supernatant was transferred to a fresh tube. Lysates were stored at -70°C.

Hunt buffer

20 mM Tris/HCl pH 8,0

100 mM NaCl

1 mM EDTA

0.5% NP-40

0,5 mM phenylmethanesulfonyl fluoride (PMSF) and complete protease inhibitor cocktail were added freshly before use

10x PBS: see chapter 2.1.1

stock solution: 200 mM PMSF in ethanol

NaCl, ACS reagent, ≥99.0%	Sigma-Aldrich
NP-40	Sigma-Aldrich
PMSF, ≥98.5% (GC)	Sigma-Aldrich
Trizma® base Biotechnology Performance Certified, ≥99.9%	Sigma-Aldrich
KCl, analysis grade	Merck
complete protease inhibitor cocktail, 1 tablet per 50 ml solution	Roche
Table top centrifuge 5415R	Eppendorf

2.6.4 Bradford Assay

In order to enable loading of equal of amounts of protein onto a polyacrylamide gel, a Bradford assay was carried out to measure relative protein content of protein lysates. The binding of the protein to the coomassie dye results in a spectral shift from the reddish/brown form to the blue form of the dye. The optimal wavelength to measure the blue color from the coomassie dye-protein complex is at 595 nm.

1 µl of protein lysate and 1 ml Bradford reagent were mixed in a disposable plastic cuvette and OD₅₉₅ was determined using a spectral photometer.

The sample which had the lowest OD₅₉₅ value was used as basis for normalization, i.e. a loading volume of 8 µl was designated to this sample, and the loading volumes of the other samples were calculated according to their OD₅₉₅ value.

Coomassie (Bradford) Protein Assay Kit	Pierce
disposable plastic cuvette	Sterilin
Hitachi U-2000 spectral photometer	Hitachi

2.6.5 Denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated according to their molecular weight by denaturing SDS-PAGE. Gels were fabricated by preparing 10 ml of a resolving gel containing 6-10% acrylamide, 380 mM Tris pH 8.8 and 1% SDS. Polymerisation was induced by adding 1% ammonium persulfate and 0,8% *N,N,N',N'*-Tetramethylethylenediamine (TEMED). The resolving gel was cast between two glass plates cleaned with 70% ethanol and separated by 1,5 mm spacers, overlaid with 2-propanol and left to polymerise for 20 to 30 min. After polymerisation, 2-propanol was decanted and residues carefully removed with a Whatman filter paper without

disturbing the gel. The resolving gel constituted approximately 80% of the total gel volume, while the stacking gel constituted the rest.

The stacking gel contained 5% acrylamide, 125 mM Tris pH 6.8 and 1‰ SDS in a total volume of 3 ml. Polymerisation was induced as described for the resolving gel with 1‰ ammonium persulfate and 1‰ TEMED. The stacking gel was cast on top of the resolving gel, a comb was placed between the two glass plates to leave slots for loading, and the gel left to polymerize for 20 to 30 min. After polymerisation, the polyacrylamide gel was placed inside the electrophoresis apparatus filled with Tris-glycine electrophoresis buffer and loading slots were rinsed with Tris-glycine electrophoresis buffer. If not used immediately, gels were wrapped with damp tissue and stored in a plastic bag at 4°C for up to one week.

A maximum of 8 µl of protein lysates was mixed with 2x SDS gel loading buffer and denatured at 95°C for 3 min. Negatively charged SDS binds to proteins, which subsequently form negatively charged protein-SDS complexes. The now uniformly charged proteins therefore travel along the same direction – from cathode to anode - in an electric field.

6 µl of PageRuler™ Prestained Protein Ladder molecular weight standard and denatured samples were loaded onto the polyacrylamide gel and a constant voltage of 120 V was applied for approximately 90 min until the bromphenol blue dye from the loading buffer had run out of the gel. Negatively charged and denatured protein travelled through the gel matrix towards the anode. While traveling the proteins were separated by size.

Gel components of a 6% polyacrylamide resolving gel (total volume of 10 ml)

Solution component	Volume (ml)
40% Acrylamide/Bis-acrylamide 29:1	1,5
1,5 M Tris/HCl pH 8,8	2,5
10% SDS	0,1
H ₂ O	5,8
10% ammonium persulfate	0,1
TEMED	0,008

Gel components of a 10% polyacrylamide resolving gel (total volume of 10 ml)

Solution component	Volume (ml)
40% Acrylamide/Bis-acrylamide 29:1	2,5
1,5 M Tris/HCl pH 8,8	2,5
10% SDS	0,1
H ₂ O	4,8
10% ammonium persulfate	0,1
TEMED	0,008

Gel components of a 5% polyacrylamide stacking gel (total volume of 3 ml)

Solution component	Volume (ml)
40% Acrylamide/Bis-acrylamide 29:1	0,375
1,0 M Tris/HCl pH 6,8	0,380
10% SDS	0,03
H ₂ O	2,2
10% ammonium persulfate	0,03
TEMED	0,003

PageRuler™ Prestained Protein Ladder: blue protein bands at 170, 130, 100, 55, 40, 35, 25, and 15 kD, red reference band at 70 kD.

2x SDS gel loading buffer:

100 mM Tris/HCl pH 6,8
 200 mM DTT
 4% SDS
 0,2% bromphenol blue
 20% glycerol
 Stored in 500 µl aliquots at -20°C

Tris-Glycine electrophoresis buffer

25 mM Tris
 250 mM glycine
 0,1% SDS

Stock solutions (in H₂O)

1,5 M Tris/HCl pH 8,8
 1,0 M Tris/HCl pH 6,8
 10% SDS
 40% Acrylamide/Bis-acrylamide 29:1
 10% ammonium persulfate

Trizma® base Biotechnology Performance Certified, ≥99.9%
 DTT *BioUltra*, for molecular biology, ≥99.5%
 SDS (Sodium *n*-Dodecyl Sulfate), molecular Biology Grade
 Bromphenol Blue
 Glycerol, for molecular biology, ≥99%
 Glycine, *ReagentPlus*®, ≥99%
 TEMED, analysis grade
 Ammonium persulfate, for molecular biology, powder, ≥98%
 40% Acrylamide/Bis-acrylamide 29:1

Sigma-Aldrich
 Sigma-Aldrich
 Calbiochem
 Sigma-Aldrich
 Sigma-Aldrich
 Sigma-Aldrich
 Merck
 Sigma-Aldrich
 Sigma-Aldrich

2-Propanol, ACS reagent, ≥99.5%
PageRuler™ Prestained Protein Ladder
PowerPac HC Power Supply
Mini-PROTEAN Electrophoresis System

Sigma-Aldrich
Fermentas
Bio-Rad
Bio-Rad

2.6.6 Blotting and detection

After completing the electrophoresis, the polyacrylamide gel was taken out of the apparatus and the stacking gel was separated from the resolving gel and discarded.

The resolving gel was placed upon a Whatman 3 MM filter wetted with transfer buffer. The filter paper with the resolving gel on top was placed on a fibre pad (part of the blotting apparatus) also wetted in transfer buffer. A piece of nitrocellulose membrane was cut to the same size as the resolving gel, wetted with transfer buffer and placed on the resolving gel in a manner that bubbles were not trapped between resolving gel and membrane. Another piece of Whatman 3 MM filter paper wetted with transfer buffer was placed upon the nitrocellulose membrane and bubbles were removed by smoothing over the Whatman 3 MM filter paper with a gel releaser. A wetted fiber pad was placed on top and bubbles removed in the same manner. The stack was enclosed in the gel holder cassette, which was submerged in transfer buffer in the blotting apparatus, with the nitrocellulose membrane facing the anode and the resolving gel facing the cathode. In order to absorb the heat generated during the blotting process, an ice-filled cooling unit was placed inside the blotting chamber. Proteins were transferred to the nitrocellulose membrane by applying a constant voltage of 100 V for one hour. After completion of the protein transfer, the nitrocellulose membrane containing the separated proteins, was removed from the apparatus and air-dried. If not used promptly, the dry nitrocellulose blot was placed between 2 sheets of Whatman 3 MM filter paper and stored in a plastic bag at room temperature.

If required, the nitrocellulose membrane was cut into an upper and a lower part to enable detection of two different proteins, e.g. EVI1 at 140 kD and β -Tubulin at 55 kD. After complete drying, the nitrocellulose membrane was shortly submerged in PBS-T and then incubated in 40 ml / 64 cm² blocking solution for 30 min at gentle rocking to block unspecific binding sites. Primary antibodies were freshly diluted in blocking solution:

- mouse anti-HA.11 clone 16B12 at 1:10 000
- rabbit anti-EVI1 C50E12 at 1:1 000
- mouse anti- β -Tubulin clone TUB 2.1 at 1:2 500

2 ml of diluted antibody in blocking solution per 64 cm² nitrocellulose membrane were added dropwise onto a piece of parafilm placed into a 150 mm petri dish. Excess blocking solution was shaken off the blot, which was placed onto the parafilm with the protein side facing the diluted antibody solution in a manner that bubbles did not get trapped. Wetted tissues were

placed at the sides of the petri dish to create a humidified chamber. The petri dish was closed and incubated overnight at 4°C.

Used antibody solution was discarded. Unbound antibody was removed by 3 washes with 1x PBS-T for 5 min each at room temperature on a rocking platform. Horse radish peroxidase (HRP) conjugated secondary antibody was freshly diluted in blocking solution:

- HRP - goat anti-mouse at 1:50 000
- HRP - goat anti-rabbit at 1:50 000

2 ml of diluted antibody in blocking solution per 64 cm² nitrocellulose membrane were added dropwise onto a piece of parafilm placed into a 100 mm petri dish. The nitrocellulose membrane was placed onto the parafilm with the protein side facing the diluted antibody solution in a manner that bubbles did not get trapped. Wetted tissues were placed at the sides of the petri dish to create a humidified chamber. The petri dish was closed and incubated at room temperature for 1-2 h. Used antibody solution was discarded. Unbound antibody was removed by 3 washes with 1x PBS-T for 5 min each at room temperature on a rocking platform. 2 ml Super Signal West Femto substrate for HRP per 64 cm² nitrocellulose membrane were placed on plastic wrap. After the final wash excess 1x PBS-T was shaken off the nitrocellulose membrane, which was then placed on the prepared plastic wrap with the protein side facing the Super Signal West Femto substrate for 3 min. Excess substrate was shaken off and the nitrocellulose membrane was wrapped in fresh plastic wrap and put into an exposition cassette. In the dark, X-ray films were exposed to the blot for various times ranging from 5 sec to 15 min. After exposition, X-ray films were submerged in developer solution for 5 min, then shortly rinsed with H₂O, and then submerged in fixer solution for 5 min. After a final wash in H₂O X-ray the films were air dried.

Transfer buffer

39 mM glycine

120 mM Tris

10x PBS: see chapter 2.1.1

PBS-T:

0,1% TWEEN-20 in 1x PBS

Blocking solution:

5% non-fat dried milk in PBS-T

Glycine, *ReagentPlus*[®], ≥99%

Sigma-Aldrich

Trizma[®] base Biotechnology Performance Certified, ≥99.9%

Sigma-Aldrich

TWEEN-20, for molecular biology

Sigma-Aldrich

Non-fat dried milk powder "fixmilch instant"

regular supermarket

Bio Trace NT nitrocellulose blotting membrane

Pall

Primary antibodies:

mouse anti-HA.11 clone 16B12	Covance
rabbit anti-EVI1 C50E12	Cell Signaling Technology
mouse anti- β -Tubulin clone TUB 2.1	Sigma-Aldrich
Secondary antibodies:	
goat anti-mouse HRP	Jackson ImmunoResearch
goat anti-rabbit-HRP	Jackson ImmunoResearch
Super Signal West Femto kit	Pierce
X-ray film CL-XPosure Film	Pierce
Dental X-ray fixer and developer solution	Kodak
Mini Trans-Blot Cell	Bio-Rad
PowerPac HC Power Supply	Bio-Rad

2.7 Chromatin immunoprecipitation (ChIP) of NTERA-2 cells

To analyze the interaction between endogenous proteins and chromatin, native NTERA-2 cells were used for ChIP assays. 2 d prior to the intended start of the ChIP experiment, 8×10^6 cells per 150 mm plate were seeded in 20 ml growth medium and left to settle for one day. To analyze the interaction between protein products derived from exogenously expressed HA tagged full length *EVI1*, NTERA-2 cells grown in 150 mm dishes were transiently transfected with the respective plasmids using the calcium phosphate method for NTERA-2 cells as described in chapter 2.1.3.3. 24 h before the start of the ChIP experiment native or transiently transfected cells were treated with 10 μ M ATRA or an equal volume of the solvent DMSO as negative control as described in chapter 2.1.2.

In order to cross-link proteins to DNA, 37% formaldehyde was added to a final concentration of 1% directly to the medium in which the cells had grown in a fume hood. The cover of the plate was put back on, the plate shaken gently and incubated for 10 min at 37°C in a non-humidified incubator. Formaldehyde was quenched by addition of 1 M glycine to a final concentration of 125 mM, the plate was shaken gently and incubated for 5 min at room temperature. The cells were washed twice with 10 ml ice cold 1x PBS containing complete protease inhibitor cocktail and scraped off the dishes with a cell scraper. Loose cells were collected in 10 ml ice cold 1x PBS containing complete protease inhibitor cocktail and transferred to a 15 ml tube. Cells were pelleted by centrifugation at 2000 rpm for 5 min at 4°C, and the cell pellet was resuspended in 1 ml ChIP lysis buffer at room temperature to avoid SDS precipitation of the lysis buffer.

Chromatin was sheared directly after the addition of lysis buffer with 8 cycles of 15 s each at 90% duty cycle with the power output scale on 5 using a 3 mm microprobe in a 15 ml tube. Between shearing cycles samples were placed on ice for 2 to 3 min to prevent overheating. In case of foaming, the sonication of the respective sample was immediately interrupted and

the sample was placed on ice to cool down. Sonication was continued then under the same conditions but with the power output scale reduced to 4 or 3. Sonicated chromatin was transferred to 1,5 ml cups and centrifuged at 13000 rpm for 10 min at 10 °C in a table top centrifuge. The supernatant containing the sheared chromatin was transferred to a fresh cup. For normalization, an aliquot of the chromatin was diluted 1:100 in H₂O and OD₂₈₀ was determined using a spectral photometer. For an OD₂₈₀ value of 0,1 100 µl of chromatin were used per ChIP experiment, e.g. for an OD₂₈₀ value of for instance 0,089 112 µl of chromatin were transferred to a fresh 1,5 ml cup. Thus all samples were normalized based on their OD₂₈₀ value. Ice cold 1x ChIP buffer supplied with the OneDay ChIP kit was added in an amount that the chromatin was diluted ten-fold, e.g. 1008 µl of 1x ChIP buffer were added to 112 µl of chromatin to give a total amount of 1120 µl. 8 µg of specific antibody or unspecific immunoglobulins from the same species the specific antibody was generated in were added to the diluted chromatin and agitated overnight at 4°C. Sheared chromatin which was not used immediately was stored at -20°C. After long-term storage for approximately 6 months it was observed that some samples became very viscous which precluded pipetting them. Those samples were not used for further ChIP experiments.

Further processing of the samples was performed using the OneDay ChIP kit according to the manufacturer's instructions. 40 µl protein A beads supplied with the OneDay ChIP kit per sample were washed prior to use with 1 ml ice cold 1x ChIP buffer, the tube was inverted twice and the beads were centrifuged for 2 min at 3000 rpm at 4°C in a table top centrifuge. The supernatant was discarded and the washing procedure was repeated once. Washed beads were kept on ice until further use. Overnight immunoselected antibody-chromatin mix was centrifuged for 10 min at 13000 rpm and 4°C in a table top centrifuge and the supernatant was transferred to the washed protein A beads which were agitated for 1 h at 4°C. Precipitated immune complexes bound to protein A beads were harvested by centrifugation for 2 min at 3000 rpm and 4°C in a table top centrifuge, and the supernatant was discarded. Unspecifically bound material was removed by 3 washes with ice cold 1x ChIP buffer. For the first wash, 1 ml 1x ChIP buffer was added to the beads, the tube was inverted twice and the beads were centrifuged for 2 min at 3000 rpm at 4°C in a table top centrifuge. The supernatant was discarded. For the second wash, the beads were resuspended in 1 ml 1x ChIP buffer and then transferred to 15 ml tubes into which 10 ml 1x ChIP buffer had been provided. The beads were pipetted carefully on top of the 10 ml 1x ChIP buffer and then left to sink down to the bottom of the tube during a 5 min incubation on ice. The beads were then centrifuged for 2 min at 1000 rpm and 4°C and the supernatant was discarded. For the third wash, the beads were resuspended in 1 ml 1x ChIP buffer and transferred to 1,5 ml screw cap tubes. The beads were centrifuged for for 2 min at 3000 rpm

and 4°C in a table top centrifuge, and the supernatant was discarded. From this point the procedure was continued at room temperature and barrier pipette tips were used.

To reverse protein-DNA cross-linking and to recover DNA from the beads, 100 µl DNA purifying slurry provided in the OneDay ChIP kit were added to the beads, the screw cap tubes were closed tightly to prevent bursting open during boiling in a waterbath for 10 min. The samples were taken out of the boiling waterbath and cooled to room temperature. 1 µl of proteinase K provided in the OneDay ChIP kit was added, followed by an incubation at 55°C for 30 min with vigorous agitation in a thermomixer. After another boiling step in a waterbath for 10 min the samples were centrifuged at 13000 rpm for 1 min at room temperature and 70 µl of de-cross-linked DNA were recovered and transferred to a fresh reaction tube. 130 µl H₂O provided in the OneDay ChIP kit were added to the DNA purifying slurry, the samples were vortexed and centrifuged at 13000 rpm for 1 min at room temperature. 130 µl supernatant were combined with the first eluate to give a total volume of 200 µl. Recovered DNA was stored at -20°C.

The antibodies successfully used in ChIP were rabbit-anti-RXR (sc-774) for detection of RXR α , RXR β , RXR γ , which also worked in immunoblotting, and rabbit-anti-RAR α (sc-551), which was not tested in immunoblotting. Antibodies unsuccessfully used in ChIP but which worked in immunoblotting were rabbit-anti-N-CoR (sc-8994) and rabbit-anti-CBP (sc-369). Further antibodies unsuccessfully used both in ChIP and immunoblotting were rabbit-anti-RAR (sc-773) for detection of RAR α , RAR β , RAR γ , rabbit-anti-RXR α (sc-553), rabbit-anti-NcCoR (ab24552), rabbit-anti-CBP (ab2832) and mouse-anti- RNA Polymerase II clone CTD4H8. Mouse-anti-HA.11 clone 16B12 was used successfully in immunoblotting but due to the lack of an appropriate positive control for ChIP it is not clear whether the negative ChIP results obtained with this antibody are to be accounted to incapability for ChIP or actual non-occupancy of the antigen to the investigated DNA region.

2.7.1 Recovery of the input sample

An aliquot of sheared chromatin not undergoing the immunoprecipitation process was recovered as “input” sample. Per input sample the same amount of chromatin was used as in the corresponding sample which underwent the immunoprecipitation process. Chromatin cross-linking in this sample was reversed by adding 300 µl of ChIP elution buffer and incubation at 65°C overnight. Following this, the protein was digested by adding 4 µl of 10 mg/ml proteinase K and an incubation at 55°C for 1 hour. Subsequently samples were cooled to room temperature.

DNA was recovered by phenol/chloroform extraction and ethanol precipitation using glycogen as a carrier. 600 µl Phenol:Chloroform:Isoamyl Alcohol 25:24:1 were added to the de-cross-linked chromatin, the mixture was vortexed for 5 sec and then centrifuged at

13000 rpm for 10 min at room temperature. Approximately 400 µl of the aqueous phase was transferred to a fresh cup and 800 µl of 96% ethanol, 12 µl of 3 M sodium acetate and 1 µl of 20 mg/ml glycogen as carrier were added. After vortexing for 5 sec DNA was pelleted by centrifugation at 13000 rpm for 20 min at 4°C. The supernatant was discarded, the pellet was washed with 1 ml 70% ethanol and centrifuged at 13000 rpm for 5 min at 4°C. The supernatant was discarded, the pellet air dried and the DNA was dissolved in 200 µl H₂O. Recovered DNA was stored at -20°C.

To check proper shearing of chromatin, 10 µl of input DNA were subjected to agarose gel electrophoresis (see chapter 2.3). Fragment sizes usually showed a DNA smear ranging in size from 100 bp to 2 kb, with a maximal intensity at 500 bp.

2.7.2 ChIP PCR

Immunoprecipitated DNA and decrosslinked ("input") DNA was used as template for PCR with primers flanking

- the *EVI1* DR5 RARE CGACCTTTTGTGACCT between position +182 and +198 relative to the beginning of *EVI1* exon 1a
- the *EVI1* DR1 RARE AGGTGATGTGAAC between position +624 and +636, relative to the beginning of *EVI1* exon 1a
- several sites in the putative *MDS1* promoter region at -1,5 kb, -0,7 kb and +0,1 kb relative to the transcription start site
- the *RARβ* RARE as positive control
- *EVI1* exon 3L as negative control

5 µl of chromatin were used per 20 µl PCR reaction with reagents and conditions equal to those described in chapter 2.4.4. Primers for ChIP-PCR with sequence, annealing temperatures, location and accession numbers are listed in the appendix in table 6.4. 10 µl of the obtained PCR products were analyzed by agarose gel electrophoresis (see chapter 2.3).

-

ChIP lysis buffer.

1% SDS
10 mM EDTA
50 mM Tris pH 8,1
complete protease inhibitor cocktail
freshly prepared

ChIP elution buffer:

2% SDS
100 mM NaHCO₃
10 mM DTT
200 mM NaCl
Freshly prepared

1 M glycine

Formaldehyde, for molecular biology, 36.5-38% in H ₂ O	Sigma-Aldrich
96% Ethanol, Ph Eur, BP	Merck
Glycine, <i>ReagentPlus</i> [®] , ≥99%	Sigma-Aldrich
SDS, molecular Biology Grade	Calbiochem
EDTA for molecular biology, 99.0-101.0%	Sigma-Aldrich
Trizma [®] base Biotechnology Performance Certified, ≥99.9%	Sigma-Aldrich
NaHCO ₃ , analysis grade	Sigma-Aldrich
DTT, <i>BioUltra</i> , for molecular biology, ≥99.5%	Sigma-Aldrich
Proteinase K (10mg/ml in H ₂ O), for molecular biology	Sigma-Aldrich
Glycogen 20 mg/ml, RNA grade	Fermentas
Phenol:Chloroform:Isoamyl Alcohol 25:24:1	Sigma-Aldrich
complete protease inhibitor cocktail, 1 tablet per 50 ml solution	Roche
Antibodies:	
rabbit-anti-RXR (sc-774) for detection of RXR α , RXR β , RXR γ	Santa Cruz
rabbit-anti-RAR α (sc-551)	Santa Cruz
mouse anti-HA.11 clone 16B12	Covance
unspecific rabbit and mouse IgG	Santa Cruz
OneDay ChIP kit	Diagenode
Cell scraper	Iwaki
Branson Sonifier S-450	Branson
Centrifuge Megafue 2.2R	Heraeus
Table top centrifuge 5415R	Eppendorf
Thermomixer comfort	Eppendorf

3 Results

3.1 Neuronal differentiation of NTERA-2 cells

The disruption of *Evi1* causes severe malformations in the central nervous system (CNS) of mouse embryos (Hoyt PR, 1997). Furthermore, *Evi1* orthologs play a role in neuronal differentiation in other species like *Xenopus* (Garriga G, 1993) and *Drosophila* (Moore, et al., 2002). The importance of *Evi1* for neuronal development was further corroborated with the finding that *Evi1* was upregulated during the all-*trans* retinoic acid (ATRA) induced neuronal differentiation of the murine embryonal carcinoma cell line P19, and that overexpression of *Evi1* was sufficient to produce a neuronally differentiated phenotype and the expression of neuronal marker proteins in this cell line (Kazama, et al., 1999). ATRA is to date the only known positive regulator of *EV11* (Russell, et al., 1994; Xi ZF, 1997).

Induction of *EV11* mRNA 5' end variants *EV11_1a* and *EV11_1b* – independently of a neuronal differentiation procedure - had already been reported in the human teratocarcinoma cell line PA1. However, this cell line is very difficult to transfect, which impedes further experiments, especially reporter gene experiments (Aytekin, et al., 2005). Therefore, the influence of ATRA on the expression of *EV11* was to be confirmed in another similar cell line, which is easier to transfect, i.e. in the human teratocarcinoma cell line NTERA-2. Furthermore, and like described for a variety of other human and mouse teratocarcinoma cell lines (P. J. McCaffery, 2003), NTERA-2 cells have the ability to undergo neuronal differentiation through ATRA treatment (Leyboldt, et al., 2002; Megiorni, et al., 2005).

These findings prompted me to use the ATRA mediated neuronal differentiation of NTERA-2 cells as an *in vitro* model system to study the expression of *EV11* and *MDS1/EV11* during this process.

3.1.1 Morphology of neuronally differentiated NTERA-2 cells

The neuronal differentiation procedure of NTERA-2 cells comprises two phases. During the first phase, cells are plated in high density on bacterial grade tissue culture dishes, which prompts them to form aggregates and impedes proliferation. From day 2 to day 19 the cell aggregates are treated with 10 μ M ATRA with regular changes of medium and dishes. The second phase starts on day 19 with the plating of the cell aggregates on poly-D-lysine and laminin coated dishes and in medium containing the mitosis inhibitors Cytosine β -D Arabinofuranoside (AraC) and uridine (Leyboldt, et al., 2002; Megiorni, et al., 2005). During this phase cells develop characteristic neuron-like morphology. By phase contrast microscopic inspection (fig 3.1), neuronally differentiated NTERA-2 cells show neurite like and branched structures protruding from cell aggregates in contrast to the epithelial appearance of undifferentiated cells.

The experiment was carried out three times with similar observations.

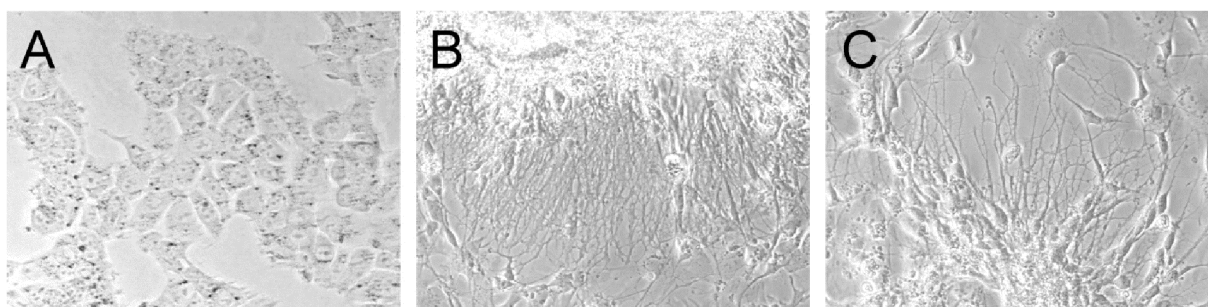


Fig. 3.1 Phase contrast microscopy of (A) undifferentiated and (B, C) neuronally differentiated NTERA-2 cells on day 23, i.e. 4 days after plating onto poly-D-lysine and laminin coated dishes at a magnification of 100x.

3.1.2 Neuronally differentiated NTERA-2 cells express the neuronal marker protein β III tubulin

In order to verify the neuronal phenotype also on a molecular basis, immunofluorescence was carried out with neuronally differentiated NTERA-2 cells (prepared as described in chapter 3.1.1) on day 24, i.e. 5 days after plating onto poly-D-lysine and laminin coated coverslips. Cells were stained with an antibody against the neuronal marker β III tubulin and an Alexa 568 conjugated secondary antibody.

While β III tubulin is only weakly expressed in undifferentiated NTERA-2 cells, it is markedly expressed in neuronally differentiated cells (fig 3.2). Additionally, this staining brought further evidence that the neurite like structures form extensive networks, like observed in phase contrast microscopy (fig. 3.1).

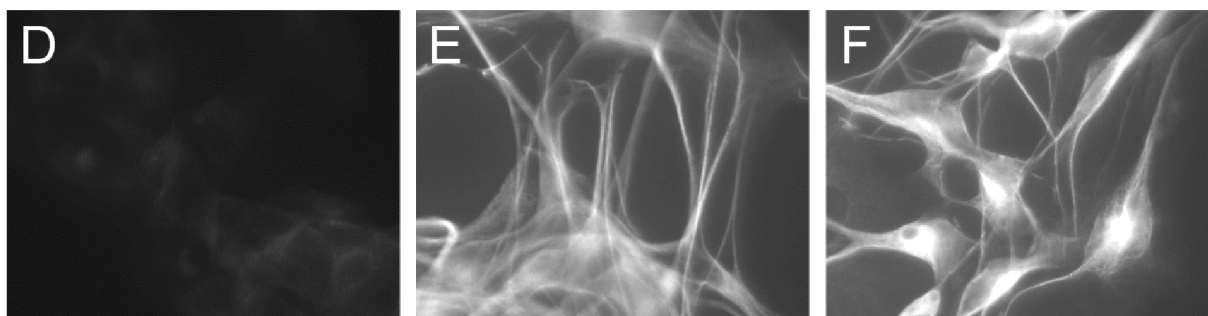


Fig. 3.2 Immunofluorescence of (D) undifferentiated and (E, F) neuronally differentiated NTERA-2 cells on day 24, i.e. 5 days after plating on poly-D-lysine and laminin coated coverslips using a β III tubulin primary antibody and an Alexa 568 conjugated secondary antibody. Images were recorded at a magnification of 400 x.

The neuronal differentiation experiment was carried out altogether three times, immunofluorescence of the neuronal marker β III tubulin was carried out in two of them. The results of the other immunofluorescence experiment were similar to the results shown in fig. 3.2).

3.1.3 Neuronally differentiated NTERA-2 cells express the neuronal marker genes *GluR*, *NeuroD* and *Tau*

In order to evaluate the expression of neuronal markers to further verify proper neuronal differentiation, total RNA was extracted on days 0, 1, 2, 4, 7, 10, 13, 16, 19, 20, 22, 24, 26 and 28 from NTERA-2 cells subjected to the neuronal differentiation procedure as described in chapter 3.1.1. The RNA was reverse transcribed and the obtained cDNA used as template for semiquantitative PCR with primers (table 6.1) specific for a number of well characterized neuronal markers, e.g. Glutamate receptor (*GluR*), Neuronal differentiation (*NeuroD*) and *Tau*. *GluR* is a channel for glutamate, the most common mammalian neurotransmitter. *NeuroD* belongs to the neural basic helix-loop-helix (bHLH) transcription factors, which are sequentially expressed during the development of the nervous system and which ensure regular neurogenesis. *Tau* is part of the cytoskeleton of neurons and has an essential role in the outgrowth of axons and dendrites. (Megiorni, et al., 2005). Amplification of *cyclophilinD* served as control for the amount of cDNA in each sample.

As expected, all of the investigated neuronal markers showed pronounced upregulation, albeit at different stages (fig. 3.3). *GluR* was abruptly upregulated during the ATRA-treatment phase between days 4 and 7 and apart from a slight drop on day 16 mRNA levels continued to rise until the end of the neuronal differentiation phase. *NeuroD* was the earliest to be upregulated; already on day 2, i.e. one day after the start of ATRA treatment, mRNA levels increased and remained high throughout the whole ATRA treatment phase. With the beginning of the differentiation phase, mRNA levels decreased slightly but then rose again until the end of the differentiation phase. *Tau* mRNA showed basal expression and was upregulated on day 7, i.e. 6 days after the start of ATRA treatment, with slow kinetics and remained elevated until the end of the neuronal differentiation phase.

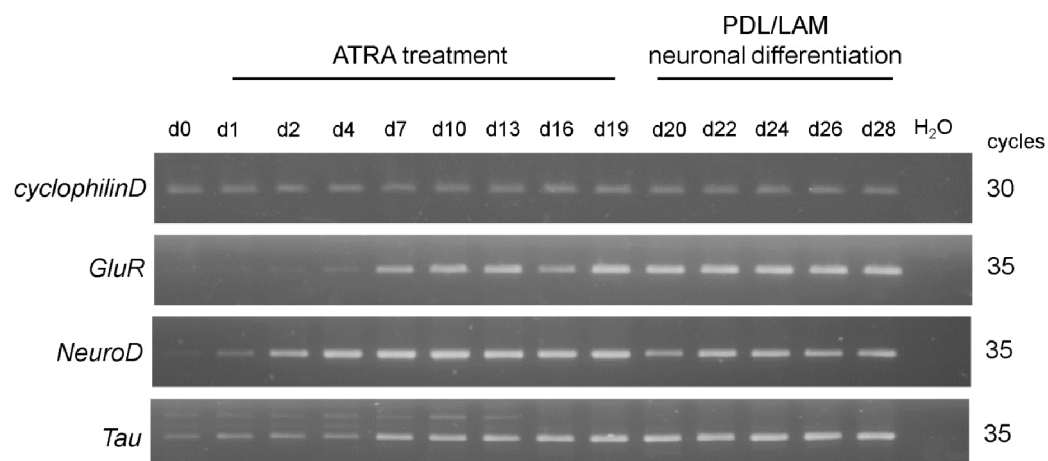


Fig. 3.3 Analysis of expression of neuronal markers *GluR*, *NeuroD* and *Tau* during neuronal differentiation of NTERA-2 cells assessed by semiquantitative PCR. The amplification of *cyclophilinD* served as control for the amount of cDNA in each sample. The number of PCR cycles is indicated on the right, time points and

experimental phase is indicated on top. PDL/LAM denote poly-D-lysine and laminin, the substrates on which cell aggregates are plated at the beginning of the differentiation phase.

According to Megiorni et al upregulation of *GluR* was described to set in on day 5, upregulation of *NeuroD* on day 2 and upregulation of *Tau* between days 5 and 10 (Megiorni, et al., 2005). The experimental findings largely correspond to the described findings.

The experiment was carried out altogether three times, twice with the described time points for RNA extraction, and once with time points day 0, 1, 7, 14, 21, 23, 26 and 28.

In the experiment with the above described number of time points kinetic of *GluR* regulation was comparable to the experiment shown in fig. 3.3, except that there was no slight drop on day 16 but on day 28. Kinetics of *NeuroD* and *Tau* regulation were similar to the shown experiment. In the experiment with reduced number of investigated time points *NeuroD* expression was at a maximum on day 7 and then gradually decreased until day 28. *GluR* levels rised abruptly between days 1 and 7 and remained high until day 28. *Tau* levels began to rise on day 1 and gradually increased until day 28. Despite minor variations regarding some details the results were largely reproducible between repeated experiments.

3.1.4 Expression of cEVI and MDS1/EVI1 during neuronal differentiation of NTERA-2 cells

In order to monitor the expression of cEVI1, a region common to all known *EVI1* mRNA 5' end variants, and MDS1/EVI1 during the course of neuronal differentiation, real time quantitative reverse transcriptase PCR (RTQ-RT-PCR) was carried out using the same cDNAs as described in chapter 3.1.3. Primers used to amplify cEVI, a region common to all known *EVI1* mRNA 5' end variants, and MDS1/EVI1, were designed to be suitable for RTQ-RT-PCR (table 6.2) and SYBR Green technology. Relative expression values and standard deviations were calculated according to the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), using *cyclophilinD* as control for the amount of cDNA in each sample, and the 0 h time point as reference sample.

cEVI1 levels began to rise with the start of ATRA treatment. A decrease set in between day 4 and day 7, which continued until day 19 (fig 3.4). At the onset of the actual neuronal differentiation, i.e. on day 20, which is one day after plating the aggregated cells onto poly-D-lysine and laminine substrate, cEVI1 levels started to rise again until day 28. The experiment could usually not be continued further since massive cell death set in beyond this time point. MDS1/EVI1 was upregulated on day 4 of ATRA treatment, and altogether considerably slower and to a lesser extent compared to cEVI1. A plateau was reached between day 7 and day 16, then a decline set in which continued during the neuronal differentiation phase until day 28. Remarkably, during the neuronal differentiation phase cEVI1 and MDS1/EVI1

behaved conversely, with cEVI1 being upregulated and MDS1/EVI1 being downregulated even below the starting level.

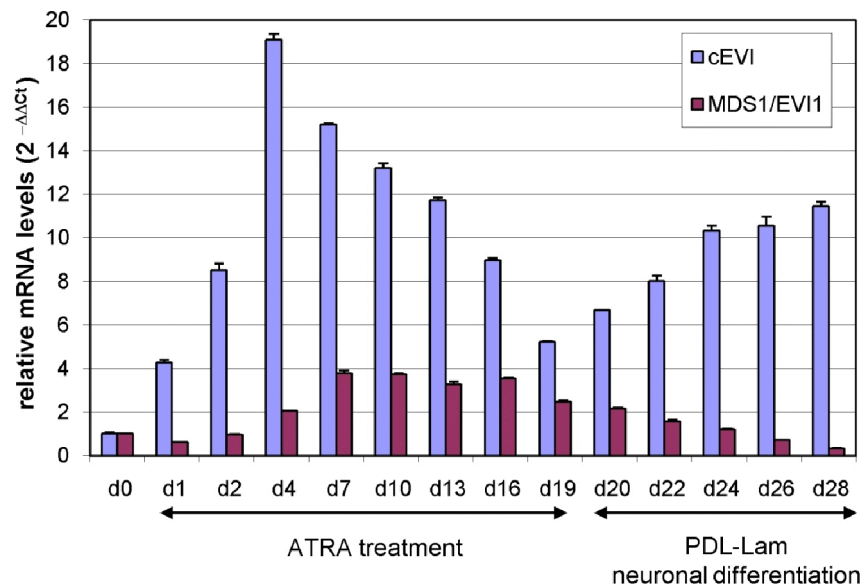


Fig. 3.4 Analysis of cEVI1 and MDS1/EVI1 expression during neuronal differentiation of NTERA-2 cells assessed by RTQ-RT-PCR. Relative expression values and standard deviation of triplicate measurements were calculated according to the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). PDL/LAM: poly-D-lysine and laminin, the substrate on which cell aggregates are plated at the beginning of the differentiation phase. Of note, due to the nature of the $\Delta\Delta C_t$ method only the mRNA levels of one amplicon during different experimental conditions can be compared, but not the levels of 2 amplicons, since the total amount of mRNA is not calculated.

The experiment was carried out altogether three times, twice with the described time points for RNA extraction, and once with time points day 0, 1, 7, 14, 21, 23, 26 and 28.

In the experiment with the in chapter time points days 0, 1, 2, 4, 7, 10, 13, 16, 19, 20, 22, 24, 26 and 28, cEVI1 and MDS1/EVI1 levels rose gradually until day 10, and both slightly decreased on day 13. However, on day 16, cEVI1 levels rose again until day 19, and decreased on day 20 at the onset of the differentiation phase, while MDS1/EVI1 levels remained constant during days 16 to 20. During the differentiation phase, cEVI1 levels increased between days 20 and 26, and slightly dropped on day 28, while MDS1/EVI1 levels gradually decreased until day 28. Taken together, during the differentiation phase the kinetics of cEVI1 and MDS1/EVI1 expression were largely reproducible between the experiment shown in fig. 3.4 and the repeated experiment, but kinetics differed during the aggregation phase between days 4 and 19. In the experiment with reduced number of investigated time points both cEVI1 and MDS1/EVI1 levels rose until day 14. On day 21, i.e. in the differentiation phase, both cEVI1 and MDS1/EVI1 levels had drastically decreased again compared to the high levels on day 14. cEVI1 levels then continually increased until day 28, while MDS1/EVI1 levels showed a slight increase on day 23 and then decreased again until day 28.

Despite deviation in kinetics, the expression data of cEVI1 and MDS1/EVI1 during neuronal differentiation of NTERA-2 cells were in summary largely reproducible with respect to an upregulation of both cEVI1 and MDS1/EVI1 during the ATRA treatment phase, and an upregulation of cEVI1 and downregulation of MDS1/EVI1 during the differentiation phase.

3.2 Induction of *EVI1* mRNA by ATRA in NTERA-2 cells

So far, the only known physiological regulator of *EVI1* in mammalian cells is ATRA, and as shown in chapter 3.1, *EVI1* mRNA is induced in NTERA-2 cells during neuronal differentiation. This prompted me to investigate the ATRA mediated induction of *EVI1* in detail, i.e. with respect to kinetics and if specific *EVI1* mRNA 5' end variants were differentially regulated. The induction of *EVI1* mRNA 5' end variants EVI1_1a and EVI1_1b by ATRA had already been reported in the human teratocarcinoma cell line PA1 (Aytekin, et al., 2005). However, this cell line is very difficult to transfect, which impedes further experiments, especially reporter gene experiments. Besides the capability for neuronal differentiation, NTERA-2 cells are more amenable for transfection than PA-1 cells. These characteristics make NTERA-2 cells a good *in vitro* model system to study the expression of *EVI1*.

3.2.1 *EVI1* mRNA is induced by ATRA in a time dependent manner in NTERA-2 cells

First, I wanted to investigate whether *EVI1* mRNA was also induced in regular cell culture conditions for NTERA-2 cells, i.e. adherent growth in cell culture grade dishes, and if induced, how early and with which kinetic the induction would take place. 10 μ M ATRA was added directly to cell culture medium for 0 h, 3 h, 6 h, 9 h, 15 h, 18 h, 24 h or 48 h, as negative control an equal volume of the solvent DMSO was added for 24h. Cells were harvested by directly lysing them with Trizol reagent. RNA was extracted, residual genomic DNA was removed by DNase I treatment, and the RNA then subjected to cDNA synthesis. The cDNA was used as template in semiquantitative PCR using primers which amplify a region common to all known *EVI1* mRNA 5' end variants (cEVI) (table 6.1). The primers used according to Russell et al. span an alternate splice site and produce PCR products of 254 bp and 281 bp (Russell, et al., 1994)

Upregulation of cEVI1 expression was observed already after 3h of ATRA treatment, and cEVI1 levels continued to rise until 48 h of ATRA treatment, while the addition of the solvent DMSO had no effect on cEVI1 expression (Fig 3.5, upper panel).

In order to find out which of the specific *EVI1* mRNA 5' end variants was upregulated, semiquantitative PCR was carried out with primers specific for the known *EVI1* mRNA 5' end variants (table 6.1) (Fig 3.5). Amplification of the variant EVI1_1c was omitted since this transcript is generally expressed at very low levels and hard to detect (Aytekin, et al., 2005;

Vinatzer U, 2003). As a positive control, cDNA derived from *EVI1* expressing MPD cells was used (Vinatzer U, 2003). Induction of the *EVI1*_1a, *EVI1*_1b, *EVI1*_1d and *EVI1*_3L variants started already after 3 h of ATRA treatment and continued until the end of treatment after 48 hours. Induction of the MDS1/*EVI1* variant started after 48 h of ATRA treatment.

The *EVI1*_1a, *EVI1*_1b and *EVI1*_1d variants are only very faintly expressed with 0 h ATRA treatment and 24 h DMSO treatment, while there appears to be a basal expression of the *EVI1*_3L and MDS1/*EVI1* variants with 0 h ATRA treatment and 24 h DMSO treatment. This might be due to the higher number of PCR cycles applied to amplify these variants.

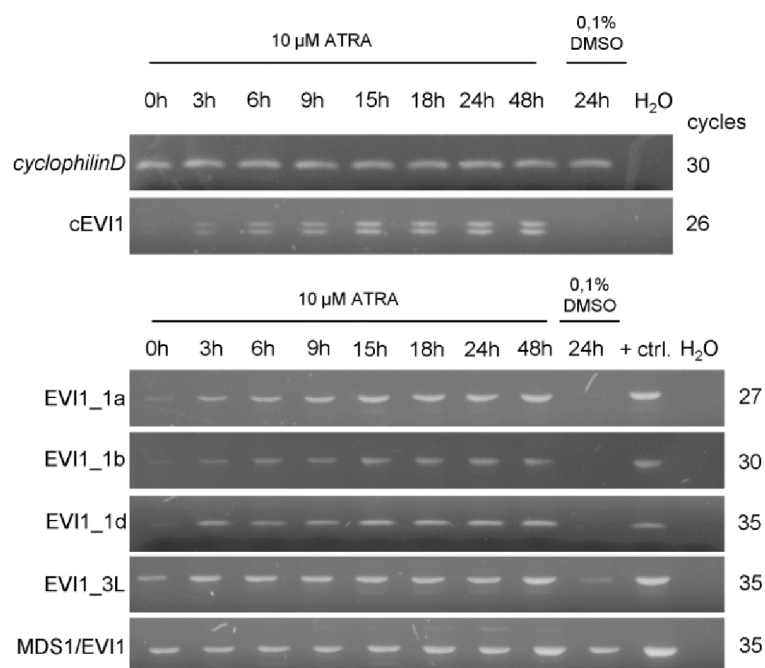


Fig. 3.5 Time course analysis of *EVI1* expression using semiquantitative PCR. The upper panel shows amplification of *cyclophilinD* as control for the amount of cDNA in each sample, and of *cEVI*. The lower panel shows the amplification of specific *EVI1* mRNA 5' end variants. The number of PCR cycles is indicated to the right.

A similar experiment was carried out with 0 h, 24 h, 48 h, 72 h, and 96 h incubation times for 10 μM ATRA (data not shown). In this experiment, *cEVI* and the *EVI1* mRNA 5' end variants *EVI1*_1a, *EVI1*_1b, *EVI1*_1d and *EVI1*_3L were distinctly upregulated after 24 h of ATRA treatment, and their levels remained high until 96 h of ATRA treatment. The MDS1/*EVI1* variant was slightly upregulated after 48 h, and distinctly after 72 h and 96 h of ATRA treatment.

For a more precise analysis of the upregulation of *EVI1* mRNA by ATRA, RTQ-RT-PCR was carried out with cDNA which was generated as described previously, only this time with time points 0 h, 1,5 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h ATRA treatment and 96 h treatment with an equal volume of the solvent DMSO. Primers used to amplify *cEVI* and *EVI1*

mRNA 5' end variants were designed to be suitable for RTQ-RT-PCR (table 6.2) and SYBR Green technology. Relative expression values and standard deviations were calculated according to the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), using *cyclophilinD* as control for the amount of cDNA in each sample, and the 0h time point as reference sample.

The levels of cEVI, EVI1_1a, EVI1_1b and EVI1_3L began to rise already after 3 h, the level of EVI1_1d began to rise 6 h after the addition of ATRA (fig. 3.6). The levels of these variants continued to rise until 48 h of ATRA treatment, and remained constant until 96 h of ATRA treatment. Throughout the increasing expression, the EVI1_1a variant had the highest, and the EVI1_1d the smallest induction factor, though all variants were upregulated with resembling kinetics.

The MDS1/EVI1 mRNA variant, however, was upregulated with different kinetics. After 24 h of ATRA treatment a very slight increase of expression set in, which became more distinct after 48 h and continued to rise until the 96 h time point.

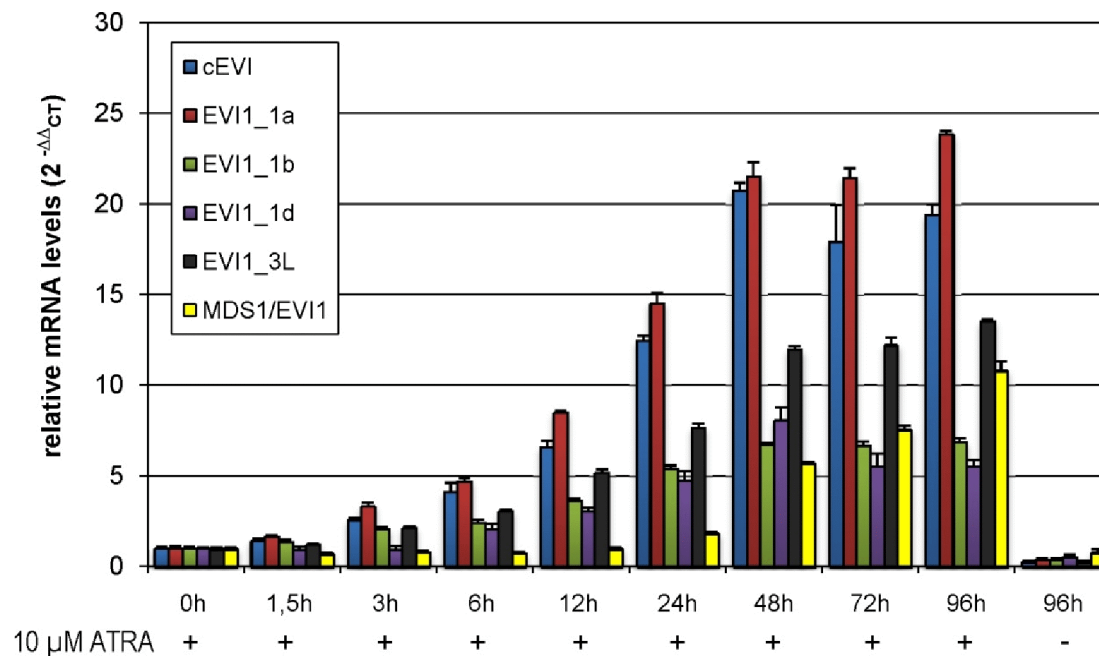


Fig. 3.6 Analysis of *EVI1* expression after time course ATRA treatment of NTERA-2 cells assessed by RTQ-RT-PCR. Cells were treated with 10 μ M ATRA for the indicated times, or an equal volume of the solvent DMSO for 96 h. Relative expression values and standard deviation of triplicate measurements were calculated according to the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

The experiment was repeated once with comparable results. As in the experiment shown in fig. 3.6, the levels of cEVI, EVI1_1a, EVI1_1b and EVI1_3L began to rise already 3 h after addition of ATRA but EVI1_1d levels rose only 12 h after addition of ATRA. The increase for these variants continued until 72 h of ATRA treatment and remained constant until the 96 h

time point. Like in the experiment shown in fig. 3.6, the MDS1/EVI1 variant was slightly upregulated after 24 h of ATRA treatment and gradually rised until the 96 h time point.

Of note, as mentioned in the previous chapter, the $\Delta\Delta C_t$ method only permits a comparison of induction factors of different amplicons, the relative expression values do not represent absolute amounts of mRNA. Since cEVI1 represents the sum of all *EVI1* mRNA 5' end variants, and some of these are induced to larger degrees than others, cEVI1 is not expected, and in fact not observed, to exhibit the largest induction factors of the amplicons.

3.2.2 EVI1 protein is induced by ATRA in a time dependent manner in NTERA-2 cells

In order to show ATRA mediated induction of *EVI1* also on the protein level immunoblot analysis was carried out. Protein extracts were prepared from NTERA-2 cells treated with 10 μ M ATRA for 0 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h, or for 96 h with an equal volume of the solvent DMSO, separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose. An EVI1 specific antibody detected three bands whose sizes corresponded to the reported protein variants of EVI1, i.e. MDS1/EVI1 at approximately 170 kD (Fears, et al., 1996), EVI1 at approximately 140 kD (Matsugi, et al., 1990) and the EVI1 Δ 324 variant at approximately 100 kD (Bordereaux D, 1990) (fig. 3.7). Both EVI1 and the EVI1 Δ 324 variant were induced with similar kinetics, with a very faint band appearing after 12 h of ATRA treatment, which increased in intensity until 96 h of ATRA treatment. The EVI1 Δ 324 band was consistently fainter than the EVI1 band, which was the most prominent of all variants throughout the time course experiment. The MDS1/EVI1 band at 170 kD band was the faintest, and was detectable only at the 72 h and 96 h time points. As control for the loading of equal amounts of protein the lower part of the immunoblot was probed with an antibody against β -tubulin which detected an approximately equally dense 55 kD band in every sample.

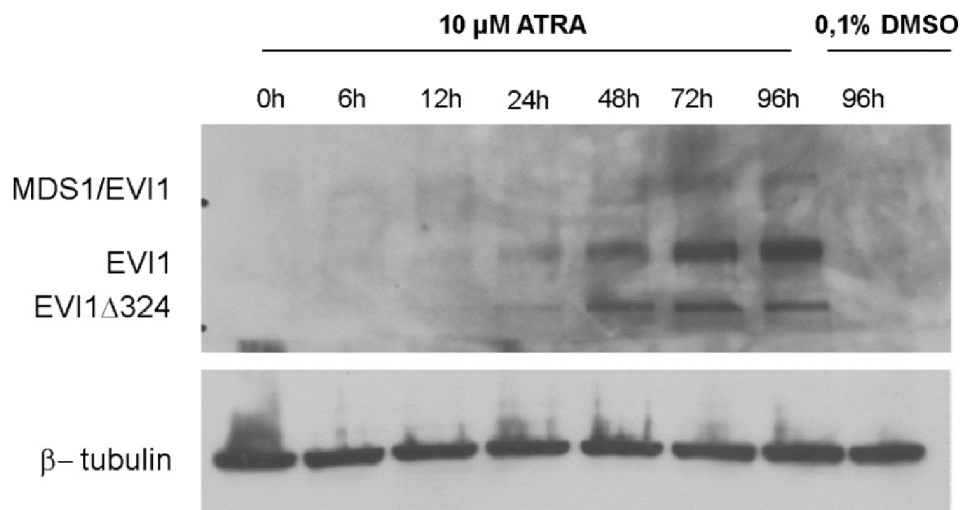


Fig. 3.7 Analysis of EVI1 protein expression after treating NTERA-2 cells with 10 μ M ATRA or an equal volume of the solvent DMSO. Proteins were extracted and subjected to immunoblot analysis using an antibody against EVI1 and against β -tubulin as loading control.

The experiment was repeated once. The other immunoblot shows less background but a faint upregulation of EVI1 could be detected already after 6 h of ATRA treatment. However, the band intensity did not increase until 24 h of ATRA treatment, but then increased abruptly after 48 h of ATRA treatment, and remained high until 96 h. Likewise, the EVI1 Δ 324 band appeared faintly after 24 h of ATRA treatment, then increased abruptly after 48 h and remained high until 96 h. A faint MDS1/EVI1 band was visible at 72 h and 96 h of ATRA treatment. Taken together, the upregulation of MDS1/EVI1, EVI1 and EVI1 Δ 324 was confirmed but the increase was less continuous than in the immunoblot shown in fig. 3.7.

3.2.3 The EVI1 mRNA is induced by ATRA in a dose dependent manner in NTERA-2 cells

In order to find out how sensitively the *EVI1* gene was upregulated in response to ATRA, a dose response experiment was carried out. NTERA-2 cells were treated with ATRA in concentrations ranging from 1 nM to 10 μ M or an equal volume of its solvent DMSO for 15 h and cells were harvested and processed as described in chapter 3.1.1.1. Since in the time course experiment all of the investigated *EVI1* mRNA 5' end variants had behaved in a similar way, an assay distinguishing between the variants was omitted. cEVI1 was already induced by as little as 1 nM ATRA, and reached maximal levels at 10 μ M ATRA (fig. 3.8).

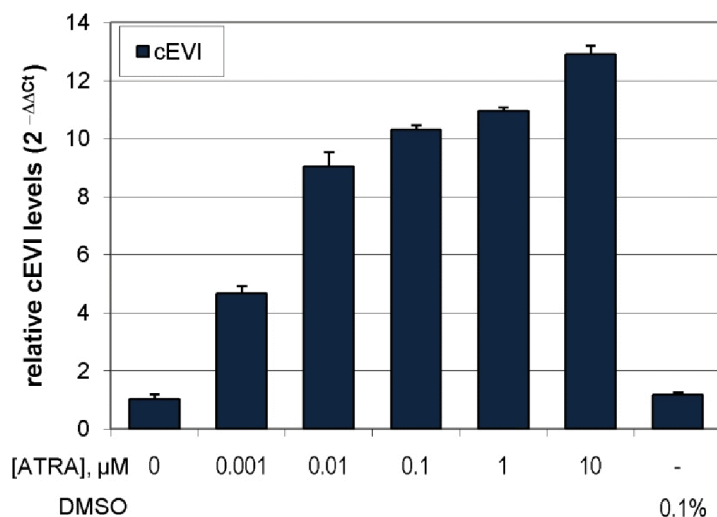


Fig. 3.8 Analysis of cEVI1 expression after treating NTERA-2 cells with the indicated amounts of ATRA or its solvent DMSO for 15 h assessed by RTQ-RT-PCR. Relative expression values and standard deviation of triplicate measurements were calculated according to the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

The experiment was repeated once with a very similar result, with a maximum induction factor of approximately 11 at an ATRA dose of 10 μM for 15 h.

3.3 Identification of a retinoic acid response element (RARE) in the *EV11* regulatory region

Since *EV11* was strongly and rapidly upregulated in response to ATRA, and previous experiments had shown that its induction, at least in PA-1 cells, was independent of new protein synthesis (Aytekin, et al., 2005), it was hypothesized that the *EV11* promoter contains a RARE. In order to identify a putative RARE in the presumptive regulatory region of the *EV11* gene, reporter gene assays were carried out.

3.3.1 Reporter gene assays of the *EV11* promoter region, the region surrounding the transcriptional start site of exon 1a and intronic region

Three fragments that, taken together, covered most of the genomic region from positions -2.6 kb to +3.9 kb relative to the 5'-end of *EV11* exon 1a were cloned into the luciferase reporter vectors pGL3basic or pGL3promoter. The constructs were termed -2629/-72, +15/+1106 and +1258/+3903. Positions refer to the transcription start site of *EV11_1a* according to Genbank accession number BX640908.

Using the transfection reagent DAC-30, these constructs were transfected into NTERA-2 cells along with the plasmid pRL-TK which constitutively expresses renilla luciferase. pRARE-tk-luc, which contains the *tk* minimal promoter and two copies of the RARE of the human *RAR β* gene in pGL2 (kindly provided by Dr. Hanna Harant, Ingenetix, Vienna, Austria) served as a positive control for the ATRA response. 24 h later cells were treated with 10 μ M ATRA or its solvent DMSO, and another 24 h later cells were lysed and assayed for luciferase activity.

Of the three constructs in the pGL3basic vector, the +15/+1106 construct, which largely corresponds to *EV11* exon 1a, responded to ATRA in reporter gene assays with an induction factor of 3.2 (fig. 3.9). The 2 other constructs showed induction factors less than 1 in response to ATRA, while the positive control pRARE-tk-luc showed 2,3 fold induction upon ATRA treatment. It was therefore concluded that +15/+1106 region contained the element which conferred ATRA responsiveness to the *EV11* gene. Of note, this element is located within the transcribed region of *EV11*.

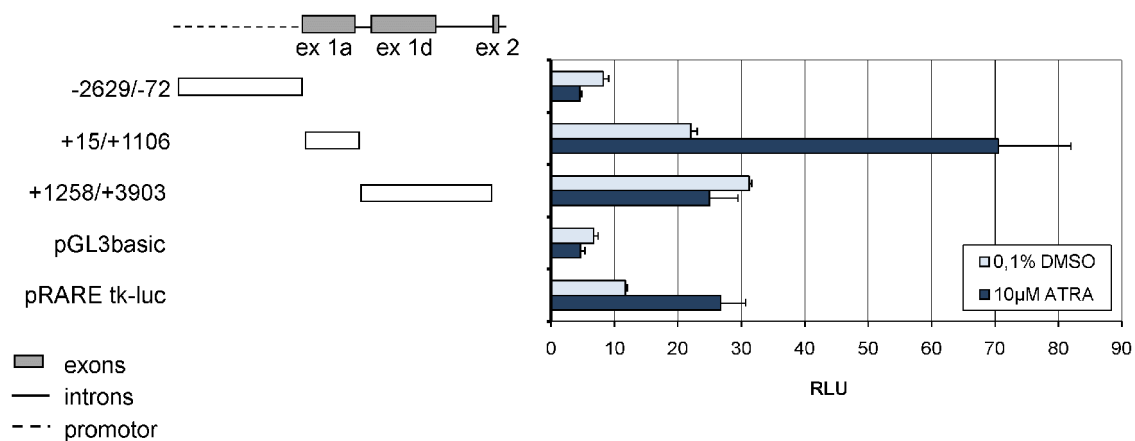


Fig. 3.9 Results of reporter gene assays with *EVI1* promoter fragments cloned into the pGL3basic vector. Shown on the left is a schematic of the constructs and their location within the *EVI1* promoter, the region surrounding the transcriptional start site of exon 1a and intronic region. Shown on the right are relative luciferase reporter gene activities of the corresponding constructs. The indicated reporter plasmids were transfected into NTERA-2 cells, which were then treated with 10 μM ATRA for 24 h, lysed, and luciferase activities were measured. Relative light units (RLU) were derived by normalizing firefly to renilla luciferase activities. Error bars represent the standard deviations between duplicate measurements.

The experiment was repeated once with a similar result: the +15/+1106 construct showed an induction factor of 3,1 while the 2 other constructs showed induction factors of less than one in response to ATRA. The positive control pRARE-tk-luc showed an induction factor of 2.3.

Similar results were obtained in reporter gene assays carried out as described with the constructs -2629/-72, +15/+1106 and +1258/+3903 cloned into the pGL3promoter vector (fig. 3.10). Again, the construct +15/+1106 showed a response ATRA treatment, albeit with a somewhat reduced induction factor of 2,6. The other 2 constructs showed induction factors of less than 1, and the positive control pRARE-tk-luc showed 1,5 fold induction.

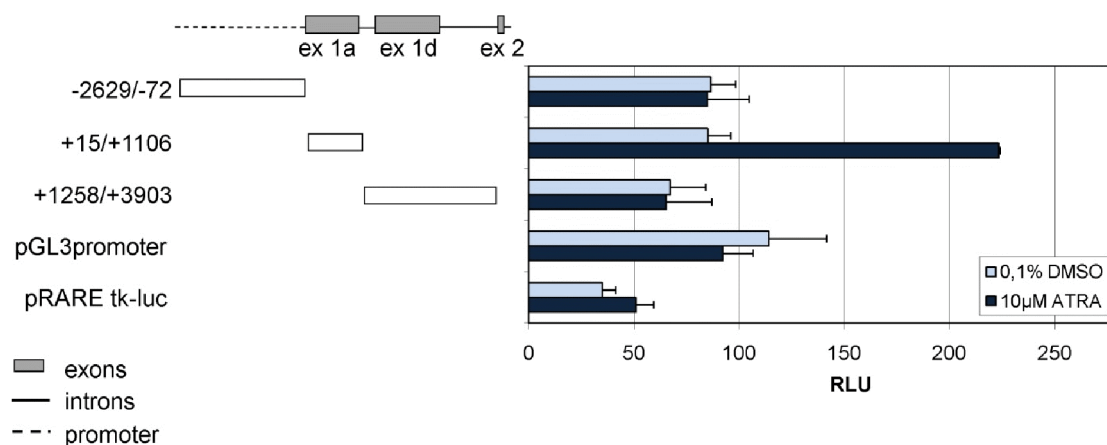


Fig. 3.10 Results of reporter gene assays with *EVI1* promoter fragments cloned into the pGL3promoter vector. Schematic and procedure of the reporter gene assay as described in the legend to fig. 3.9.

The experiment was repeated once with a similar result: an induction factor of 1,9 for the +15/+1106 construct, smaller than 1 for the two other constructs and 1.9 for the pRARE-tk-luc positive control. Since the ATRA response was more pronounced in the pGL3basic constructs, all further constructs for reporter gene assays were cloned into this vector.

3.3.2 *In silico* analysis of the *EVI1* +15/+1106 region to identify putative RAREs

An *in silico* analysis of *EVI1*(+15/+1106) was carried out in order to determine whether this region contained conserved and thus predictable RAREs. Analysis using the on-line programmes “Match” (www.gene-regulation.com/cgi-bin/pub/programs/match) and “TFSEARCH” (www.cbrc.jp.htbin/nph-tfsearch) revealed no potential RAREs in this region. The programme “Patch” (www.gene-regulation.com/cgi-bin/pub/programs/patch) predicted a DR2 RARE CTCCCTTTCTCCCT between position +290 and +303, and a DR1 RARE AGGTGATGTGAAC between position +624 and +636, relative to the beginning of *EVI1* exon 1a. The programme “TESS” (www.cbil.upenn.edu/cgi-bin/tess) predicted a potential DR5 RARE CGACCTTTTTGTGACCT (inverted) between position +182 and +198 relative to the beginning of exon1a. In summary, the results of the *in silico* analysis of *EVI1*(+15/+1106) suggested the presence of three potential RAREs.

3.3.3 Refined reporter gene analysis of *EVI1* (+15/+1106)/pGL3basic and derivative constructs

Based on the predictions from the *in silico* analysis of the *EVI1*(+15/+1106) fragment, a series of 5' - deletion constructs were engineered.

First, the predicted RAREs of *EVI1*(+15/+1106) were sequentially deleted from the 5' end, thus yielding the constructs +86/+1106, +255/+1106, +325/+1106, +587/+1106, +653/+1106. The obtained constructs were then used in reporter gene assays as described in chapter 3.3.1. The construct +86/+1106 was still ATRA-responsive but already the deletion of the first RARE, the inverted DR5 RARE CGACCTTTTTGTGACCT, abolished the ATRA response (fig. 3.11). This strongly suggested that this is the one RARE mediating the ATRA response of the *EVI1* gene.

Subsequently, further constructs were engineered, i.e. both 5' and 3' deletions (+86/+274) and (+140/+227), and one carrying a mutated DR5 RARE (+15/+1106 mut).

The construct *EVI1*(+86/+274)/pGL3basic, which contained the DR5 RARE, was still responsive to ATRA, while *EVI1*(+140/+227)/pGL3basic, which also contained this RARE but was somewhat shorter, was not (fig 3.11). This suggested that this construct possibly lacked binding sites for further cooperating transcription factors or the basal transcription machinery, which were neither present in the pGL3basic vector. Mutating the second half site of the inverted DR5 RARE from CGACCTTTTTGTGACCT to CGACCTTTTTGCTTTAG, as

suggested in a study by Jiang et al. (Jiang, et al., 2005), lead to a complete loss of the ATRA response, which clearly corroborated the importance of the DR5 RARE. Of note, the basal activity was elevated compared to that of the corresponding wild type construct, which might be due to loss of the transrepressional effect exerted by corepressors of unliganded but DNA bound retinoic acid receptors (Chambon, 1996).

Since the production of the transfection reagent DAC-30 used in the initial reporter gene assays was discontinued, subsequent transfections were carried out using FuGENE®6, this was also the case for the reporter gene assay presented in fig. 3.11. Ever since the employment of this protocol change the ATRA mediated induction factors of the luciferase activities increased approximately 3-fold.

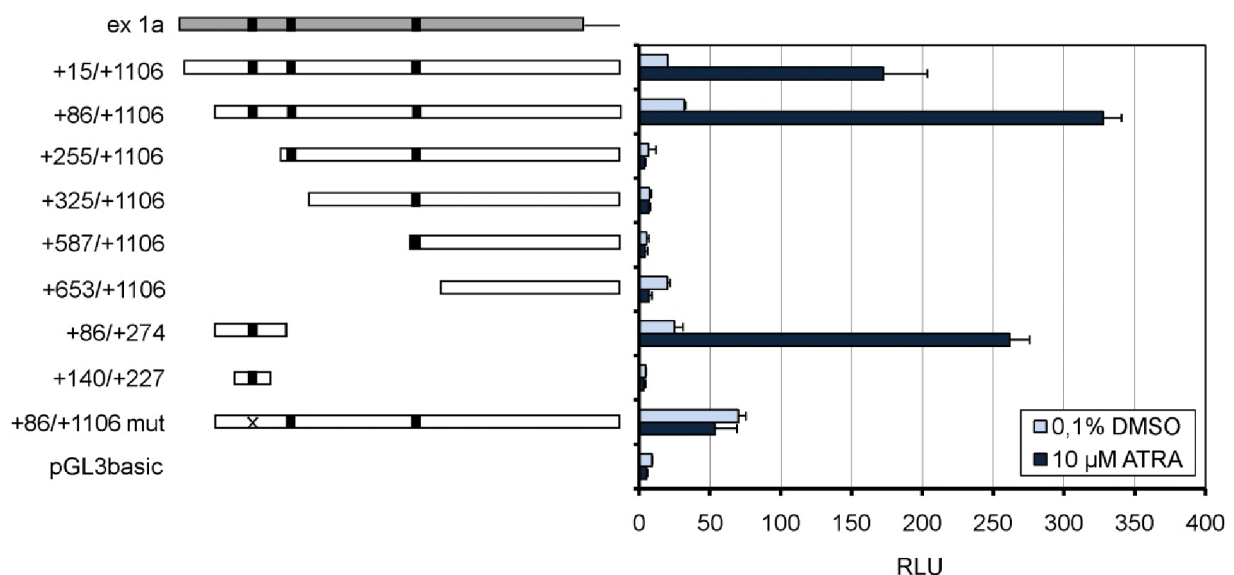


Fig. 3.11 Results of reporter gene assays of EVI1(+15/+1106)/pGL3 and its derivative constructs. Shown to the left is a schematic of *EVI1* exon 1a (grey box), the EVI1(+15/+1106) construct and its derivative constructs (white boxes). Black boxes denote predicted RAREs. In EVI1(+86/+1106)mut/pGL3basic, the second half site of the *EVI1* RARE was mutated (indicated by a cross). Shown to the right are relative luciferase reporter gene activities of the corresponding constructs. RLU values were calculated as described in the legend to fig. 3.9.

The results were confirmed in two further separate experiments, one set with 5' deletion constructs +86/+1106, +255/+1106, +325/+1106, +587/+1106, +653/+1106, and another with constructs +86/+274, +140/+227, and +15/+1106 mut. While the +86/+1106 and the +86/+274 construct were responsive to ATRA treatment, all further 5' deletion constructs, the +140/+227, and +15/+1106 mut were not.

3.3.4 The DR5 RARE is located within the transcribed region of *EVI1* exon1a

The *EVI1* DR5 RARE is located within a region located with an expressed sequence tag (EST), e.g. BX640908, which implies that it is located within the transcribed region of the *EVI1* gene. Though not undescribed, the presence of a transcription factor binding site within a transcribed region still constitutes an unusual circumstance.

In order to confirm that the *EVI1* DR5 RARE is part of the ATRA induced transcript, NTERA-2 cells were incubated with 10 μ M ATRA for 72 h, or were left untreated, cells were lysed and RNA was isolated, and treated with DNase I prior to cDNA synthesis. The cDNA served as template for a PCR with primers surrounding the DR5 RARE. The amplicon was elevated in response to ATRA, which demonstrated that the ATRA induced transcripts of *EVI1* indeed begin in part or entirely upstream of this RARE (fig. 3.12). As control, PCR with an intron-spanning primer pair located in a more proximal region of *EVI1_1a* was performed. This amplicon was also elevated after 72h ATRA treatment, which confirmed the induction of *EVI1*. The effectiveness of the DNaseI treatment was verified with primers for the microsatellite marker D22S686. Absence of a PCR product for this primer pair when using cDNA, but presence of a PCR product when using genomic DNA as template confirmed complete absence of contaminant genomic DNA in the cDNA. Amplification of the housekeeping gene *cyclophilinD* served as control for the amount of cDNA in each sample. The experiment was carried out once.

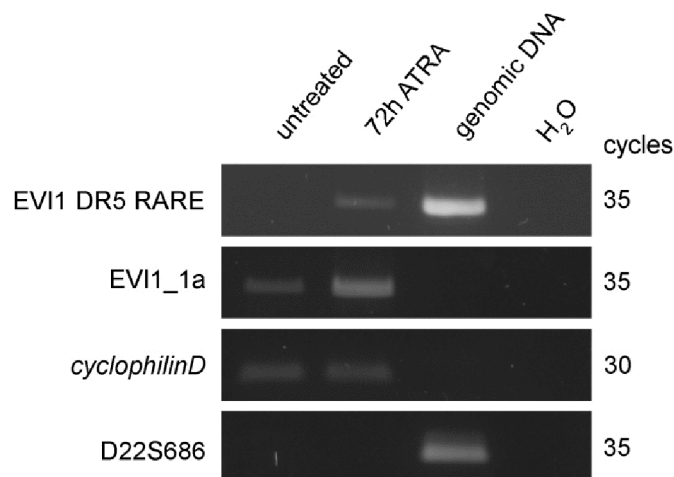


Fig. 3.12 RT-PCR confirming that the DR5 RARE of the *EVI1* gene is located within its transcribed region. The number of PCR cycles is indicated to the right.

3.4 Retinoic acid receptor (RAR) and retinoid X receptor (RXR) binding to the *EVI1* DR5 RARE

In order to determine whether the DR5 RARE in the *EVI1* 5'UTR was indeed bound by retinoic acid receptors in intact cells, and therefore a functional RARE, chromatin immunoprecipitation (ChIP) experiments were carried out. Prior to the ChIP experiment the expression of all isoforms of RAR and RXR was analysed in order to determine which of them was expressed in ATRA treated NTERA-2 cells and was therefore eligible as an antigen in ChIP. The expression of RAR α , β and γ and RXR α in NTERA-2 cells - either basally or induced by ATRA – had been shown previously (Moasser MM, 1995; Simeone A, 1990). These findings were to be verified and extended to RXR β and RXR γ .

3.4.1 Expression of RARs and RXRs in NTERA-2 cells

NTERA-2 cells were treated with 10 μ M ATRA or an equal volume of its solvent DMSO for the indicated times, cells were harvested by directly lysing them with Trizol reagent. RNA was extracted, and then subjected to cDNA synthesis. RT-PCR was carried out with intron spanning primers located in different functional regions of the *RAR* and *RXR* paralogs, respectively, to prevent the possibility of unspecific amplification due to high homology between paralogs. Amplification of *cyclophilinD* served as control for the equal amount of cDNA in each sample. All *RAR* and *RXR* isoforms were basally expressed in NTERA-2 cells (fig. 3.13). *RAR* β was upregulated in response to ATRA treatment, which is consistent with published results (Moasser MM, 1995). The expression of the other *RAR*s and *RXR*s remained unaltered through ATRA treatment. Thus, any of the *RAR*s and *RXR*s might be involved in mediating the ATRA response of the *EVI1* gene.

The experiment was carried out once.

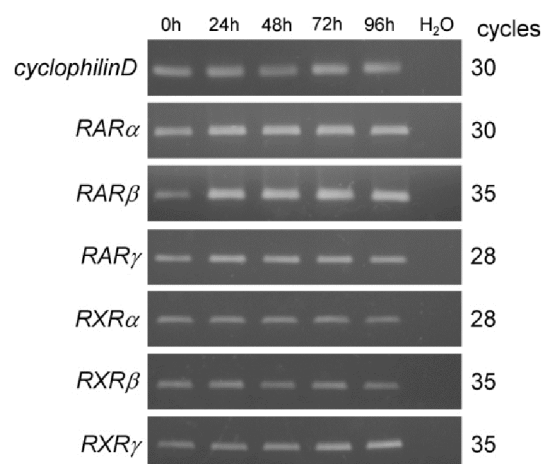


Fig. 3.13 Semiquantitative RT-PCR confirming the expression of all *RAR* and *RXR* isoforms in NTERA-2 cells. The number of PCR cycles is indicated to the right.

3.4.2 The *EVI1* DR5 RARE is bound by canonical RA and RX receptors

For ChIP experiments, NTERA-2 cells were treated with 10 μ M ATRA or an equivalent volume of DMSO for 24 h. DNA and proteins bound to it were cross linked by addition of formaldehyde, cells were lysed, and chromatin was sheared by sonication. DNA fragments which were bound by retinoic acid receptors, were immunoprecipitated using a RAR α and a pan-RXR antibody. Both antibodies detected protein bands of the expected size for RAR α and RXR paralogs in immunoblotting (data not shown). Chromatin was then de-cross-linked, DNA recovered and used as template in PCR with primers flanking the regions of interest (table 6.1.4).

The presence of a distinct PCR product generated with primers flanking the *EVI1* DR5 RARE and DNA from chromatin immunoprecipitated with a RAR α and a pan-RXR antibody revealed that RAR α , as well as RXR α , RXR β or RXR γ were associated with the *EVI1* DR5 RARE both in the absence and presence of ATRA (fig. 3.14). Binding of receptors to RAREs independently of the availability of ligand is in accordance with the current model of retinoic acid induced transcriptional regulation (Bastien and Rochette-Egly, 2004). In contrast to this, only a faint PCR product was obtained with primers flanking the *EVI1* DR1 RARE AGGTGATGTGAAC between position +624 and +636 relative to the beginning of *EVI1* exon 1a indicating that this RARE is not bound by RAR α or RXR isoforms. This is consistent with the fact that this RARE does not mediate responsiveness to ATRA in luciferase reporter assays.

A distinct PCR product was obtained for the RARE of the *RAR β* gene promoter, which was used as a positive control. Only a faint PCR product was obtained with primer pairs specific for *EVI1* exon 3L approximately 11 kb downstream of the DR5 RARE, which served as negative control amplicon. Only faint bands were observed when negative control immunoprecipitates obtained with unspecific immunoglobulins which confirms the specificity of the interactions.

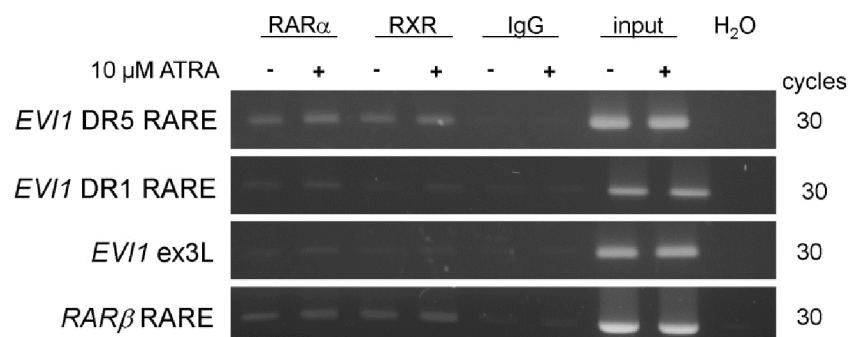


Fig. 3.14 ChIP of NTERA-2 cells treated with ATRA or DMSO for 24 h using an antibody specific to RAR α , a pan-RXR antibody, or nonspecific rabbit IgG as a negative control. Immunoprecipitated chromatin and input DNA as a positive control were amplified with primers flanking the indicated loci (table 6.1.4). The number of PCR cycles is indicated to the right.

The experiment was repeated once with a largely similar result, with the exception that consistently in all the samples with ATRA treatment the PCR product appeared somewhat more intense than in the samples with DMSO treatment, which indicates that the normalisation of chromatin amounts might have been imprecise.

In conclusion the first exon of the *EVI1* gene contains a consensus DR5 RARE motif that necessary and sufficient to mediate ATRA responsiveness in luciferase reporter assays and that is bound by canonical RA and RX receptors.

3.5 Regulation of the *MDS1/EVI1* promoter

3.5.1 Reporter gene assays of the *MDS1* promoter comprising part of the 5' UTR, part of the coding region and the first intron

Like *EVI1*, the *MDS1/EVI1* variant was also upregulated in response to ATRA, albeit with considerably slower kinetics than the other *EVI1* mRNA 5' end variants. In order to investigate how ATRA conferred induction of *MDS1/EVI1*, reporter gene assays as described in chapter 3.3.1 were carried out with 4 overlapping fragments, which together covered the genomic region from position -3.0 to +2.5 kb relative to the transcriptional start site of *MDS1/EVI1*, were cloned into the luciferase reporter vector pGL3basic. The *MDS1/EVI1* promoter was assumed to be identical to that of the *MDS1* promoter.



Fig. 3.15 Schematic of *MDS1/EVI1* promoter constructs in relation to the putative *MDS1/EVI1* promoter region. Dashed line: *MDS1/EVI1* putative promoter region, black box: *MDS1* exon 1, continuous line: *MDS1* intron 1, arrow: transcription start site, black bar within -920/+357 construct: predicted DR2 RARE according to the programme "Patch" (www.gene-regulation.com/cgi-bin/pub/programs/patch).

These constructs - termed MDS1(-2968/-1975), MDS1(-2027/-915), MDS1(-920/+357) and MDS1(+310/+2456) (fig. 3.15) - were transfected into NTERA-2 cells and reporter gene assays carried out as described in chapter 3.3.1, but with the exception that the incubation time for ATRA was prolonged to 48h because in the ATRA time course experiment described in chapter 3.2.1 it had become apparent by RTQ-RT-PCR that the *MDS1/EVI1* variant was only very weakly induced after 24 h but distinctly after 48 h of ATRA treatment. None of the 4 fragments conferred ATRA responsiveness to the luciferase reporter (fig. 3.16). The construct MDS1(-920/+357) exhibited the highest basal activity compared to the other constructs indicating that this region was presumably important for the expression of *MDS1/EVI1*, yet this was not mediated directly by ATRA. Therefore, the observed upregulation of the *MDS1/EVI1* variant by ATRA, which was observed in ATRA treatment experiments, was concluded to be indirect.

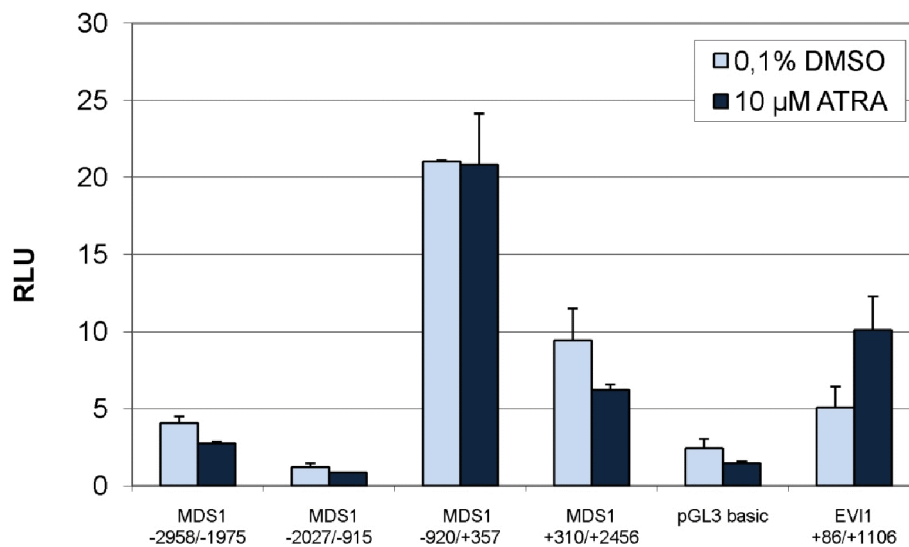


Fig. 3.16 Results of reporter gene assays of *MDS1/EVI1* promoter constructs comprising part of the 5' UTR, part of the coding region and the first intron. EVI1(+86/+1106)/pGL3basic, which had shown upregulation in response to ATRA in previous experiments, was included as positive control. RLU values were calculated as described in the legend to fig 3.9.

The reporter gene assay shown in fig. 3.16 was carried out using the transfection reagent DAC-30, the experiment was repeated once using the transfection reagent FuGENE®6, with very similar results. None of the constructs showed responsiveness to ATRA. Of note, the included positive control for the ATRA response, EVI1(+86/+1106)/pGL3basic, showed an induction factor of only 2,0, which had been 3,2 and 3,1 in previous experiments (chapter 3.3.1, fig. 3.9). This might be due to the prolonged incubation with ATRA, which seems less than optimal for this construct. A similar low induction factor of 2,5 was observed in the repetition of the experiment using FuGENE®6.

3.5.2 Neither EVI1, nor ATRA through EVI1 regulate the MDS1/ EVI1 promoter

Since ATRA does not directly regulate the *MDS1* promoter we speculated that maybe the rapidly induced EVI1 protein could be involved in mediating the slower ATRA response of the *MDS1* promoter. Cotransfection experiments were carried out in NTERA-2 cells using *MDS1* promoter constructs described in chapter 3.5.1 as reporter constructs together with the *EVI1* expression plasmid haEVI1/pEFzeo (Vinatzer U, 2001). This plasmid contains the *EVI1* cDNA with an N-terminal HA epitope tag and a Kozak consensus for translation initiation under the control of the *eIF1 α* promoter. Respective empty vectors, i.e. pGL3basic and pEFzeo, were used as negative controls. The plasmid pRL-TK, which expresses renilla luciferase constitutively, was also cotransfected to allow normalisation of obtained RLU values. The reporter plasmids EVI1(+86/+1106)/pGL3basic and pRARE-tk-luc, which contains two copies of the human RAR β RARE and the *tk* minimal promoter in pGL2, served as positive controls for the ATRA response. The plasmid pERELuc, which contains two copies of an EVI1 DNA binding site (Vinatzer U, 2001), served as positive control for regulation by *EVI1*. 24 h after transfection cells were treated with 10 μ M ATRA or an equal volume of DMSO and another 24 h later cells were lysed and subjected to luciferase assays. Neither ATRA, nor exogenously expressed *EVI1*, nor ATRA together with exogenously expressed *EVI1* induced any of the *MDS1* promoter constructs (fig. 3.17). However, the included positive controls behaved in an unexpected way. EVI1(+86/+1106)/pGL3basic showed the expected induction of luciferase activity through ATRA treatment, but the induction was diminished when additionally *EVI1* was overexpressed. Likewise, pRARE-tk-luc showed the expected induction upon ATRA treatment, but in contrast to EVI1(+86/+1106)/pGL3basic it showed enhancement of the ATRA response when additionally *EVI1* was overexpressed. This rules out the possibility of an unspecific squelching effect through supraphysiological EVI1 concentration as an explanation for the negative feedback of EVI1 on its own promoter.

As expected, overexpression of *EVI1* reduced the activity of pERELuc, but unexpectedly, ATRA treatment also reduced the activity of pERELuc. A likely explanation for this is the ATRA mediated upregulation of endogenous *EVI1*. Notably, ATRA treatment in conjunction with *EVI1* overexpression reduced activity even a bit further. This supports the assumption that EVI1 protein induced by ATRA in NTERA-2 cells is functional as a transcription regulator.

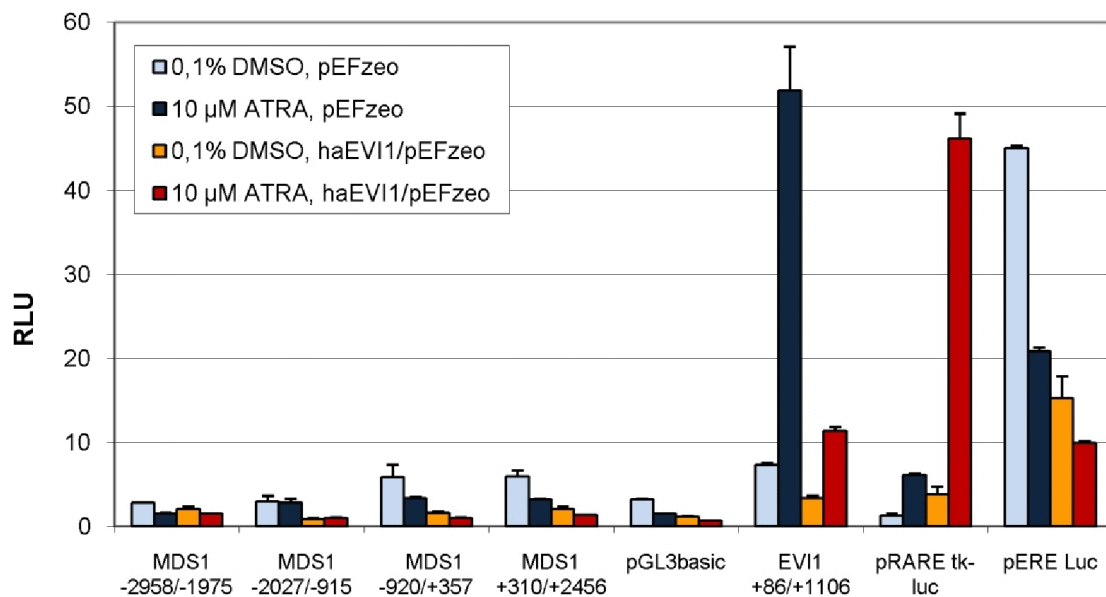


Fig. 3.17 Results of reporter gene assays of cotransfection of presumptive *MDS1*/*EVI1* promoter, 5' UTR, coding region, and intron as reporter constructs, haEVI1/pEFzeo as cotransfected construct and respective empty vectors as controls. RLU values were calculated as described in the legend to fig 3.9.

The experiment was repeated once with similar results: none of the *MDS1* promoter constructs showed induction upon ATRA treatment, *EVI1* overexpression, or both, and the unexpected behaviour of the positive controls pRARE tk-luc and pERE Luc was confirmed. These results suggest that *EVI1* is capable of acting as a modulator of the ATRA response, both in a negative and a positive way, and the modulation even affects its own promoter. The surprising findings challenge to dissect this phenomenon further, with respect to what domains of *EVI1* and which consensus binding sites are necessary for the modulation.

3.6 Modulation of the ATRA response by EVI1

Cotransfection experiments as described in chapter 3.5.2 were carried further to see if MDS1/EVI1 protein was also capable of exerting the observed modulating effect of the ATRA response. As reporter constructs, EVI1(+86/+1106)/pGL3basic and the smallest ATRA responsive construct containing the *EVI1* DR5 RARE, EVI1(+86/+274)/pGL3basic, were chosen to investigate the counteraction of the ATRA response of the EVI1 promoter (presented in chapter 3.6.1), and the reporter construct pRARE tk-luc was chosen to investigate the enhancement of the *RAR* β RARE (presented in chapter 3.6.2).

3.6.1 EVI1 and MDS1/EVI1 repress the ATRA response of the *EVI1* DR5 RARE

Reporter constructs EVI1(+86/+1106)/pGL3basic, EVI1(+86/+274)/pGL3basic or the empty pGL3basic vector were cotransfected with the expression plasmids haEVI1/pEFzeo or haMDS1/EVI1/pEFzeo or empty pEFzeo vector, and the reporter gene assay was continued as described in chapter 3.5.2.

Like *EVI1* overexpression, *MDS1/EVI1* overexpression was capable of abolishing the ATRA mediated increase of luciferase activity for the EVI1(+86/+1106)/pGL3basic construct (fig. 3.18). The abolishing effect of *EVI1* overexpression in conjunction with ATRA treatment was also observed with the smallest ATRA responsive construct EVI1(+86/+274)/pGL3basic, indicating that the *EVI1* DR5 RARE might be directly involved in this effect.

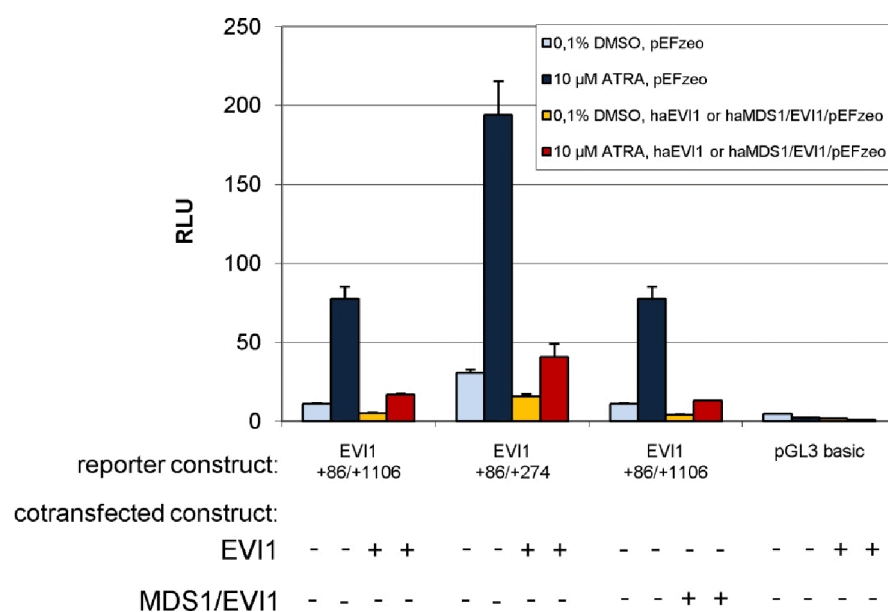


Fig. 3.18 Results of reporter gene assays with various *EVI1* promoter constructs as reporter, haEVI1/pEFzeo, haMDS1/EVI1/pEFzeo or empty pEFzeo vector as cotransfected constructs, and empty pGL3basic vector as control. RLU values were calculated as described in the legend to fig. 3.9.

The experiment was repeated once with the exception that *MDS1/EVI1* overexpression was not included. However, *EVI1* overexpression yielded a reproducible repressive effect on the ATRA response of the *EVI1* DR5 RARE for both the *EVI1*(+86/+1106)/pGL3basic and the *EVI1*(+86/+274)/pGL3basic construct.

3.6.2 *EVI1* and *MDS1/EVI1* enhance the ATRA response of the *RAR β* RARE

Cotransfection experiments were carried out as described in chapter 3.5.2 with the reporter construct pRARE tk-luc or the respective empty vector pGL2 tk-luc together with expression plasmids haEVI1/pEFzeo or haMDS1/EVI1/pEFzeo and the empty pEFzeo vector as control. Like *EVI1* overexpression, *MDS1/EVI1* overexpression was capable of enhancing the ATRA mediated increase of luciferase activity for the pRARE tk-luc construct (fig. 3.19).

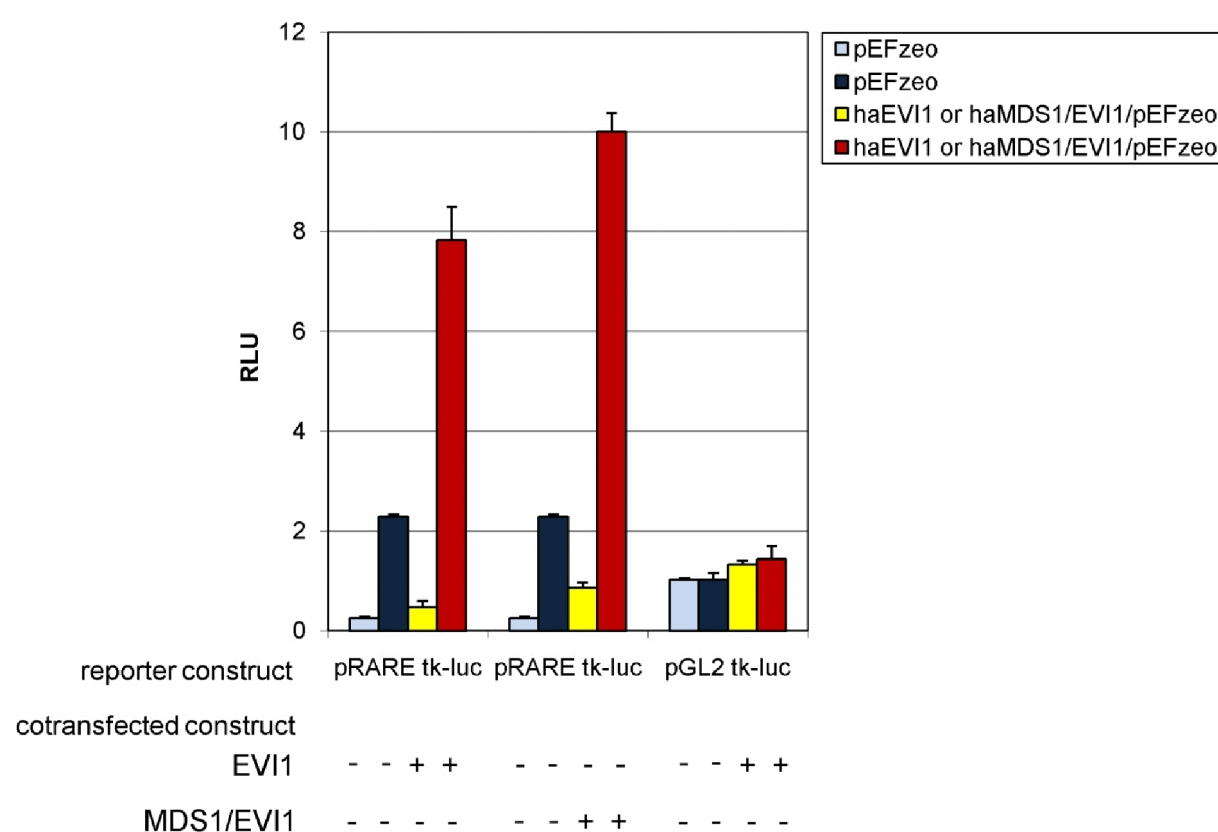


Fig. 3.19 Results of reporter gene assays with pRARE tk-luc as reporter construct, haEVI1/pEFzeo, haMDS1/EVI1/pEFzeo or empty pEFzeo vector as cotransfected constructs and empty pGL2 tk-luc vector as control. RLU values were calculated as described in the legend to fig. 3.9.

The experiment was repeated once. Both *EVI1* and *MDS1/EVI1* overexpression yielded a reproducible effect on the enhancement of the ATRA response of pRARE tk-luc.

3.7 Generation and properties of *EVI1* deletion constructs

In order to identify regions in the *EVI1* protein that contribute to positive and negative modulation of the ATRA response, a series of *EVI1* deletion constructs was engineered, in which functional domains were sequentially deleted. All constructs were derived from the expression construct ha*EVI1*/pEFzeo (Vinatzer U, 2001), and retained the amino terminal hemagglutinin (HA) epitope tag and the Kozak consensus for translation initiation. Constructs either retained a predicted nuclear localisation sequence (NLS) between positions 421-434, which is located within the IR (intervening region), or carried an engineered tandem repeat of the SV40 large T antigen NLS at the carboxy terminus.

EVI1 consists of an amino terminal zinc finger region (ZF1), intervening region (IR), repression domain (RD), carboxy terminal zinc finger region (ZF2) and an acidic region (AR). A series of carboxy terminal deletions yielded the Δ AR construct (deletion of AR), the Δ AR-ZF2 construct (deletion of both the AR and ZF2), the ZF1-IR construct (deletion of AR, ZF2 and RD) and the ZF1 construct (deletion of AR, ZF2, RD and IR). Further constructs had an amino terminal deletion like Δ ZF1 (deletion of ZF1) or both amino terminal and carboxy terminal deletions like IR-RD (deletion of ZF1, AR and ZF2) and IR (deletion of ZF1, AR, ZF2 and RD).

Prior to using the *EVI1* deletion constructs in reporter gene experiments they were tested by (i) immunofluorescence if their protein products showed proper intracellular localisation, and by (ii) immunoblotting if their protein products had the expected size.

3.7.1 Intracellular localisation of endogenous *EVI1* and of the *EVI1* deletion constructs

In order to be able to compare the intracellular localisation of the engineered *EVI1* deletion constructs to that of endogenous *EVI1* immunofluorescence analysis was carried out. NTERA-2 cells were treated with 10 μ M ATRA for 72h to induce *EVI1* expression, or were treated with an equal volume of DMSO for 72h as control. Cells were fixed and immunostaining was carried out with a primary antibody against *EVI1* and an Alexa 568 conjugated secondary antibody. As negative control cells were stained only with secondary antibody. DNA was counterstained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) in order to visualise the nucleus. Cells were microscopically examined at a magnification of 400x.

DMSO treated cells revealed only faint nuclear staining similar to the background observed in the negative control experiment, while ATRA treated and consequently *EVI1* expressing cells showed a clear nuclear signal (fig. 3.20). This is in agreement with published observations (Matsugi, et al., 1990).

The experiment was carried out once.

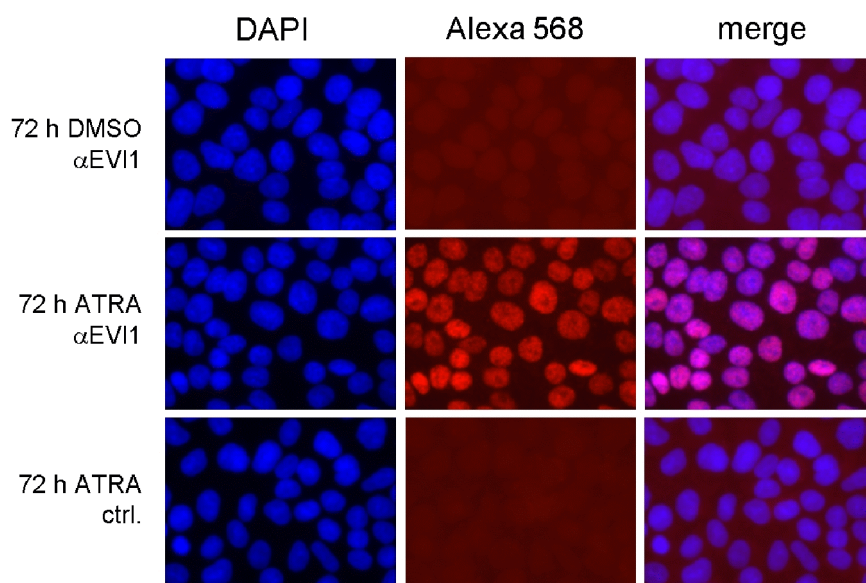


Fig. 3.20 Immunofluorescence of endogenous EVI1 in NTERA-2 cells. Cells were stained with an EVI1 antibody and an Alexa 568 conjugated secondary antibody (first two rows). As control experiment only secondary antibody was used (third row). Nuclei were counterstained with DAPI.

NTERA-2 cells grown on coverslips were transiently transfected with haEVI1/pEFzeo and the derived *EVI1* deletion constructs Δ AR, Δ AR-ZF2, ZF1-IR, ZF1, Δ ZF1, IR-RD, IR and the empty pEFzeo vector. As another negative control untransfected cells were used. 48h later cells were fixed and stained with a primary anti-HA antibody and a FITC-conjugated secondary antibody. DNA was counterstained with DAPI in order to visualise the nucleus. Cells were microscopically examined at a magnification of 400x.

Full length exogenously expressed *EVI1* and most *EVI1* deletion constructs produced a nuclear signal like that of endogenous EVI1 (fig. 3.20), however, some did not, like those containing only the ZF1 or the IR domain (fig 3.21). This was probably due to inefficient nuclear transport, and those constructs had to be excluded from further analyses.

According to the Expasy database (www.expasy.ch/sprot/), EVI1 contains a predicted nuclear localisation sequence (NLS) comprising the amino acids KEKFKENGKMFKDK between positions 421-434, which is located within the IR. This presents a feasible explanation as to why the ZF1 construct, which lacks the IR, did not produce a predominantly nuclear signal, on the other hand this could not be accounted for with the IR construct. A possible explanation could be that the IR resumes a different conformation when expressed without adjacent domains, which might disturb recognition of the NLS.

In order to obtain a ZF1 construct which would yield a nuclear localisation a tandem repeat of the SV40 large T antigen nuclear localization sequence as described by Fischer-Fantuzzi *et al* (Fischer-Fantuzzi and Vesco, 1988) was inserted before the stop codon of the ZF1 construct. After transient transfection of this construct into NTERA-2 cells and

immunostaining as described, nuclear localisation was restored (fig 3.22), and thus the first ZF1 construct could be replaced by the ZF1-NLS construct.

Immunofluorescence experiments were carried out once for each construct.

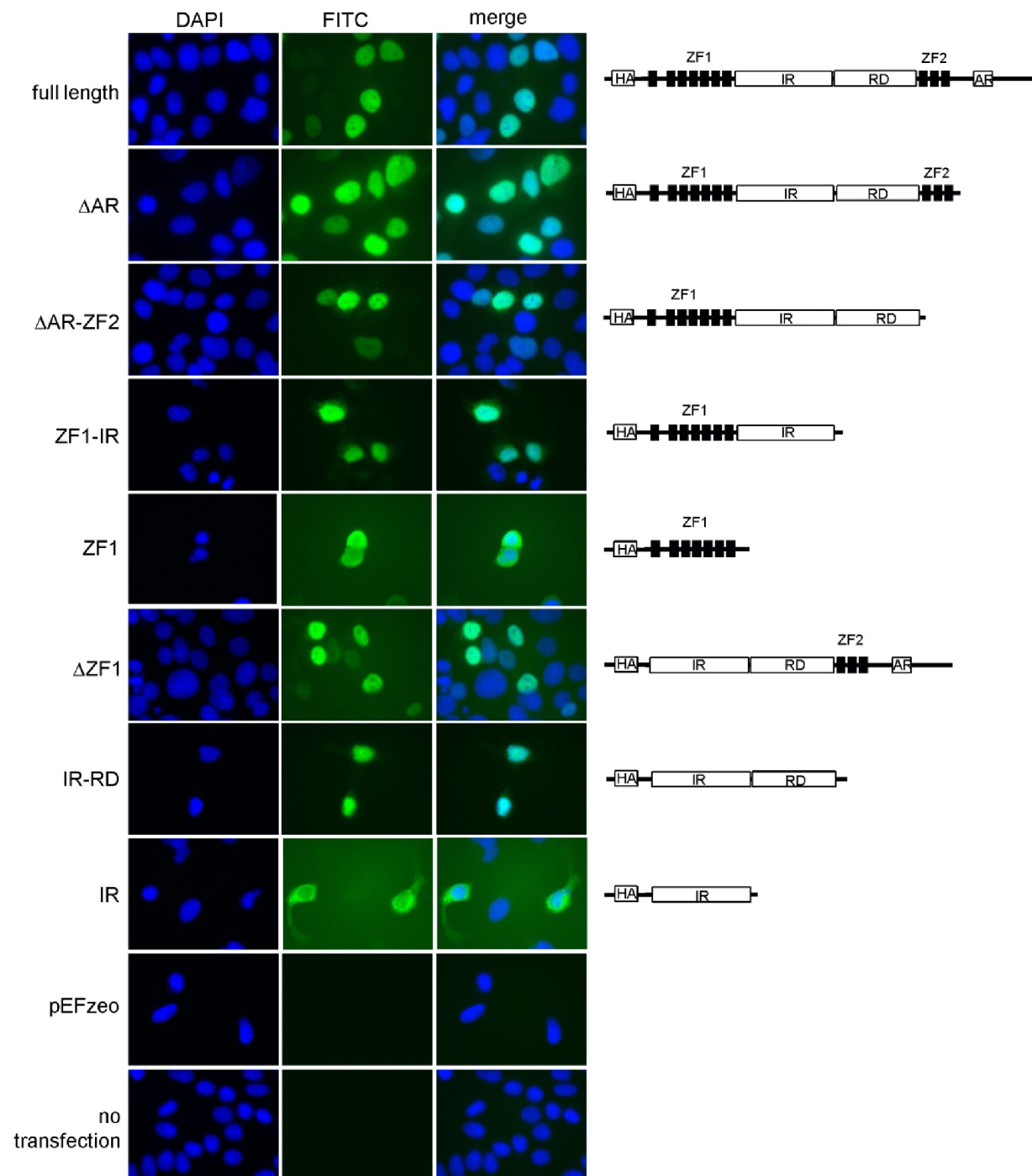


Fig. 3.21 Immunofluorescence of HA-tagged full length exogenously expressed *EVI1* and HA-tagged *EVI1* deletion constructs after transient transfection into NTERA-2 cells. Cells were stained with an antibody against the HA-tag and a FITC conjugated secondary antibody, and nuclei were counterstained with DAPI. Empty vector transfected and untransfected cells (last row "no transfection") served as negative control. Shown to the right is a schematic of the transfected constructs. HA = HA-tag, ZF1 = amino terminal zinc finger region, IR = intervening region, RD = repression domain, ZF2 = carboxy terminal zinc finger region, AR = acidic region.

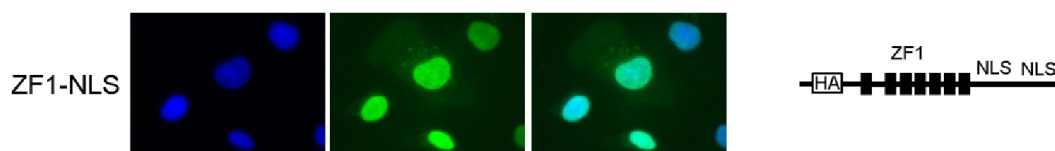


Fig. 3.22 Immunofluorescence of ZF1-NLS construct after transient transfection into NTERA-2 cells. Cells were processed as described in fig. 3.21. To the right a schematic of the transfected construct. HA = HA-tag, ZF1 = amino terminal zinc finger region, NLS = nuclear localisation signal (not drawn to scale).

In order to investigate if protein products derived from *EV11* deletion constructs had the correct size, these constructs were transiently transfected into NTERA-2 cells, which were harvested 48 h later. Lysates were analysed by immunoblotting using an antibody against the HA – tag.

All constructs produced bands of approximately the expected size (listed in table 3.1), though the products were consistently somewhat larger than expected (fig. 3.23). An explanation for this might be that in the calculation of the molecular weight according to <http://www.expasy.org/tools/peptide-mass.html> possible posttranslational modifications were not taken into consideration. The difference in band intensity is possibly due to a better transfection efficiency and/or stronger expression of smaller proteins in comparison with larger proteins, and/or better transfer efficiency of the smaller proteins during the blotting procedure, since with decreasing molecular weight of the expressed protein the bands become more intense.

The experiment was carried out once.

construct	Number of amino acids, excluding HA-tag	Number of amino acids, including HA-tag	Calculated molecular weight, including HA-tag [kD]
full length	1051	1078	121,53
Δ AR	818	845	95,50
Δ AR-ZF2	728	755	84,74
ZF1-IR	508	535	59,98
ZF1-NLS	281	308	35,83
Δ ZF1	781	808	90,60
IR-RD	458	485	53,81

Table 3.1 listing of the number of amino acids of *EV11* deletion constructs and respective calculated molecular weights

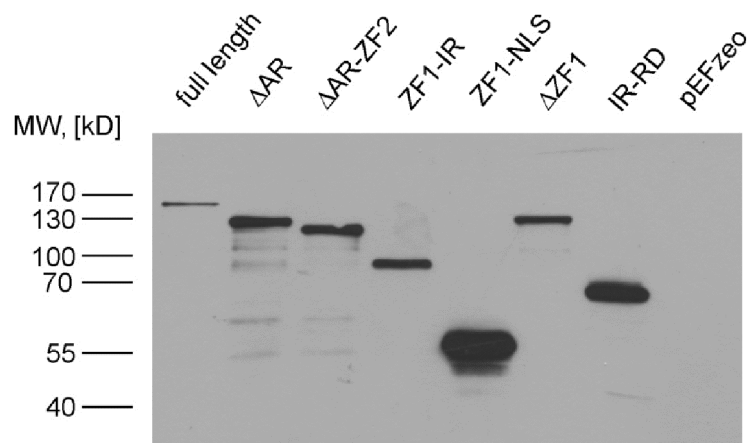


Fig. 3.23 Immunoblot analysis of *EVI1* deletion construct expression. Sizes of the bands of a molecular weight marker (MW) are shown on the left

3.8 EVI1 protein domains involved in modulating the ATRA response of the *EVI1* promoter and the *RARβ* RARE

To test which domains of EVI1 were involved in modulating the ATRA response of the *EVI1* promoter and the *RARβ* RARE, *EVI1* deletion constructs or the empty pEFzeo vector were cotransfected into NTERA-2 cells along with the reporter plasmids (i) EVI1(+86/+1106)/pGL3basic to investigate the repressive effect of EVI1 on the ATRA response of the EVI1 promoter (presented in chapter 3.8.1), or (ii) pRARE-tk-luc to investigate the enhancement of the ATRA response of the *RARβ* RARE by EVI1 (presented in chapter 3.8.2). The plasmid pRL-TK, which expresses renilla luciferase constitutively, was also cotransfected to allow normalisation of obtained RLU values. Cells were treated for 24h with 10 μ M ATRA or an equal volume of DMSO and then lysed and assayed for luciferase activity.

3.8.1 EVI1 protein domains involved in repressing the ATRA response of the *EVI1* promoter

Deletion of functional domains at either terminus of EVI1, i.e. of the first zinc finger domain (Δ ZF1), or of the acidic region (Δ AR), or of both acidic and second zinc finger domain (Δ AR-ZF2) did not abolish the repressive effect of EVI1 on the ATRA response of EVI1(+86/+1106)/pGL3basic (fig. 3.24). However, concomitant deletion of those 3 mentioned domains i.e. in the IR-RD construct, reduced but did not abolish the repressive effect on the ATRA response of EVI1(+86/+1106)/pGL3basic. Two constructs that were devoid of the repression domain (RD), i.e. the ZF1-IR and the ZF1-NLS constructs, even enhanced the ATRA response of EVI1(+86/+1106)/pGL3basic, the ZF-IR construct only slightly, but the ZF1-NLS construct distinctly. The RD was thus clearly important for the negative feedback of EVI1 on its own promoter.

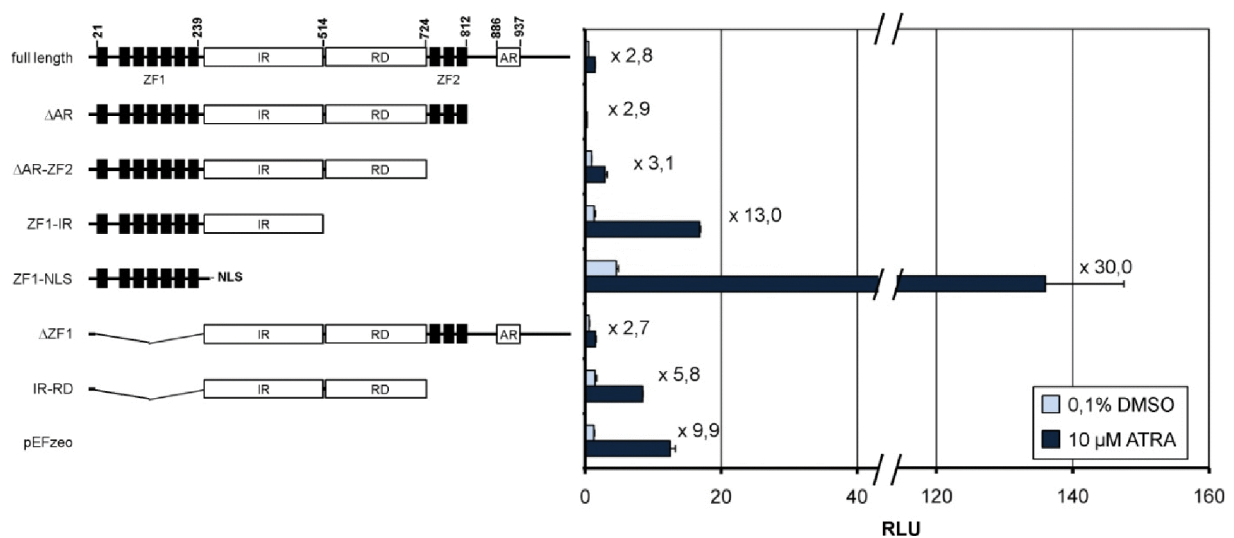


Fig. 3.24 Results of reporter gene assays of cotransfection of EVI1(+86/+1106)/pGL3basic as reporter plasmid, and the indicated *EVI1* deletion constructs or empty pEFzeo vector as cotransfected plasmids. RLU values were calculated as described in the legend to fig. 3.9.

The experiment was repeated once with generally similar results but slightly diverging induction factors. Full length EVI1 and constructs with a deletion of the acidic region (Δ AR), of both acidic and second zinc finger domain (Δ AR-ZF2), of the first zinc finger domain (Δ ZF1) counteracted the ATRA response of EVI1(+86/+1106)/pGL3basic. This coincides with the shown experiment. The ZF1-NLS construct still increased the ATRA response of EVI1(+86/+1106)/pGL3basic, though with an induction factor of 18,6 not as massively as in the shown experiment. Unlike in the shown experiment, the ZF-IR construct did not alter the ATRA response of EVI1(+86/+1106)/pGL3basic, and the IR-RD construct reduced the ATRA response of EVI1(+86/+1106)/pGL3basic to the same extent as full length EVI1. However, it was confirmed that the RD was important for the negative feedback of EVI1 on its own promoter.

3.8.2 EVI1 protein domains mediating the enhancement of the ATRA response of the *RAR β* RARE

Deletion of any domain of the EVI1 protein disturbed the enhancement of the ATRA response by EVI1, albeit to varying extent (fig. 3.25). While deletion of the AR had only a modest effect on the enhancement of the ATRA response by EVI1, the deletion of both the AR and ZF2 eliminated it. An EVI1 protein lacking the ZF1 or encoding only the ZF1 did not alter the ATRA response of pRARE-tk-luc. However, an EVI1 protein comprising only ZF1 and IR, or IR and RD, reduced the ATRA response of pRARE-tk-luc below the level which is achieved when only empty pEFzeo vector is cotransfected with pRARE-tk-luc.

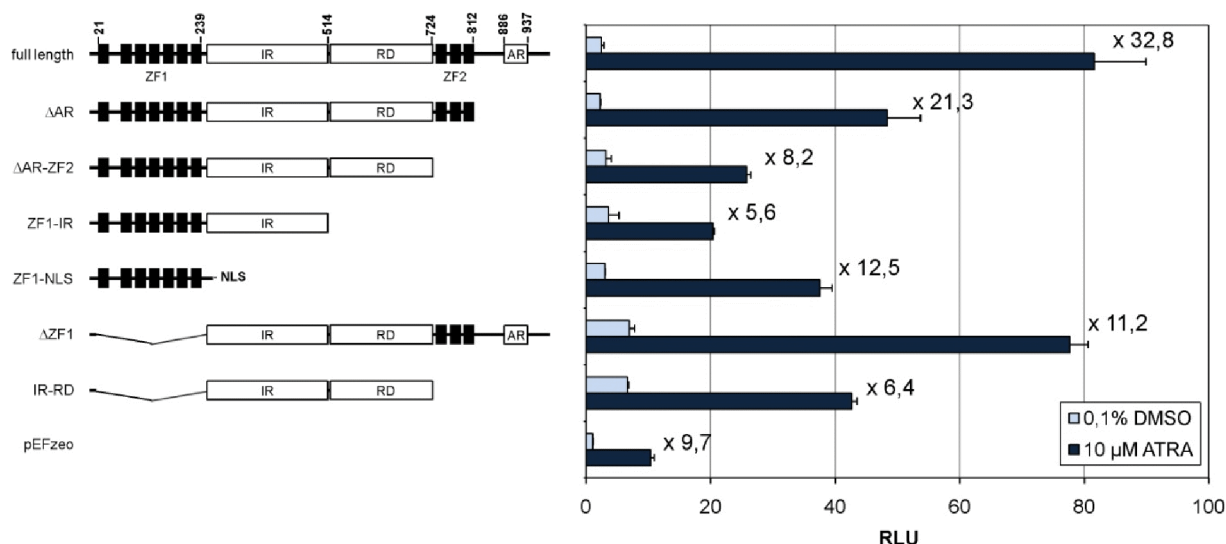


Fig. 3.25 Results of reporter gene assays of cotransfection of pRARE tk-luc as reporter plasmid, and the indicated *EVI1* deletion constructs or empty pEFzeo vector as cotransfected plasmids. RLU values were calculated as described in the legend to fig. 3.9.

The experiment was repeated twice with partially conflicting results. While in the experiment shown in fig. 3.25 full length *EVI1* yielded the strongest enhancement of the ATRA response of the *RARβ* RARE, the deletion of the AR and ZF2 domain enhanced the ATRA response of the *RARβ* RARE the most in another experiment. In the second repetition of the experiment, the deletion of the AR domain enhanced the ATRA response of the *RARβ* RARE most distinctively. However, the experiment shown in fig. 3.25 was chosen as representative experiment because the enhancement of the ATRA response of the *RARβ* RARE by full length *EVI1* with an induction factor of almost 33 was closest to what has been observed in other experiments, e.g. shown in fig. 3.17, fig. 3.19 and fig. 3.28. In summary, a specific functional domain of *EVI1* which was specifically and distinctively important for the enhancement of the ATRA response of the *RARβ* RARE could not be pinpointed.

Taken together, several or all *EVI1* protein domains contributed to the enhancement of the ATRA response by *EVI1*.

3.8.3 The ZF1 domain of *EVI1* exerts no dominant negative effect over full length *EVI1*

Since the ZF1-NLS construct caused a rather striking enhancement of the ATRA response of reporter plasmid *EVI1*(+86/+1106)/pGL3basic, which was in contrast to the downregulating effect of the other *EVI1* constructs, we asked whether this might be due to a dominant negative effect. A dominant negative effect may arise in cases of multiprotein complexes, in which the dysfunction of one of the involved proteins leads to dysfunction of the whole complex even though the other proteins function normally. Possibly, *EVI1* binds to the promoter region of the *EVI1* gene and participates in limiting its ATRA response to avoid

uncontrolled overexpression by attenuating the action of the transcription machinery. Then, by losing all domains except the ZF1 domain, this attenuating effect might be lost and thus give way to unrestrained transcription.

Cotransfections were carried out as described in chapter 3.8 with EVI1(+86/+1106)/pGL3basic as reporter plasmid and either haEVI1/pEFzeo, ZF1-NLS/pEFzeo or both as cotransfected plasmids. All plasmids were transfected in equal masses, e.g. EVI1(+86/+1106)/pGL3basic constituted one third, haEVI1/pEFzeo and ZF1-NLS/pEFzeo likewise. If only one of the latter plasmids was cotransfected, empty pEFzeo vector was used for filling up to the total DNA amount.

Like in previous experiments, the ATRA response of EVI1(+86/+1106)/pGL3basic was repressed when haEVI1/pEFzeo was cotransfected, but strongly enhanced when ZF1-NLS/pEFzeo was cotransfected (fig. 3.26). When all three plasmids were cotransfected together, the ATRA response of EVI1(+86/+1106)/pGL3basic was repressed rather than enhanced, which suggested that the ZF1-NLS/pEFzeo mediated enhancement of the ATRA response was not due to a dominant negative effect.

The experiment was carried out once.

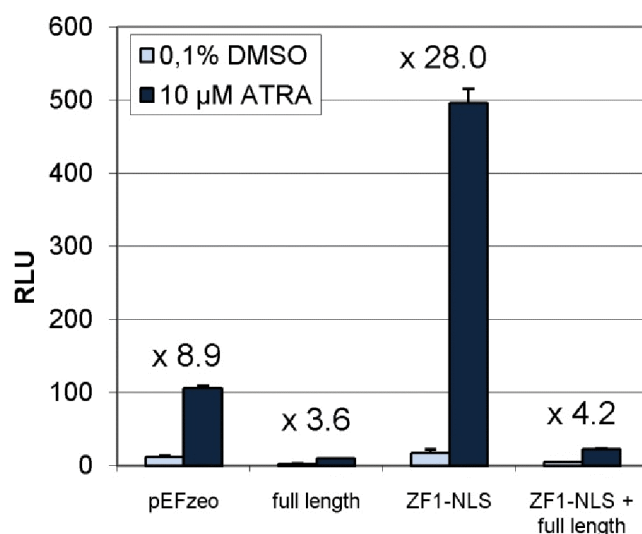


Fig. 3.26 Results of reporter gene assay with EVI1(+86/+1106)/pGL3basic as reporter plasmid and cotransfected EVI1 full length and/or ZF1-NLS expression construct as indicated. RLU values were calculated as described in the legend to fig. 3.9.

3.9 The role of the CtBP binding site of EVI1 for the modulation of the ATRA response of the *EVI1* promoter and the *RAR β* RARE

The repression domain (RD), which is necessary to mediate the ability of EVI1 to interfere with the ATRA response of its own promoter (chapter 3.8.1), contains two closely spaced binding sites for the corepressor C-terminal binding protein (CtBP). The more carboxy terminal one at position 586-587 was reported to be functionally more important (Izutsu, et al., 2001). We hypothesized that recruitment of CtBP may play a role for the repressive effect of EVI1 on its own promoter. To investigate this, an *EVI1* construct in which amino acids DL at positions 586 and 587 were changed to AS, or the respective *EVI1* wild type construct (kind gifts of Dr. Mineo Kurokawa, University of Tokyo, Japan) (Izutsu, et al., 2001) were cotransfected into NTERA-2 cells with EVI1(+86/+1106)/pGL3basic as reporter plasmid (presented in chapter 3.9.1). The plasmid pRL-TK, which expresses renilla luciferase constitutively, was also cotransfected to allow normalisation of obtained RLU values. Cells were treated for 24h with 10 μ M ATRA or an equal volume of DMSO and then lysed and assayed for luciferase activity. The loss of a corepressor binding site of EVI1 would be expected to weaken the ability of EVI1 to counteract the ATRA response of EVI1(+86/+1106)/pGL3basic.

As a control experiment, the above mentioned EVI1 wild type and the EVI1 construct with mutated CtBP binding site were cotransfected into NTERA-2 cells with pRARE-tk-luc as reporter plasmid, and the procedure continued as described (presented in chapter 3.9.2). The loss of a corepressor binding site of EVI1 would be expected to have much influence on the enhancement of the ATRA response of the *RAR β* RARE.

3.9.1 CtBP has only a moderate influence on the repression of the ATRA response of the *EVI1* promoter

Mutation of the CtBP binding site at position 586-587 from amino acids DL to AS on the background of the full length EVI1 protein only moderately decreased the ability of EVI1 to counteract the ATRA response (fig. 3.27).

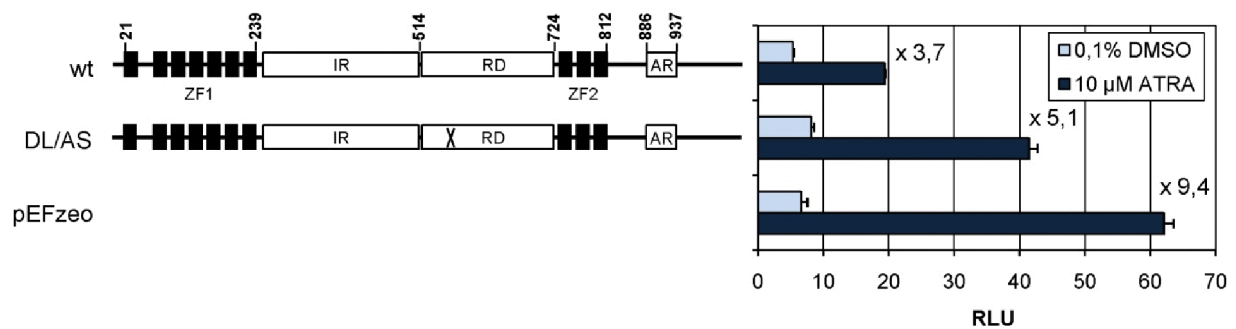


Fig. 3.27 Results of reporter gene assays of cotransfection of EVI1(+86/+1106)/pGL3basic as reporter plasmid, and *EVI1* wild type (wt), or *EVI1* with mutated CtBP binding site (DL/AS) as cotransfected constructs, and empty pEFzeo vector as control. RLU values were calculated as described in fig. 3.9.

These results were confirmed in a repetition of the experiment in which the mutated EVI1 notably but not fully counteracted the ATRA response to a similar extent as in the shown experiment. This suggests that CtBP was probably partially, but not fully responsible for the transcriptional repression by EVI1 in this context. However, EVI1 interacts with other corepressors, e.g. HDAC1 (Vinatzer U, 2001), SUV39H1 (Spensberger and Delwel, 2008), which might also participate in the repression of the ATRA response by EVI1.

3.9.2 The CtBP binding site of EVI1 appears to play a role for the enhancement of the ATRA response of the *RARβ* RARE through EVI1

Unexpectedly, mutation of the CtBP binding site at position 586-587 from amino acids DL to AS on the background of the full length EVI1 protein decreased the ability of EVI1 to enhance the ATRA response of the *RARβ* RARE compared to the enhancing activity of the *EVI1* wild type construct (fig. 3.28).

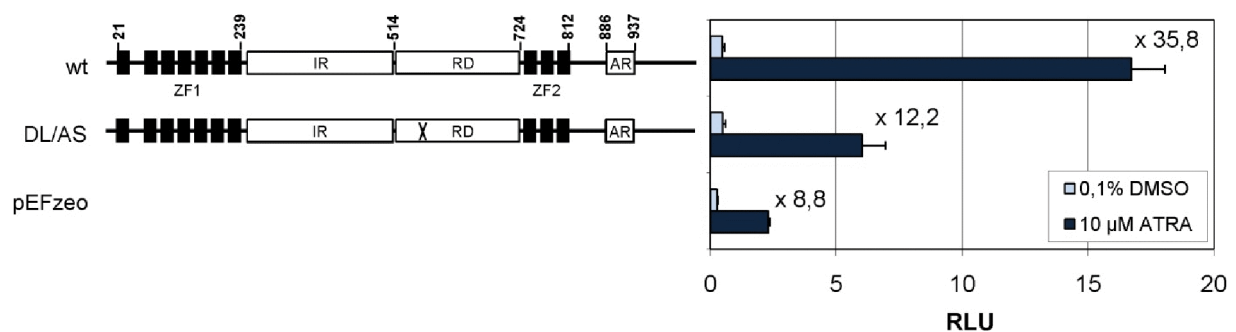


Fig. 3.28 Results of reporter gene assays of cotransfection of pRARE tk-luc as reporter plasmid, and wild type (wt), or mutated CtBP binding site (DL/AS) as cotransfected constructs, and empty pEFzeo vector as control. RLU were calculated as described in the legend to fig. 3.9.

The experiment was repeated twice with reproducible results (the measuring point with empty pEFzeo vector is identical to the one shown in fig. 3.6.2).

The finding is rather peculiar because disturbing corepressor binding would not be expected to reduce the ATRA response of the *RARβ* RARE. Besides the reported abolishment of CtBP binding (Izutsu, et al., 2001) the DL/AS mutation might affect the tertiary structure of EVI1 or the interaction with other proteins.

3.10 EVI1 does not directly bind to the regulatory regions of ATRA target genes

In order to determine whether EVI1 possibly binds directly to the *EVI1* or *RARβ* promoters in intact cells to exert its modulating effect of the ATRA response, ChIP assays were carried out. NTERA-2 cells grown in 15 cm dishes were transfected with haEVI1/pEFzeo expression plasmid or respective empty pEFzeo vector as control, and 24 h later cells were treated with 10 μM ATRA or an equal volume of its solvent DMSO. Another 24 h later cells were harvested and subjected to the ChIP procedure described in chapter 3.4.2 using an antibody against the HA tag or unspecific immunoglobulins. ChIP PCRs were carried out with primers flanking the *EVI1* DR5 RARE and the *EVI1* DR1 RARE, the primer pair for the negative control PCR was located in *EVI1* ex 3L, and the primer pair for the positive control was flanking the *RARβ* RARE (table 6.1.4). Direct target genes of EVI1 have been described for mouse, e.g. *GATA-2* (Yuasa, et al., 2005), but these findings have not yet been confirmed in humans, therefore a suitable positive control PCR was not available.

No distinct PCR product was obtained for any of the investigated promoter regions (fig. 3.29). This might signify that a direct binding of EVI1 to the DR5 RARE, or to the DR1 RARE of its own promoter or to the *RARβ* RARE seems to be unlikely but it could also be possible that these findings might relate to technical problems, e.g. the antibody was not suitable for ChIP or did not recognize a formaldehyde crosslinked epitope.

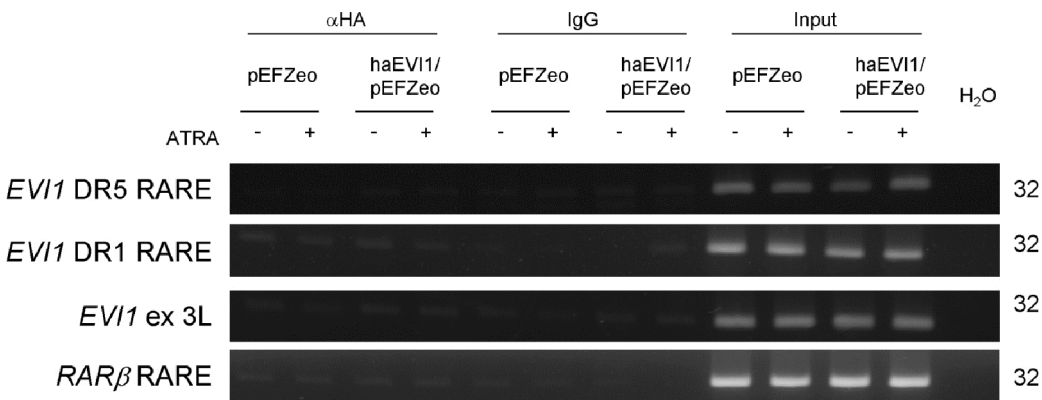


Fig. 3.29 NTERA-2 cells were transfected with haEVI1/pEFzeo or empty pEFzeo vector, and treated with ATRA or DMSO for 24 h. Chromatin immunoprecipitation (ChIP) was carried out using an antibody specific to the HA tag, or nonspecific mouse IgG as a negative control. Immunoprecipitated chromatin and input DNA as a positive control were amplified with primers flanking the indicated loci (table 6.1.4). The number of PCR cycles is indicated to the right.

The experiment was repeated with a slight modification of the cross-linking step: prior to the standard formaldehyde cross-linking step which covalently links DNA to proteins, cells were treated with disuccinimidyl glutarate (DSG) to covalently cross-link proteins to each other within multiprotein complexes. Supposedly, this two-step method is expected to enable the immunoprecipitation of proteins which are indirectly bound to DNA via other proteins. However, the results of this experiment were the same.

In a similar experiment, untransfected but also for 24h with 10 μ M ATRA or an equivalent volume of DMSO treated NTERA-2 cells were used for ChIP with an antibody against EVI1 or unspecific immunoglobulins. Likewise, an interaction between EVI1 and the DR5 RARE or the DR1 RARE of its own promoter or to the *RAR β* RARE could not be detected in the consequent ChIP PCR (data not shown).

4 Discussion

4.1 All-*trans* retinoic acid (ATRA) induces *EVI1* mRNA variants in NTERA-2 cells

The rapid and sensitive upregulation of *EVI1* and the expression of all isoforms of *retinoic acid receptor* (*RAR*) and *retinoic X receptor* (*RXR*) make NTERA-2 cells a very suitable model cell system to study the ATRA mediated regulation of *EVI1*. Furthermore, the cells are transfectable by standard lipofection reagents and yield acceptable transfection efficiency, which allows cotransfection of several plasmids and which is an important prerequisite for reporter gene assays.

Previous studies in the human teratocarcinoma cell line PA-1 cells have revealed that certain *EVI1* 5' end variants, i.e. *EVI1_1a* and *EVI1_1b*, are induced selectively by ATRA (Aytekin, et al., 2005). These findings were to be extended and verified in NTERA-2 cells. However, in this cell line all of the investigated *EVI1* mRNA variants showed similar induction upon ATRA treatment, albeit with different kinetics and to different extents. Thus, the upregulation of a specific *EVI1* mRNA variant through ATRA appears to be partly cell dependent.

Of note, PA-1 cells were treated with 100 nM ATRA (Aytekin, et al., 2005), while NTERA-2 cells were treated with 10 μ M ATRA, which is the concentration most widely used for this cell line (Kerley, et al., 2001; Spinella MJ, 1998). The *EVI1_1a* variant showed the strongest induction levels in both PA-1 (Aytekin, et al., 2005) and NTERA-2 cells, the *EVI1_1b* variant was induced to a lower extent in both cell lines. Remarkably, while in NTERA-2 cells *EVI1* mRNA levels remained upregulated until 96 h of ATRA treatment, levels started to decline already after 24 h in PA-1 cells, with the *EVI1_1b* variant decreasing more rapidly than the *EVI1_1a* variant (Aytekin, et al., 2005). This might be due to degradation of ATRA in cell culture medium, which naturally comes into effect earlier when the starting concentration is low, as it was by a factor of 100 lower for PA-1 cells than for NTERA-2 cells. The more rapid decrease of *EVI1_1b* mRNA in PA-1 cells might indicate that this variant possibly requires higher ATRA concentration for induction.

EVI1 mRNA was already induced with as little as 1 nM ATRA in NTERA-2 cells. In comparison, the high-performance liquid chromatography (HPLC) measured concentration of ATRA in the developing mouse embryo at 10.5 dpc varies from a minimum of 7.9 nM in the forebrain to a maximum of 122.4 nM in the spinal cord (Horton C, 1995). Assuming that physiological ATRA concentration are similar between humans and mice, this shows that the *EVI1* promoter is indeed very sensitive towards ATRA in NTERA-2 cells.

The results of the time-dependent induction of *EVI1*, assessed by real time quantitative-reverse transcriptase-PCR (RTQ-RT-PCR) analyses on the mRNA level and by immunoblot on the protein level are in good agreement with each other with respect to the strong and

rapid induction of *EVI1* upon incubation with ATRA and with respect to the difference in kinetics of upregulation of *MDS1/EVI1* compared to the other *EVI1* 5' mRNA variants.

4.2 A retinoic acid response element (RARE) is present in the 5'-UTR of *EVI1*

The rapid upregulation of the *EVI1* mRNA in NTERA-2 cells through ATRA lead to the hypothesis that the *EVI1* promoter may contain a RARE. Promoter elements mediating this induction had not been identified in the *EVI1* promoter up to now.

Using reporter gene assays, the promoter element mediating the ATRA response of the *EVI1* gene was attributed to a classical, inverted DR5 RARE CGACCTTTTGTGACCT between position +182 and +198 relative to the beginning of exon 1a, which is located within the transcribed region of the *EVI1* gene. This element and its immediate surroundings were necessary and sufficient to confer ATRA responsiveness to a heterologous luciferase reporter gene. The DR5 RARE was constitutively bound by RAR and RXR in intact cells.

In silico analysis of the *EVI1* promoter region using different on-line available programs with different search parameters has been very useful in identifying the RARE mediating the ATRA response of the *EVI1* promoter. This approach is suitable to find classical RAREs but irregular RAREs are likely to escape, and it should be taken into account that not every predicted RARE is necessarily a functional RARE. At any rate, this search has given a decisive clue for the design of a construct with a mutated RARE, which subsequently allowed the precise localization of the RARE mediating the ATRA response of the *EVI1* promoter.

Of note, the basal activity of the construct carrying a mutated RARE was elevated compared to that of the corresponding wild type construct. Unliganded and DNA bound retinoid acid receptors bind nuclear receptor corepressor (NCoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), which leads to transrepression of basal level promoter activity observed in transfected cells (Chambon, 1996). Mutation of the RARE abrogates binding of retinoid acid receptors and subsequently causes a loss of the transrepressional effect, which gives a feasible explanation for the increased basal activity of the mutated construct and further underlines that this RARE is functional.

EVI1(+140/+227)/pGL3basic, even though containing the DR5 RARE, did not show ATRA mediated induction of reporter gene activity in luciferase assays. The reason for this is unclear, the fragment might be too short to harbour all of the necessary factors for transcription initiation. Of note, the pGL3basic vector does not contain eukaryotic promoter or enhancer sequences which could compensate for this.

Even so, in summary the results of the deletion and mutation analysis of the *EVI1* promoter constructs give conclusive evidence that the DR5 RARE of the *EVI1* promoter is functional.

Unlike the majority of promoter elements utilized by RNA polymerase II, the DR5 RARE mediating the ATRA response of the *EV11* gene is located within the transcribed region of one of the alternative first exons.

Consensus binding sites for transcriptional regulators, though in the majority of cases located upstream of the transcription initiation site of RNA polymerase II, have also been reported to be located within the transcribed region for a many types of transcriptional regulators, e.g. p53 binding sites within the intronic and coding regions of the *MKP1* gene (Yang H, 2004), *Sp1* binding sites within the 5' UTR of the *GlyR β* gene (Tintrup H, 2001), or CREB binding sites within the 5' UTR of the *ZNF268* gene (Guo, et al., 2006). Moreover, response elements for nuclear receptors have also been found in intragenic regions, among them multiple functional androgenic response elements (AREs) within the coding region of the *androgen receptor (AR)* gene (Grad, et al., 1999) or a vitamin D response element (VDRE) within the coding region of the *GADD45* gene (Jiang, et al., 2003).

An intragenic DR5 RARE has been described for the *CD38* gene, to which retinoic acid receptors directly bind, and transcription is initiated upon the addition of ATRA (Kishimoto, et al., 1998). Thus the presence of a RARE within the transcribed region of a gene presents an unusual but not novel finding.

The promoter of the *RAR β* gene has been intensely investigated to clarify the mechanisms of retinoic acid mediated transcription upregulation. The *RAR β* gene is regulated by promoters P1 and P2. P1 is the more 5' promoter, it is not ATRA inducible and directs transcription of *RAR β 1*, which is a fetal isoform and is also expressed in small-cell lung cancer (Xu, 2007). P2 is ATRA inducible and contains a classical DR5 RARE (de Thé H, 1990); it directs transcription of the *RAR β 2* and *RAR β 4* transcript variants. *RAR β 2* is the most abundant and the major retinoic acid inducible isoform, while *RAR β 4* is generated by alternative splicing from the same primary transcript as *RAR β 2* but is translated into an amino terminally truncated RAR β (Sommer, et al., 1999; Xu, 2007). Additional isoforms, e.g. *RAR β 5* and *RAR- β 1'*, have been identified in human cancer cells (Peng, et al., 2004a; Xu, 2007). The etiology of *RAR β 4* upregulation in cancer cells remains unknown (Xu, 2007).

In addition to the known *RAR β 2* transcript further shorter transcript variants have been identified which were all induced by ATRA in several breast cancer cell lines, albeit to different extents. It has been hypothesized that the P2 promoter of the *RAR β* gene, which directs transcription of the *RAR β 2* transcript, also directs transcription of the shorter variants. Of note, all of the variants are translated into the same protein (Peng, et al., 2004b). It therefore seems possible that in analogy to the P2 promoter of the *RAR β* gene the transcription of several *EV11* 5' mRNA variants is initiated at the DR5 RARE of the *EV11* promoter.

Since the *EVI1* DR5 RARE is the only functional RARE within a region of -2.6 to +3.9 kb relative to the transcription start site of *EVI1* exon 1a, it is presumably the RARE mediating the upregulation of most of the alternative first exon variants in response to ATRA, with an exception possibly for the *EVI1_3L* variant remains, since the transcription start site of this variant is approximately 11 kb downstream from that of the other 5' variants. The site locations for RAREs have been described as ranging from 10.1 kb upstream to 8.1 kb downstream of the transcription start site of ATRA regulated genes (Balmer and Blomhoff, 2005).

4.3 ATRA does not directly regulate the *MDS1/EVI1* promoter

4 overlapping constructs derived from a 5.5 kb region surrounding the transcription start site of the *MDS1/EVI1* gene were cloned in front of a luciferase reporter gene. However, none of them showed increased luciferase activity in response to ATRA in reporter gene assays, including the one construct comprising exon 1 with a predicted DR2 RARE according to "Patch Search" (www.gene-regulation.com/cgi-bin/pub/programs/patch). It must therefore be concluded that the *MDS1/EVI1* gene is not a direct ATRA target gene, as also suggested by its late induction.

Since ATRA did not directly regulate the *MDS1* promoter we speculated that maybe the rapidly induced *EVI1* protein could be involved in mediating the slower ATRA response of the *MDS1* promoter. However, reporter gene assays showed that *MDS1/EVI1* was neither regulated by *EVI1* alone nor by *EVI1* in addition to ATRA. It cannot be excluded that regulatory elements outside the region investigated might confer direct responsiveness to ATRA and/or *EVI1* to the *MDS1/EVI1* promoter but all in all the mechanism of the ATRA mediated induction of *MDS1/EVI1* remains unclear.

4.4 *EVI1* modulates the ATRA response of two ATRA target genes

The *EVI1* DR5 RARE not only mediated transcriptional induction in response to ATRA, but also conferred negative feedback by the *EVI1* protein on its own promoter. This may serve to limit the extent and/or duration of its induction by retinoic acid. On the other hand, *EVI1* enhanced the ATRA response of a reporter gene driven by the *RAR β* RARE. *EVI1* is thus able to repress or enhance gene induction by ATRA in a promoter specific manner. Furthermore, this dual modulatory action argues against a nonspecific squelching effect due to supraphysiological amounts of *EVI1* protein present during the overexpression of *EVI1* through transient transfection as an explanation for the negative feedback of *EVI1* on its own promoter.

Autoregulatory loops have been described for a number of genes, such as *Gfi1b* (Vassen, et al., 2005), *Ovo1* (Nair, et al., 2007) and *Snail1* (Peiro, et al., 2006), and in most cases the protein product of the respective gene binds to its own promoter and represses transcription. However, the autoregulatory loop of the *EVI1* promoter appears to be a lot more complex than that, since the feedback loop is dependent on the presence of ATRA. In silico analysis using various online programmes to predict putative transcription factor binding sites (see chapter 3.3.2) did not predict EVI1 consensus DNA binding sites in the vicinity of the *EVI1* DR5 RARE or the *RAR β* RARE.

In agreement with this, ChIP experiments using an antibody against the HA epitope tag in transfected NTERA-2 cells failed to reveal an interaction between the EVI1 protein and the *EVI1* DR5 RARE, *RAR β* RARE or a predicted DR2 RARE and further sites of the presumptive promoter region of the *MDS1/EVI1* gene. The negative ChIP results may on the one hand be due to technical problems related to, e.g., suitability of the antibody to recognize a cross-linked epitope or a possible inaccessibility of the epitope. On the other hand, modulation of the ATRA response by EVI1 may not require interactions between EVI1 and DNA but rather with other cofactors recruited to the transcription start site.

A further complex feedback loop which involves ATRA and Hoxb4 has been described to regulate and fine-tune the expression of the ATRA target gene *Rar β* during mouse hindbrain segmentation. However, this feedback loop not only requires the *Rar β* RARE but also a distal enhancer. While *Rar β* expression is induced by ATRA in the early phase of hindbrain development, direct binding of Hoxb4 to the distal enhancer is required for the maintenance of *Rar β* expression in later stages (Serpente, et al., 2005). In contrast to this, feedback regulation at the *EVI1* promoter appears to take place only at the DR5 RARE, thus a similar mechanism seems to be precluded.

The behaviour of EVI1 in this case is rather reminiscent of that of ligand-dependent nuclear receptor coregulator proteins, such as receptor-interacting protein 140 (RIP140), and ligand-dependent corepressor (LCoR). Among those, RIP140 is the most intensely characterized coregulator.

RIP140 is a widely expressed ligand dependent corepressor for nuclear receptors such as estrogen receptor α (ER α), thyroid hormone receptor (TR), RAR and RXR, VDR, peroxisome proliferator-activated receptor α (PPAR α) and liver X receptor α (LXR α). The interaction of RIP140 thus mainly results in active suppression of the agonist-bound nuclear receptor. Recent reports have shown that RIP140 is also able to repress transcription independently of its interaction with ligand bound nuclear receptors. (Augereau, et al., 2006). Similar to *EVI1*, *RIP140* mRNA is induced by ATRA in a number of cell lines, including NTERA-2 cells, and the rapid induction soon after ATRA administration suggests that *RIP140* is a direct ATRA target gene (Kerley, et al., 2001).

The suppressive properties of RIP140 are due to its ability to recruit corepressors such as histone deacetylase (HDAC) and C-terminal binding protein (CtBP) and to displace coactivators such as p300/CBP-associated factor (P/CAF) from liganded RAR/RXR heterodimers. RIP140 thus seems to work as a platform for the recruitment of additional proteins required for chromatin remodeling (Parker, et al., 2003). It contains 4 repression domains, of which the first interacts with HDACs and the second with CtBP, which in turn also interacts with histone deacetylases. Furthermore, it contains 10 leucine-rich (LXXLL) motifs, which mediate the binding of RIP140 to nuclear receptors in a ligand dependent manner. The LXXLL motif binds to a cleft in the ligand-binding domain of nuclear receptors, when a ligand is bound. Motif 10, which is slightly divergent since it is LXXML, is reported to bind preferentially to retinoic acid receptors (Augereau P, 2006; Steel, et al., 2005).

The mechanisms RIP140 applies for suppression are in part of a competitive nature. For example, the RIP140 binding site to RAR/RXR partially overlaps that of coactivators of the p160 family, which are subsequently displaced (Augereau P, 2006). Furthermore, RIP140 competes with the ligand-dependent coactivator P/CAF for binding to liganded RAR/RXR (Chen, et al., 2004). Another suppressive mechanism RIP140 employs is the recruitment of HDAC1 and HDAC3. RIP140 mediated suppression of retinoic acid induced upregulation of its target genes can be alleviated by the HDAC inhibitor Trichostatin A (TSA) (Wei, et al., 2001; Wei, et al., 2000). RIP140 also directly interacts with CtBP which in turn recruits HDACs (Castet, et al., 2004; Vo, et al., 2001).

A further, but not extensively characterized, ligand dependent nuclear receptor corepressor is LCoR (ligand-dependent corepressor). LCoR also contains a LXXLL motif, which mediates binding to the nuclear receptor, 2 functional CtBP binding sites and binding sites for HDACs. The repressive activity of LCoR can be abolished by addition of TSA and can subsequently be considered as HDAC dependent. Of note, the repressive activity of LCoR has only been investigated with respect to the ER (Fernandes, et al., 2003).

According to the Expasy database (www.expasy.ch/sprot/), EVI1 contains neither contains helical leucine rich LXXLL motifs nor the divergent LXXML motif but shares other features with RIP140 and LCoR, like CtBP interaction domains (Chakraborty, et al., 2001) and HDAC interaction domains (Vinatzer U, 2001). Interestingly, both EVI1 and RIP140 share the ability to modulate the effects of ATRA signalling while simultaneously being a target of it. Of note, P/CAF does not contain LXXLL motifs and still binds to liganded RAR/RXR heterodimers (Chen, et al., 2004), thus the presence of this motif does not seem to be a prerequisite for binding of a protein to ligand bound retinoic acid receptors.

In summary, EVI1 interacts with some of the coregulators which also interact with ligand-dependent nuclear receptor coregulator proteins. This might provide a clue for the mechanism through which EVI1 modulates the ATRA response of retinoic acid target genes.

However, the mere ability for interaction with these factors does not suffice to attribute the function of a ligand-dependent nuclear receptor coregulator protein to the EVI1 protein.

It also might be possible that EVI1 counteracts its own ATRA response not by binding to retinoid receptors or other factors recruited to the *EVI1* DR5 RARE, and thus interfering directly at the transcription start site, but rather by sequestering important cofactors and preventing them from binding to the *EVI1* promoter. Such mechanisms have been described for the EVI1 mediated impairment of GATA-1 and PU.1 regulated promoters: the interaction between EVI1 and GATA-1 blocked GATA-1 from binding to DNA and did not involve binding of EVI1 to DNA (Laricchia-Robbio, et al., 2006), and similarly EVI1 blocked the interaction of PU.1 with crucial cofactors rather than displacing PU.1 from the DNA (Laricchia-Robbio, et al., 2009).

Mouse models gave evidence that RIP140 plays a crucial role in the ovary and in adipose biology, or more precisely, in female fertility and energy homeostasis. Unlike EVI1, RIP140 is not required for viability but essential for female fertility since female mice devoid of RIP140 fail to ovulate even when undergoing superovulation (Steel, et al., 2005). Considering the distinct expression of *EVI1* in oocytes (Morishita K, 1990b) and the ability of EVI1 to interact with the same cofactors as LCoR and RIP140 it is tempting to speculate that EVI1 might also play a role in oocyte development, in which steroid hormones play an important role. Apart from its proposed role of interfering with TGF β signalling in ovarian cancer (Sunde, et al., 2006), EVI1 might also interfere with steroid hormone nuclear receptor regulated transcription in ovarian cancer. Thus it would be interesting to see whether EVI1 also modulates the activity of other nuclear receptors, e.g. ER, in a ligand-dependent way.

4.4.1 Considerations regarding putative mechanisms for the modulation of the ATRA response by EVI1

In some biological assays EVI1 and MDS1/EVI1 show antagonistic properties, while in some they do not (Wieser, 2007). With respect to the modulatory effects on the retinoic acid response of the *EVI1* DR5 RARE and the *RAR β* RARE they behave likewise, and the presence of the PR domain in the MDS1/EVI1 variant does not alter the extent or direction of the modulation. The positive and negative effects of EVI1 on the ATRA response were mediated through different protein domains. While the repression domain (RD) of EVI1 was important for the repression of the ATRA response of the *EVI1* promoter, several or all domains of EVI1 contributed the enhancement of the ATRA response of the *RAR β* RARE.

Only those constructs containing the EVI1 RD and the intervening region (IR) were able to counteract the ATRA induction of the *EVI1* promoter, albeit to varying extent. Expression of the isolated IR+RD reduced the ATRA response of the *EVI1* promoter but full repression required also the domains adjacent to the IR and RD, e.g. the amino terminal zinc finger domain (ZF1) or the carboxy terminal zinc finger domain together with the acidic region (AR). The adjacent domains might be required to maintain the IR and RD in the conformation they adopt in the context of the full length protein. The region between the two zinc finger domains can therefore be considered as necessary but only partially sufficient for the repression of the ATRA response.

The IR, which is deleted in the $\Delta 324$ splice variant, has been reported to contribute to the repressive activity of EVI1 on certain promoters (Kilbey A, 1998). The RD has the ability to interact with the corepressor CtBP via a functional CtBP binding motif PLDLS at amino acid position 584 to 588 (Izutsu, et al., 2001), and further studies confirmed that the repressive effects of EVI1 were at least partially mediated through the interaction with CtBP at this binding motif (Chakraborty, et al., 2001). It therefore appears to be consistent with other findings reported in the literature that the RD and IR are of all other functional domains EVI1 the most important for the repressive effects of EVI1 on the ATRA induction on its own promoter.

A point mutation which disrupted the PLDLS consensus binding sites for the corepressor protein CtBP in the EVI1 RD (Izutsu, et al., 2001) reduced the negative modulation of the ATRA response by EVI1. One might therefore conclude that this effect depends at least partially on binding of CtBP, however, the very same point mutation also exhibited a reduced ability to enhance the ATRA response of the *RAR β* RARE. As the activity of enhancing the ATRA response of the *RAR β* RARE is unlikely to be mediated by a corepressor but rather maybe by a coactivator, CtBP is likely not responsible for negative modulation of the ATRA response by EVI1. Instead, this effect may require the recruitment of other corepressors which functionally interact with EVI1, like HDAC1 (Vinatzer U, 2001) or the histone methyl transferase SUV39H1 (Spensberger and Delwel, 2008).

For the enhancement of the ATRA response of the *RAR β* RARE the structural integrity of the EVI1 protein appeared to be crucial since deletion of any domain already reduced the activating effect. Deletion of the acidic region, which had been implicated in transcriptional activation by EVI1 (Tanaka, et al., 1994), caused the least reduction of the ATRA response enhancement of *RAR β* gene. Further deletion of the aminoterminal zinc finger domain (ZF1) then abolished the ATRA response of the *RAR β* gene, as did the deletion of the carboxyterminal zinc finger domain (ZF2). The modulation of the ATRA response by EVI1 possibly requires multiple and complex interactions with other cofactors.

The compelling dual modulatory action of EVI1 on the ATRA response of retinoid target genes naturally raises the question which mechanisms determine whether transcription of a target gene is repressed or enhanced.

A clue as to how the switch between “repression” and “augmentation” is achieved might come from the *MDS1/EVI1* paralogue *PRDM16* (*MEL*), which shows rather interesting properties with respect to nuclear receptors. *PRDM16* directly interacts via its zinc-finger regions with the nuclear receptor $\text{PPAR}\gamma$, which is a master regulator of adipogenic differentiation. In adipose tissue, *PRDM16* functions as a coregulatory protein which simultaneously stimulates certain genes required for differentiation into brown adipose tissue like *PGC-1 α* and *UCP1*, and represses genes required for differentiation into white adipose tissue like *resistin*. This dual behaviour is achieved via regulated docking of the corepressor CtBP. Like EVI1, *PRDM16* has 2 CtBP binding sites, of which the more carboxy terminal PLDLS motif binds both CtBP1 and CtBP2. The $\text{PPAR}\gamma$ -*PRDM16* complex then recruits CtBPs to the promoters of genes typically expressed in white adipose tissue, like *resistin*, which are subsequently repressed, but this complex recruits coactivators *PGC-1 α* and *PGC-1 β* to promoters of genes typically expressed in brown adipose tissue. The binding of *PGC-1 α* / *PGC-1 β* or CtBP to *PRDM16* appears to be mutually exclusive and thus they are probably competing for binding sites to *PRDM16*. However, how promoter specificity is thus achieved still remains unclear. Of note, binding of *PRDM16* to $\text{PPAR}\gamma$ is not dependent on the presence of its ligand, but the coactivating function of *PRDM16* is increased upon binding of the ligand rosiglitazone (Kajimura, et al., 2008; Seale, et al., 2008; Seale P, 2007).

Given the distinct similarity between the two paralogs *PRDM16* and *MDS1/EVI1* a similar mechanism for the switch between “repression” and “augmentation” could be assumed for the mechanism by which EVI1 and/or *MDS1/EVI1* modulates the transcription of retinoid target genes. While this model – representing a platform for competition of coactivators with the corepressor CtBP at the promoter bound nuclear receptor – may be useful in explaining why EVI1 with a mutated CtBP binding site within the repression domain (EVI1 DL/AS mutant) does not counteract ATRA mediated *EVI1* induction as efficiently as wild type EVI1, it fails to explain why EVI1 DL/AS also exhibits a reduced ability to enhance the ATRA response of the *RAR β* RARE.

The mere ability of EVI1 to modulate the response of ATRA target genes towards repression as well as towards augmentation possibly lies within its interaction with both repressors of transcription like HDACs (Vinatzer U, 2001), CtBP (Izutsu, et al., 2001) and SUV39H1 (Spensberger and Delwel, 2008) as well as with activators of transcription such as CBP/p300 and PCAF (Chakraborty, et al., 2001). Finally it might depend on the conformation of the multiprotein complex and the positions of cofactors relative to each other whether the ATRA

response is repressed or enhanced. In contrast to EVI1, other corepressors like RIP140 mainly interact with net transcription rate decreasing factors like CtBP (Castet, et al., 2004; Vo, et al., 2001), HDACs (Wei, et al., 2001; Wei, et al., 2000).

4.4.2 Significance of the modulatory effect of EVI1 on ATRA target genes

The currently preliminary findings regarding the ability of EVI1 to modulate the ATRA response of the *EVI1* gene and of the *RAR β* RARE only allow limited speculations as to what the biological significance of this effect may be, and call for more detailed studies aiming to find out whether there are more ATRA target genes whose ATRA mediated induction is modulated by EVI1, and how promoter specificity is achieved. Furthermore, it would be interesting to see if this ligand dependent modulatory action also extends to other nuclear receptors apart from retinoic acid receptors.

Possibly, the modulation of the response of ATRA target genes has an implication during embryonic development, which usually depends on temporally and spatially restricted expression of its regulators. To ensure that this complex interplay remains functional, feedback control, or general control of gene expression, is important for developmental regulators, since otherwise developmental abnormalities or tumour formation occur. Since ATRA target genes, and among them EVI1, fulfill important functions during embryogenesis it is not surprising that they are regulated in a complex and interdependent way. The activity of ATRA target genes can be controlled at different levels, e.g. retinoid synthesis and cellular transport, availability of retinoid receptors and cofactors and presence of orphan receptors competing for binding sites (Folkers, et al., 1998). The presented findings have added another level of regulation of controlling the response of ATRA target genes, namely by modulating their activity in an ATRA dependent way. This regulation can be considered as a fine tuning, very likely dependent on the cellular context, and leads to the hypothesis that ATRA target genes network and synergize to exert a mutual control.

ATRA is generally believed to work in an upregulatory fashion (Balmer and Blomhoff, 2002), and the presence of ATRA would lead to a global induction of direct target genes, provided that they are in a permissive state. Therefore it makes sense that a feedback mechanism, which is “alerted” by the same regulator, then conducts and, if necessary, restricts the ATRA response to only certain target genes in order to prevent too many pleiotropic and possibly harmful effects.

4.4.3 Possible therapeutic relevance of the ATRA response modulating activity of EVI1

EVI1 acts both as a downstream target of ATRA and as an upstream regulator the ATRA response. It may therefore contribute to the biological effects of ATRA in a dual manner: firstly, *EVI1*, after being induced by ATRA, acts directly on its target genes; and secondly, EVI1 modulates the expression of other ATRA target genes, provided that ATRA is continually present, since the modulation seems to be ATRA dependent.

The latter behaviour could be the basis for a therapeutic intervention: *EVI1* overexpressing leukemias might exhibit increased sensitivity to ATRA induced differentiation.

This has already been reported in an analogous case in NTERA-2 cells, in which *RIP140*, an ATRA-dependent corepressor of retinoic acid signalling, has been silenced. The induction of the *RAR β* gene, which is considered as a tumour suppressor gene (Alvarez, et al.), as well as differentiation and G1 cell cycle arrest occurred with accelerated kinetics after ATRA treatment in *RIP140* silenced cells than in control cells (White, et al., 2003). Furthermore, *RIP140* silencing lead to increased sensitivity of NTERA-2 cells to low doses of ATRA (Heim, et al., 2007). A similar finding has been reported with the knockdown of the ATRA signalling corepressor *PRAME*, which restored ATRA sensitivity in otherwise ATRA resistant cell lines, and inhibited melanoma growth (Epping, et al., 2005).

Conversely, the overexpression of *EVI1* in addition to ATRA treatment might have the similar effect as knocking down an inhibitor of ATRA signalling, namely enhancing sensitivity to ATRA, upregulate the tumour suppressor *RAR β* , promote differentiation and impede unrestrained growth of tumour cells. However, this speculative assumption needs to be substantiated by further experiments, but if the hypothesis can be verified, new treatment possibilities may emerge for myeloid leukemia with *EVI1* overexpression.

For further possibilities of therapeutic exploitation of it would be useful to know which ATRA target genes can be modulated by overexpression of *EVI1*. A microarray analysis would be the method of choice to clarify this.

4.5 *EVI1* and neuronal differentiation

During a 4 week neuronal differentiation of NTERA-2 cells, which consisted of an initial aggregation phase with ATRA treatment and a subsequent ATRA-free differentiation phase, cEVI, a region common to all known *EVI1* 5' mRNA variants, and MDS1/EVI1, were differentially regulated.

A rapid upregulation of cEVI1 was observed at the beginning of the aggregation phase. This was followed by a decline of expression from the middle to the end of the aggregation phase. At the onset of the differentiation phase cEVI1 was upregulated again. MDS1/EVI1 levels continually increased during the aggregation phase and decreased during the differentiation phase.

4.5.1 Antagonistic regulation of *EVI1* and *MDS1/EVI1* during neuronal development

In contrast to the upregulation of cEVI1 during the neuronal differentiation phase, the MDS1/EVI1 variant was downregulated. This implies that *EVI1* might play a role during neuronal differentiation, while *MDS1/EVI1* might interfere with this process and thus the two variants may act as antagonists. This behaviour is in contrast to that regarding the modulating effect of the ATRA response, where *EVI1* and *MDS1/EVI1* act likewise, and it argues for a context and/or cell type dependent behaviour of these two variants.

Expression of *Prdm* paralogs – to which *MDS1/EVI1* belongs as being synonym with *PRDM3* - during mouse neurogenesis has shown interrelated and complex interplay, with some paralogs being expressed in spatially restricted manner with mutual sharp borders. This suggests that members of the *Prdm* family are involved in neuronal specification and that they may repress each other (Kinameri, et al., 2008).

Several lines of evidence suggest a relationship between *MDS1/EVI1* and Notch signalling. The *Drosophila* ortholog of *MDS1/EVI1*, *Hamlet*, instructs neuron and glial cell fate and mediates Notch signalling in a neural lineage specific manner (Moore, et al., 2004). In *C. elegans*, the *EVI1* ortholog *egl-43* is necessary for cell fate specification and acts downstream of the *Notch* ortholog *LIN-12* (Hwang, et al., 2007; Rimann and Hajnal, 2007). During mouse neurogenesis, expression of *Prdm16* (*MEL*, or "*Mds1/Evi1* like") is regulated by the Notch-Hes pathway (Kinameri, et al., 2008).

Notch is a transmembrane receptor for the transmembrane ligands Delta and Jagged present on the surface of neighboring cells. In mammals, four Notch receptors (Notch1-Notch4) and five Notch ligands (Delta1, Delta-like3, Delta-like4, Jagged1 and -2) have been identified. Ligand-receptor interaction redirects the fate of the signal-receiving cell to a non-neuronal phenotype by inhibiting neuronal differentiation. During development this pathway is required for the maintenance and expansion of neural progenitor pool and it inhibits premature onset of neurogenesis. On the other hand, cells that express Delta or Jagged

ligands and which have neuronal potential acquire a neuronal phenotype. Accordingly, low levels of Notch signalling are a prerequisite for neuronal differentiation (Chiba, 2006; Shimojo, et al., 2008).

Considering the apparent evolutionary conserved regulation of *MDS1/EVI1* orthologs in several species through Notch signalling and the similarity between *MEL* and *MDS1/EVI1*, it can be hypothesized that Notch signalling maybe regulates *MDS1/EVI1* in humans. Decreasing Notch signalling at the onset of neuronal differentiation of NTERA-2 cells might provide an explanation for the observed decrease of *MDS1/EVI1* expression.

Since there is no ATRA present during neuronal differentiation phase there must be one or more other factors which mediate the upregulation of *EVI1*, but it remains unclear what those may be.

Despite the limitations of this differentiation model with respect possible heterogeneity of the differentiated cells and the elaborateness of the procedure, the neuronal differentiation of NTERA-2 cells serves as suitable starting model to study the role of *MDS1/EVI1* and *EVI1* during this process.

4.5.2 Is *EVI1* necessary and/or sufficient for neuronal development?

The question emerges whether *EVI1* is necessary and/or sufficient for neuronal differentiation. Generating a stable NTERA-2 cell line in which *EVI1* is silenced and then submitting these cells to neuronal differentiation could clarify whether *EVI1* is necessary for neuronal differentiation. Considering the circumstance of the downregulation of *MDS1/EVI1* during the neuronal differentiation phase it would be interesting to see if the knockdown of specifically this variant would facilitate neuronal differentiation.

Generating a stable *EVI1* overexpressing NTERA-2 cell line might clarify whether *EVI1* is sufficient for neuronal differentiation. This has already been found in the P19 cell line, in which constitutive overexpression of *Evi1* caused a change in morphology towards a neuronal phenotype. Furthermore, a consistent upregulation of neuronal markers *Map2* and *TrkA* was observed in all generated clones. This suggests that *Evi1* is sufficient to promote neuronal differentiation in this cell line (Kazama, et al., 1999).

5 Abstract

The *ecotropic viral integration site 1* (*EVI1*) gene codes for a nuclear DNA binding protein containing two domains of zinc finger clusters (ZF1 and ZF2), an intervening region (IR), a repression domain (RD), and an acidic region (AR). *EVI1* plays important roles in embryonic development and in myeloid leukemogenesis. It is transcribed into several mRNA species with variable 5'-ends. The transcription of one of these mRNA variants, MDS1/*EVI1*, initiates at the *myelodysplasia syndrome 1* (*MDS1*) promoter located distally from the *EVI1* promoter, and the transcript gives rise to an *EVI1* protein with an extended amino terminus, i.e. the MDS1/*EVI1* variant. The other *EVI1* 5' mRNA variants *EVI1_1A*, *EVI1_1B*, *EVI1_1D*, *EVI1_3L*, whose transcription start sites lie closely spaced in the region of the *EVI1* promoter, are most likely translated into the same protein, i.e. the *EVI1* protein variant. The functions of the MDS1/*EVI1* protein variant are partially different from those of the *EVI1* protein variant. So far all-*trans* retinoic acid (ATRA) is the only known physiological regulator of *EVI1* in mammalian cells.

ATRA rapidly induced the *EVI1* mRNA in the human teratocarcinoma cell line NTERA-2. This response was already detectable after 3 h with an ATRA concentration of 10 μ M and affected all *EVI1* 5' mRNA variants, including the MDS1/*EVI1* variant, albeit to variable degrees. An ATRA responsive region of approximately 200 bp was identified within exon 1a. Chromatin immunoprecipitation (ChIP) experiments confirmed constitutive binding of retinoic acid receptors RAR and RXR to a DR5 type retinoic acid response element (RARE) in this region. No RARE could be identified in the promoter region of *MDS1/EVI1*. The mRNA variants giving rise to the short *EVI1* protein are thus direct targets of retinoic acid signalling and their regulation via this pathway is mediated through an intragenic RARE, while *MDS1/EVI1* is only indirectly regulated by ATRA.

The *EVI1* as well as the MDS1/*EVI1* protein exerted a negative feedback on the induction of the *EVI1* promoter by ATRA. For this effect, the smallest ATRA responsive region of the *EVI1* promoter containing the *EVI1* DR5 RARE was sufficient, and it required the RD of the *EVI1* protein. In contrast to this, *EVI1* had the opposite effect on the RARE of the *RAR β* gene, i.e. *EVI1* enhanced the ATRA mediated induction of the *RAR β* RARE. For this effect all of the functional domains of *EVI1* were necessary.

This work has added important information about the molecular mechanisms through which *EVI1* acts, a protein with crucial roles in embryonic development and in myeloid leukemogenesis but with hitherto only partially understood molecular functions.

5.1 Zusammenfassung

Das "*ecotropic viral integration site 1*" (*EVI1*) Gen kodiert für ein nukleäres DNA-bindendes Protein, das zwei Zinkfinger Domänen (ZF1 und ZF2), eine Zwischenregion (IR), eine Repressionsdomäne (RD) und eine saure Region (AR) enthält. *EVI1* hat wichtige Funktionen in der Embryonalentwicklung und bei der Entstehung myeloischer Leukämien. Dieses Gen wird in mehrere mRNAs mit variablen 5' Enden transkribiert. Die Transkription einer dieser mRNA-Varianten, MDS1/*EVI1*, initiiert am distal vom *EVI1* Promoter gelegenen *myelodysplasia syndrome 1* (*MDS1*) Promoter, und das Transkript wird in eine Proteinvariante mit einem verlängerten N-Terminus translatiert, d.h. die MDS1/*EVI1* Proteinvariante, translatiert. Die anderen *EVI1* 5' mRNA Varianten *EVI1_1A*, *EVI1_1B*, *EVI1_1D*, *EVI1_3L*, deren Transkriptionsstartpunkte relativ nah beieinander in der *EVI1* Promoter Region liegen, werden höchstwahrscheinlich in die kurze *EVI1* Proteinvariante translatiert. Die Funktionen der MDS1/*EVI1* Proteinvariante unterscheiden sich teilweise von denen der *EVI1* Proteinvariante. Bislang ist all-*trans* Retinsäure (ATRA) der einzige bekannte physiologische Regulator des *EVI1* Gens in Säugetierzellen.

ATRA bewirkt eine rasche Induktion der *EVI1* mRNA in der humanen Teratokarzinomzelllinie NTERA-2. Diese Reaktion war schon nach 3 Stunden bei einer ATRA Konzentration von 10 µM nachweisbar und betraf alle *EVI1* 5' mRNA Varianten, inklusive der MDS1/*EVI1* Variante, wenn auch in unterschiedlichen Ausmaßen. Innerhalb von Exon 1a wurde eine etwa 200 bp große ATRA regulierbare Region identifiziert. Mittels Chromatin-Immunpräzipitation (ChIP) wurde konstitutive Bindung der Retinsäurerezeptoren RAR und RXR an ein DR5 Retinsäure-reguliertes Element (RARE) in dieser Region bestätigt. Ein funktionelles RARE konnte in der Promoterregion von *MDS1/EVI1* nicht identifiziert werden. Die mRNA Varianten, die in die kurze *EVI1* Proteinvariante translatiert werden, sind daher direkte Ziele des Retinsäuresignalweges, und die Regulation durch diesen Signalweg erfolgt durch ein intragenisches RARE, während *MDS1/EVI1* nur indirekt von ATRA reguliert wird.

Sowohl das *EVI1* als auch das MDS1/*EVI1* Protein übten eine negative Rückkopplung auf die Induktion des *EVI1* Promoters durch ATRA aus. Für diesen Effekt reichte die kleinste ATRA regulierbare Region des *EVI1* Promoters aus, und die RD des *EVI1* Proteins war erforderlich. Im Gegensatz dazu hatte *EVI1* den gegenteiligen Effekt auf das RARE des RARβ Gens, d.h. *EVI1* verstärkte die ATRA-vermittelte Induktion des RARβ RARE. Für diesen Effekt waren alle funktionellen Domänen von *EVI1* erforderlich.

Mit dieser Arbeit wurden wichtige Erkenntnisse über die molekularen Mechanismen gewonnen, durch die *EVI1* agiert, ein Protein von essentieller Bedeutung in der Embryonalentwicklung und in der Entstehung von myeloischen Leukämien, aber mit bislang nur teilweise verstandenen molekularen Funktionen.

6 Appendix

6.1 Primer sequences

6.1.1 Primers for semiquantitative PCR and for PCR with genomic DNA

Primer name	Primer sequence	acc. no.	position	AT
cEVI fwd ¹	AGCAACGTCGAATCAAGACCTGCTTCAGAT	X54989	2110– 2139	62°C
cEVI rev ¹	ACTGACTGTAAGAGCTCACTGGCCTCAGGT	X54989	2381– 2361	62°C
EVlex1a fwd ²	TATTGCTGAGTTGAGGCCATAG	AF487424	372-393	58°C
EVI1ex1b fwd ²	GGGCTGCTTATCTACGTTGC	AF487422	1 –18	58°C
EVlex3L fwd ²	AGATTTGTGAACTGGCTTGAGA	AF487423	127– 148	58°C
EVlex1a,1b,3L rev ²	TTGAAAATGCTGAGTGAGGAGT	X54989	424-403	58°C
EVI1ex1d fwd ²	CTTCTTGACTAAAGCCCTTGGA	AF164154	1234- 1255	60°C
EVI1ex1d rev ²	GTA CT TGAGCCAGCTTCCAACA	AF164154	1357- 1335	60°C
MDS1/EVI1 fwd ²	GGGGCAGGACTAGGAATATGGAC	S69002	2362– 2384	58°C
MDS1/EVI1 rev ²	TTGCCGTTCTTCGTGGATATCCG	X54989	327– 305	58°C
cycD fwd ³	ATATTGGAAAATGTGGAAGTGAAAGG	NM_005038	593-618	60°C
cycD rev ³	TCGCCAGAGCCATCTTTTG	NM_005038	711-693	60°C
RAR α fwd	GACCAGATCACCTCCTCAA	NM_001145302.1	1332- 1351	60°C
RAR α rev	TCCGCACGTAGACCTTTAGC	NM_001145302.1	1659- 1640	60°C
RAR β 2 fwd	TTCGAAGCAAGAATGCACAG	NM_016152	598-617	60°C
RAR β 2 rev	ATGGTCAAGCCAGTGAAACC	NM_016152	852-833	60°C
RAR γ fwd	CTCATCACCAAGGTCAGCAA	NM_000966.4	1049- 1069	60°C
RAR γ rev	GAAGGTCATGGTGTCTGCT	NM_000966.4	1349- 1330	60°C
RXR α fwd	AACGACCCTGTCACCAACAT	NM_002957.4	852-871	60°C
RXR α rev	TCAGCACCTGTCAAAGATG	NM_002957.4	1119- 1100	60°C
RXR β fwd	GGGACAACAAAGACTGCACA	NM_021976	904-923	60°C
RXR β rev	TCAGCTGCCTGACAGATGTT	NM_021976	1210- 1191	60°C

RXR _γ fwd	TGTGGTCAACAGTGTCTAGCA	NM_006917.3	603-622	60°C
RXR _γ rev	CCTCACTCTCAGCTCGCTCT	NM_006917.3	970-951	60°C
D22S686 fwd	TTGATTACAGAGTGGCTCTGG	G08094	77-96	58°C
D22S686 rev	TAAGCCCTGTTAGCACCACT	G08094	260-237	58°C
NeuD fwd ⁴	gccccagggttatgagactatcact	NM_002500.2	177-201	60°C
NeuD rev ⁴	ccgacagagcccagatgtagtctt	NM_002500.2	699-675	60°C
GluR fwd ⁴	AACCTGCAGAACCGCAAG	NM_021569.2	1393-1410	60°C
GluR rev ⁴	GCTTGATGAGCAGGTCTATGC	NM_021569.2	1725-1706	60°C
Tau fwd ⁴	GTAAAAGCAAAGACGGGACTGG	NM_016841.3	532-553	58°C
Tau rev ⁴	ATGATGGATGTTGCCTAATGAG	NM_016841.3	1043-1022	58°C

Table 6.1 First column: primer name, with forward primers carrying the suffix "fwd", and reverse primers the suffix "rev". Second column: Primer sequences, from 5'- to 3'-end.

cEVI1 comprises the sum of all EVI1 mRNA 5'-end variants. EVI1_1a, EVI1_1b, EVI1_1d, and EVI1_3L designate EVI1 5'-end variants starting with exons 1a, 1b, 1d, and 3L, respectively. *cycD* (*cyclophilinD*) served as an internal reference gene to control for the amount of cDNA present in each sample. Third column: Genbank accession numbers of the sequences used for primer design. Fourth column: positions of the oligonucleotides from 5' to 3', within the sequences indicated in the third column. AT = Annealing temperature.

¹ primers were previously published by Russell et al (Russell, et al., 1994).

² primers were previously published by Aytekin et al (Aytekin, et al., 2005)

³ primers were previously published by Vinatzer et al (Vinatzer U, 2003)

⁴ primers were previously published by Megiorni et al (Megiorni, et al., 2005)

6.1.2 Primers for RTQ-RT-PCR

Primer name	Primer sequence	acc. no	position
cEVI1-F ¹	ACCCACTCCTTTCTTTATGGACC	NM_005241.2	2136–2158
cEVI1-R ¹	TGATCAGGCAGTTGGAATTGTG	NM_005241.2	2273–2252
EVI1_1a-F ¹	TATTGCTGAGTTGAGGCCATAG	NM_001105078.2	981-1002
EVI1_1a-R ¹	CTTCCAACATCTGGTTGACTGG	NM_001105078.2	1074-1053
EVI1_1b-F ¹	TGGGTCGTCCGGCCTT	AF487422	80-95
EVI1_1b-R ¹	GCTTCCAACATCTGGTTGACTG	NM_005241.2	117-96
EVI1_1d-F ¹	CTTCTTGACTAAAGCCCTTGGA	NM_001105077.2	69-90
EVI1_1d-R ¹	GTACTTGAGCCAGCTTCCAACA	NM_001105077.2	191-170
EVI1_3L-F ¹	GGTATCTTAGTGTATATCTTGCCCTTTGT	AF487423	263-291
EVI1_3L-R ¹	GCGCAATGTCTGCAACTACTCT	NM_005241.2	220-199
MDS1/EVI1-F ¹	CCAGTTATGGATGGGAGATCTTAGAC	S69002	2456-2481
MDS1/EVI1-R ¹	CCAGCGAATCTAATGTACTTGAGC	NM_005241.2	143-120
cycD-F ²	ATATTGGAAAATGTGGAAGTGAAAGG	NM_005038	593-618
cycD-R ²	TCGCCAGAGCCATCTTTTG	NM_005038	711-693

Table 6.2 First column: primer name, with forward primers carrying the suffix “F”, and reverse primers the suffix “R”. Second column: Primer sequences, from 5'- to 3'-end.

cEVI1 comprises the sum of all EVI1 mRNA 5'-end variants. EVI1_1a, EVI1_1b, EVI1_1d, and EVI1_3L designate EVI1 5'-end variants starting with exons 1a, 1b, 1d, and 3L, respectively. *cycD* (*cyclophilinD*) served as an internal reference gene to control for the amount of cDNA present in each sample. Third column: Genbank accession numbers of the sequences used for primer design. Fourth column: positions of the oligonucleotides from 5' to 3', within the sequences indicated in the third column. Fifth column: Annealing temperature.

¹ primers were previously published by Aytekin et al (Aytekin, et al., 2005)

² primers were previously published by Vinatzer et al (Vinatzer U, 2003)

6.1.3 Primers for plasmid constructs

Primer name	Primer sequence	acc. no.	position	AT
MDS1prom-2968 NheI fwd	GCTAGCTAGCCAGGCACTTGAGAATG	AC007849	64908- 64924	58°C
MDS1prom-1975 Bgl II rev	GAAGATCTCCAGCAGCACCTGAAGAT	AC007849	65901- 65884	58°C
MDS1prom-KpnI 2027 fwd	GGGGTACCATTGGAGGGACAAGACCT	AC007849	65849- 65866	58°C
MDS1prom-915 rev	TACAACCTCGGGGTCAAAAG	AC007849	67095- 67076	58°C
MDS1prom-920 fwd	TGCAAGAAGGTTGTACCAAAA	AC007849	66892- 66912	58°C
MDS1prom+357 XhoI rev	ACCGCTCGAGACCTACTTGTGGCCAGTT	AC007849	68232- 68215	58°C
MDS1prom+310 Bgl II fwd	GAAGATCTACAGCATGAGATCCAAAG	AC007849	68185- 68202	55°C
MDS1prom+2456 rev	CGTATGTCTTGCTTGGCAAT	AC007849	70449- 70430	55°C
EVI1prom 3903 XhoI fwd	CCGCTCGAGTCTGGAGGGAAGAAGATGAGAAC AA	AC078985	117529- 117553	60°C
EVI1prom 1258 KpnI rev	GGGGTACCGGTGGGTTTCTCTGCCGGCTTGT	AC078985	120174- 120152	60°C
EVI1prom+15 KpnI fwd	GGGGTACCCACACCACACTTGTGCTT	BX640908	15-32	60°C
EVI1prom+1106 Bgl II rev	GAAGATCTCCGTAGAAACGGTGCCCCGGCA	BX640908	1086-1106	60°C
EVI1prom -2639 Bgl II fwd	GAAGATCTTAGAGAAGTGCTTTCAGGGT	AC078985	121503- 121522	58°C
EVI1prom -72 KpnI rev	CGCGGTACCTGGTGATGGTATTTGGCTCC	AC078985	124062- 124042	58°C
EVI1prom RAREmut fwd	GACCTTTTTGCTTTAGCTCCCGCCAG	BX640908	183-208	60°C
EVI1prom RAREmut rev	GGGAGCTAAAGCAAAAAGGTCGCCA	BX640908	203-179	60°C
EVI1prom+274 HindIII rev	GGAAGCTTCAGAGTGATCTGATCGGAAGC	BX640908	274-254	58°C
EVI1prom+140 NheI fwd	CTAGCTAGCTGCATCCATAATCAGTCTGTCC	BX640908	140-161	58°C
EVI1prom+227 Bgl II rev	GAAGATCTAAAGTGACAGCAGCCTCCTC	BX640908	227-208	58°C

EVI1prom+255 NheI fwd	GCTAGCTAGCCTTCCGATCAGATCACTCTGG	BX640908	255-275	58°C
EVI1prom+325 NheI fwd	GCTAGCTAGCGGCCGAGCAGATCCTCTTTA	BX640908	325-344	58°C
EVI1prom+587 NheI fwd	GCTAGCTAGCCGGGCTGATAAGAGCAAGAG	BX640908	587-605	58°C
EVI1 270 fwd	GCGAAGACTATCCCCATGAA	NM_05241	251-270	56°C
EVI1 1039 Stop NotI rev	GAGCGGCCGCTCATTATCCATAGCTGGGGTT C	NM_05241	1039-1019	56°C
EVI1 1118 EcoRV fwd	GAGATATCAGGCATCCTGCTGGTCTTAC	NM_05241	1099-1118	56°C
EVI1 1767 Stop NotI rev	GAGCGGCCGCTCAGGGAAACATGGAAGGGTA	NM_05241	1767-1750	56°C
EVI1 2147 fwd	CATGCAAGACCCACTCCTTT	NM_05241	2128-2147	56°C
EVI1 2427 Stop NotI rev	GAGCGGCCGCTCACTTCCGCAGAAGGTTCTCT	NM_05241	2427-2409	56°C
EVI1 2441 rev	TAGCGCTCCTTTCCCTTCC	NM_05241	2441-2423	56°C
EVI1 2698 Stop XbaI rev	CTGCTCTAGATCACGGACATGTTCCCATTCTCA	NM_05241	2698-2679	56°C
EVI1 ZF1 fusNLS rev	CTCTTCTTTTTTGGATCTTTATCCATAGCTGGC	NM_05241	1039-1023	56°C
NLS fwd	GATCCAAAAAAGAAGAGAAAGGTAGATCCAAAA AAGAAGAGAAAGGTA	NC_001669	4445-4422	60°C
NLS Stop NotI rev	AGCGGCCGCTCATACCTTTCTCTTCTTTTTTGG ATCTACCTTTCTCTTCTTTTTTGG	NC_001669	4422-4445	60°C
EVI1 ZF1 NLS Stop NotI rev	AGCGGCCGCTCATACCTTTCT	NC_001669	4422-4430	58°C

Table 6.3 First column: primer name, with forward primers carrying the suffix "fwd", and reverse primers the suffix "rev". Second column: Primer sequences, from 5'- to 3'-end. Engineered restriction sites, indicated in the primer name, are underlined, engineered stop codons are in bold letters. Third column: Genbank accession numbers of the sequences used for primer design. Fourth column: positions of the oligonucleotides from 5' to 3', within the sequences indicated in the third column. Fifth column: Annealing temperature.

6.1.4 Primers for ChIP

Primer name	Primer sequence	acc. no.	position	AT
EVI1 DR5 RARE ChIP fwd	<u>GGGGTACCC</u> CACACCACACTTGTGCTT	BX640908	15-32	58°C
EVI1 DR5 RARE ChIP rev	GA <u>AAGATCT</u> AAAGTGACAGCAGCCTCCTC	BX640908	227-208	58°C
EVI1 DR1 RARE ChIP fwd	CGGGCTGATAAGAGCAAGAG	BX640908	587-606	58°C
EVI1 DR1 RARE ChIP rev	CGATTTCCAAATGGGTCTC	BX640908	832-814	58°C
EVI1 ex3L ChIP fwd	CAGAGTTGAATCCTGCCTGGAATTCTCCAG	CB241806	243-275	56°C
EVI1 ex3L ChIP rev	GTATGGGAGCACCCCTTCTGA	CB241806	462-443	56°C
MDS1prom1 ChIP- F	gtgggcactagagccttctc	AC007849	66321- 66340	58°C
MDS1prom1 ChIP- R	aaggatgcctaccattttcc	AC007849	66531- 66512	58°C
MDS1prom2_ChIP- F	tttgaccccgagggtgtag	AC007849	67072- 67096	58°C
MDS1prom2_ChIP- R	ccatccttgggacatttcag	AC007849	67305- 67286	58°C
MDS1 DR2RARE ChIP-F	TGCTGGAGAGATAGCGAGTGT	AC007849	67906- 67926	60°C
MDS1 DR2RARE ChIP-R	TTCTTTTGCTCTCCCTCTCG	AC007849	68127- 68108	60°C
RARβ2 ChIP-F	AAGCTCTGTGAGAATCCTG	DA403684	120-137	56°C
RARβ2 ChIP-R	GGATCCTACCCCGACGGTG	DA403684	406-388	56°C

Table 6.4 First column: primer name, with forward primers carrying the suffix “fwd”, and reverse primers the suffix “rev”. Second column: Primer sequences, from 5'- to 3'-end. Engineered restriction sites, indicated in the primer name, are underlined, engineered stop codons are in bold letters. Third column: Genbank accession numbers of the sequences used for primer design. Fourth column: positions of the oligonucleotides from 5' to 3', within the sequences indicated in the third column. Fifth column: Annealing temperature.

6.1.5 Literature

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6.1.6 Lebenslauf

PERSÖNLICHE DATEN

Name	Sonja Bingemann
Geburtsdatum	30. Januar 1977
Geburtsort	Kassel, Deutschland
Nationalität	Deutsch

SCHULAUSBILDUNG

1983-1987	Grundschule Landwehrhagen
1987-1989	Orientierungsstufe II Hann. Münden
1989-1993	Grotefend-Gymnasium Hann. Münden
1993-1994	Schüleraustausch in Michigan, USA, Besuch der Chippewa Hills High School, Schulabschluß mit Klassenrang 3/150
1994-1996	Fortsetzung des Schulbesuchs auf dem Grotefend-Gymnasium, Abitur 1,9

BERUFSAUSBILDUNG

08/1996-07/1999	Ausbildung zur Biologielaborantin im Institut für Humangenetik der Universität Göttingen, Schwerpunkte: Molekular- und Zytogenetik
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STUDIUM DER MOLEKULAREN MEDIZIN

WS 1999-SS 2003	Studium der Molekularen Medizin an der Universität Erlangen-Nürnberg September 2003: Diplomprüfung Hauptfach: Humangenetik
10/2003-04/2004	Diplomarbeit im Institut für Humangenetik der Universität Erlangen-Nürnberg unter Betreuung von Dr. Cornelia Kraus Thema: „Klonierung des Gens für die Maus-Mutante md ^f “ Abschluß des Studiums der Molekularen Medizin mit der Gesamtnote 1,4

POSTGRADUALE AUSBILDUNG

- 06/2004-05/2005** Naturwissenschaftliche Dissertation am Zentrum für Anatomie und Zellbiologie der Medizinischen Universität Wien, Abteilung angewandte Anatomie, unter Betreuung von Prof. Dr. Reginald Bittner
- Thema: „Molekulare und funktionelle Charakterisierung des Genprodukts des „mdf“ Locus
- 06/2005-01/2009** Naturwissenschaftliche Dissertation am Institut für Humangenetik der Medizinischen Universität Wien unter Betreuung von Prof. Dr. Rotraud Wieser
- Thema: The oncogene, developmental regulator, and all-*trans* retinoic acid target gene *ecotropic viral integration site 1* as a dual modulator of the ATRA response
- seit 08/2008** wissenschaftliche Mitarbeiterin am Institut für Humangenetik der Medizinischen Universität Wien
- Schwerpunkte: humangenetische Diagnostik und Befundung (Marfan-Syndrom, Osteogenesis Imperfecta), Forschungsarbeiten zum Rett Syndrom und zur adulten dominanten Leukodystrophie (ADLD), Beteiligung an Lehre

PUBLIKATIONEN

Zinc finger transcription factor ecotropic viral integration site 1 is induced by all-*trans* retinoic acid (ATRA) and acts as a dual modulator of the ATRA response

Bingemann SC, Konrad TA, Wieser R

FEBS J. 2009 Nov;276(22):6810-22. Epub 2009 Oct 16

Mutation in the *Scyl1* gene encoding amino-terminal kinase-like protein causes a recessive form of spinocerebellar neurodegeneration

Schmidt WM, Kraus C, Höger H, Hochmeister S, Oberndorfer F, Branka M, Bingemann S, Lassmann H, Müller M, Macedo-Souza LI, Vainzof M, Zatz M, Reis A, Bittner RE

EMBO Rep. 2007 Jul;8(7):691-7. Epub 2007 Jun 15

A fast polymerase chain reaction-mediated strategy for introducing repeat expansions into CAG-repeat containing genes.

Laccone F, Maiwald R, Bingemann S

Hum Mutat. 1999;13(6):497-502

6.1.7 Curriculum Vitae

PERSONAL DATA

Name	Sonja Bingemann
Date of birth	January 30 th , 1977
Place of birth	Kassel, Germany
Nationality	German

SCHOOL EDUCATION

1983-1987	Elementary school Landwehrhagen
1987-1989	Middle School (Orientierungsstufe II) Hann. Münden
1989-1993	Secondary School (Grotefend-Gymnasium) Hann. Münden
1993-1994	Exchange student to Michigan, USA, attendance of Chippewa Hills High School
1994-1996	Secondary School (Grotefend-Gymnasium) Hann. Münden

IN-FIRM TRAINING

08/1996-07/1999	professional training as laboratory technician at the Institute for Human Genetics of the University of Göttingen, focus: molecular and cytogenetics
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ACADEMIC STUDIES

winter term 1999 – summer term 2003	attendance of the Molecular Medicine programme at the University of Erlangen-Nürnberg September 2003: final exams, major subject: human genetics
10/2003-04/2004	diploma thesis at the Institute for Human Genetics, University of Erlangen-Nürnberg under supervision of Dr. Cornelia Kraus topic: positional cloning of the gene of the mouse mutant “mdf”

POSTGRADUAL STUDIES

- 06/2004-05/2005** dissertation at the Centre for Anatomy and Cell Biology, Medical University of Vienna under supervision of Prof. Dr. Reginald Bittner

topic: „Molecular and functional characterisation of the gene product of the “mdf” locus
- 06/2005-01/2009** dissertation at the Institute for Human Genetics, Medical University of Vienna, under supervision of Prof. Dr. Rotraud Wieser

topic: The oncogene, developmental regulator and all-*trans* retinoic acid (ATRA) target gene ecotropic viral integration site 1 (*EV1*) as a dual modulator of the ATRA response
- since 08/2008** research assistant at the Institute for Human Genetics, Medical University of Vienna
focus: diagnostics of genetic diseases (Marfan-syndrome, Osteogenesis Imperfecta), research activities: Rett syndrome, adult dominant Leukodystrophy (ADLD), participation in teaching

PUBLICATIONS

Zinc finger transcription factor ecotropic viral integration site 1 is induced by all-*trans* retinoic acid (ATRA) and acts as a dual modulator of the ATRA response

Bingemann SC, Konrad TA, Wieser R

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