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CIZ as a fusion partner for TET-proteins in leukemia: models for leukemogenesis and interaction partners

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Index

List of abbreviations

(co)-IP	(co)-immunoprecipitation
(E)GFP	(enhanced) green fluorescent protein
(RT)-PCR	(reverse transcription) polymerase chain reaction
5'FU	5'-fluorouracil
AA	amino acid
AD	transcriptional activation domain
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
AUL	acute undifferentiated leukemia
BAC	bacterial artificial chromosome
BLAST	basic local alignment search tool
BM	bone marrow
BMPs	bone morphogenetic proteins
BMT	bone marrow transplantation
bp	basepair(s)
CBC	complete blood count
CLL	chronic lymphoblastic leukemia
CML	chronic myeloid leukemia
C_T	threshold cycle
d	days
DBD	DNA-binding domain
der(12)	derivative chromosome 12
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EC	EWSR1/CIZ fusion protein (gene)
ECM	extracellular matrix
ELISA	enzyme linked immunosorbant assay
EMSA	electrophoretic mobility shift assay
ETS	E twenty-six (type of DBD)
EWS	Ewing's sarcoma
FAB	French/American/British system of classification
FCS	fetal calf serum
GCOS	Affymetrix GeneChip Operating Software
GM-CSF	granulocyte/macrophage colony stimulating factor
GO	gene ontology
H&E	hematoxylin and eosin
hnRNP	heterogeneous nuclear ribonucleoprotein
HSC	hematopoietic stem cell

IF	intermediate filament
Ig	immunoglobulin
IGF	insulin like growth factor
IL3	interleukin 3
IL6	interleukin 6
IRES	internal ribosome entry site
kb	kilobase(s)
kDa	kilo Dalton
KH-domain	hnRNP-K homology domain
LIM-domain	Lin-11, Icl-1, Mec-3 domain
LTR	long terminal repeat
MDS	myelodysplastic syndrome
MMP	matrix metalloproteinase
MPS	myeloproliferative syndrome
NDLB	non denaturing lysis buffer
p	short chromosomal arm
PAGE	polyacrylamide gel electrophoresis
PB	peripheral blood
q	long chromosomal arm
qRT-PCR	quantitative real-time PCR
RA	retinoic acid
RAG	recombination activating genes
RGG-box	arginine-glycine-glycine repeat region
RNA	ribonucleic acid
SCF	stem cell factor
SCID	severe combined immunodeficient
SH3-domain	Src homology 3 domain
SR-family	serine/arginine family of splice factors
TBP	TATA-box binding protein
Tc	tetracyclin
TERT	telomerase reverse transcriptase
TET-family	TLS/FUS-EWSR1-TAF15 family
TF	transcription factor
TNF	tumor necrosis factor
TRAP	telomeric repeat amplification protocol
UTR	untranslated region
V	vector
WBC	white blood cell
wt	weight
Y2H	yeast two-hybrid
ZF	zinc finger

List of gene symbols

ABL1	Abelson murine leukemia viral oncogene homolog 1
AF9	translocated to MLL, 3
ALP	alkaline phosphatase
AML1	acute myeloid leukemia 1
ATP6V0A1	ATPase, H^+ transporting, lysosomal V0, subunit a, isoform1
B3GALT2	UDP-Gal:bGlcNAc b1,3-galactosyltransferase polypeptide 2
BARD1	BRCA1 associated RING domain 1
BCR	breakpoint cluster region
BMP2	bone morphogenetic protein 2
BRN3A	POU domain, class 4, transcription factor 1
BTK	Bruton agammaglobulinemia tyrosine kinase
CALML4	calmoduline-like 4
CBFA1	core-binding factor alpha A subunit
CHOP	C/EBP homologous protein
CIZ	Cas interacting zinc finger protein (=NMP4, ZNF384, TNRC1)
COL1A1	collagen, type I, alpha 1
CRK	crk sarcoma virus CT10 oncogene homolog
CRP1	cysteine rich protein 1
EAT2	EWS/FLI1 activated transcript 2
EMP1	epithelial membrane protein 1
ERG	erythroblastosis virus E26 oncogene like
ETO	translocated to AML1, 1
ETV1	ETS variant gene 1
EWSR1	Ewing sarcoma breakpoint region 1
FAK	focal adhesion kinase
FES	feline sarcoma virus
FLI1	Friend leukemia virus integration 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GATA1	GATA binding protein 1
GR1	lymphocyte antigen 6 complex, locus G
IGFBP4	insuline-like growth factor binding protein 4
ITGB3	integrin beta 3
KIT	Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
MAC1	macrophage antigen alpha
MFNG	manic fringe
MLL	myeloid/lymphoid or mixed-lineage leukemia
MMP1	matrix metalloproteinase 1 (interstitial collagenase)
MMP13	matrix metalloproteinase 13 (collagenase 3)
MMP3	matrix metalloproteinase 3 (stromelysin 1, progelatinase)

MMP7	matrix metalloproteinase 7 (matrilysin, uterine)
MMP8	matrix metalloproteinase 8 (neutrophil collagenase)
MOZ	monocytic leukemia zinc finger protein
MT1	metallothionein 1
MYC	myelocytomatosis viral oncogene homolog
OCN	osteocalcin
p130CAS	Crk-associated substrate (= CAS, BCAR1)
p16	cyclin-dependent kinase inhibitor 2A (= CDKN2A)
p53	tumor protein p53
PCBP1	poly(rC) binding protein 1 (= hnRNP-E1)
PLZF	promyelocytic leukemia zinc finger protein
PML	promyelocytic leukemia
PU.1	PU-box binding protein 1 (= SPI-1)
PYK2	protein tyrosine kinase 2 beta
RARα	retinoic acid receptor, alpha
RAS	Harvey rat sarcoma viral oncogene homolog
Rb	retinoblastoma 1
REL	reticuloendotheliosis viral oncogene homolog
R-SMAD	regulatory smad
SARFH	cabeza (*Drosophila melanogaster*)
SF1	splicing factor 1
SMAD1	SMAD, mothers against DPP homolog 1
SOCS3	suppressor of cytokine signaling 3
SPI-B	SFFV provirus integration site B
SPI-C	Spi-1/PU.1 related transcription factor, Spi-family member C
SRC	sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAM2	signal transducing adaptor molecule 2
TAF15	TBP-associated factor, RNA polymerase II
TGFβ	transforming growth factor, beta 1
TIF2	transcriptional intermediary factor 2
TLS/FUS	translocated in liposarcoma / fusion involved in t(12;16)
TM4SF3	transmembrane 4 superfamily member 3
TNFRSF9	TNF receptor superfamily, member 9
U1C	U1 snRNP protein C
VAV	vav 1 oncogene
VIM	vimentin
YB1	Y box-binding protein I
ZYX	zyxin

1 General Introduction

1.1 Cancer, hematopoiesis and leukemia

1.1.1 Cancer

Cancer is a disease of damaged genes

The human body is composed of approximately 10^{13} cells. These cells build tissues and organs without the presence of a supervising architect; the control on the complexity of the organism is bottom-up. This not only implies magnificent beauty, but also means continuous threat: if a single cell leaves the right track and starts to proliferate without control, a tumor can arise.

Cells can proliferate, differentiate, die, or retreat into a non-growing state. While this output of the cell behavior decision machinery is rather limited, its input is highly complex and results from many different sources. Internal signals that dictate cell behavior come from housekeeping information about DNA health and the metabolic status of the cell. However, the majority of fate-determining information originates from the cell surroundings. Such external signals are provided by signaling molecules like growth factors and cytokines, and physical contacts with the extracellular matrix and adjacent cells. The messages are transferred across the cell membrane through the action of specialized transmembrane receptors, or via cellular uptake of the signaling molecules themselves. A signaling pathway is then activated that consists of a network of interacting proteins, which filter, amplify and/or pass the signal towards the nucleus. There it is translated into a read-out by transcription factors, which alter the expression levels of specific target genes. Cells are thus provided with an accurate system for processing behavioral signals. All the information that is needed to build up this system is contained in the DNA, the blueprint of the cell's behavior.

When this genetic blueprint is damaged, certain components of the signal operating circuitry might malfunction and disrupt the entire decision-making process. As a consequence, the sophisticated equilibrium between cell proliferation, differentiation and cell function gets out of balance. This is how cancer begins. [Weinberg, 1998].

When looked at in more detail, tumorigenesis clearly is a multistep process and a cell gradually acquires more oncogenic properties until it is capable of full malignant growth. Cell growth is very strictly controlled and a cancer cell has to overcome at

least four constraints that are intent on limiting uncontrolled proliferation: (i) deprivation of growth stimuli, (ii) exposure to growth-inhibitory signals, (ii) induction of cell death by apoptosis, (iii) telomere shortening, which works as an internal clock that limits replicative potential and (iv) the immune system of the body [Weinberg, 1998]. Moreover, the tumor has to become capable of (v) sustained angiogenesis and (vi) tissue invasion and metastasis [Hanahan and Weinberg, 2000]. It is clear that one single mutation is not likely to render a cell fully malignant. At least four to seven 'hits' in the genome are thought to be necessary to overcome all these tumor-suppressing obstacles [Renan, 1993]. This is why, in spite of the immense number of cells in the body, cancer nevertheless is a relatively 'rare' disease.

Genetic defects in cancer

Genetic lesions at the basis of cancer vary from small, single base pair mutations to losses or doublings of complete chromosomes. Other anomalies are deletions and amplifications of chromosomal fragments and balanced aberrations where the total amount of DNA is not altered, like inversions and translocations.

DNA-damage can be caused by environmental mutagens such as UV- and X-radiation or toxic substances, but may also ensue from normal cellular processes that go wrong. Errors during DNA-replication that escape normal repair mechanisms for example, cause mutations. Double stranded DNA breaks, which are at the origin of several chromosome lesions, also occur in normal circumstances. During meiotic recombination for example, or by the activity of certain DNA-processing enzymes, such as the RAG-proteins that coordinate V(D)J-recombination in lymphoid cells, or topoisomeraseII which relaxes overwound DNA by breaking, unwinding and resealing it [Greaves and Wiemels, 2003]. Errors during repair of these breaks can generate aberrant derivative chromosomes [Ferguson and Alt, 2001].

Cancer genes

Mutated genes that are causally implicated in oncogenesis are subdivided into oncogenes, which have growth promoting functions and are activated in tumors, and tumor suppressor genes, which encode tumor controlling proteins and need to be inactivated for cell transformation.

Cancer genes usually are involved in proliferation, differentiation or apoptosis and take part in all possible stages of the signal operating circuitry: from growth signals and transmembrane receptors to intracellular signal transducers and transcription factors in the nucleus. In these strategic positions, they are well suited to influence

cell behavior. Genes involved in DNA damage repair are also hit by oncogenic mutations. After their elimination, the genome is prone to a quicker accumulation of mutations in the growth-regulating class of cancer genes. This genomic instability often is considered as an early event in tumorigenesis and might be the driving force behind further cell transformation [Sieber et al., 2003].

Since the identification of RAS as the first oncogene in a human bladder tumor [Reddy et al., 1982], [Tabin et al., 1982], more than two decades of cancer research have passed. Meanwhile, about 300 different cancer genes have been reported – or about 1% of all the genes in the human genome [Futreal et al., 2004]. Some clear patterns emerge. The most common functional domain encoded by cancer genes is the protein kinase, followed by domains involved in transcription regulation (DNA-binding, transactivation) and DNA-repair. Of all cancer genes reported so far, 90% bear somatic mutations, 20% have germ line mutations and 10% show both (figure 1A). Among known cancer genes, translocations are the most common type of somatic mutations (figure 1B) and more than 70% of cancer genes have been identified in leukemias, lymphomas and sarcomas, even though these diseases represent only 10% of human cancers (figure 1C). Whether this is merely due to the relative ease of studying these types of cancer or whether it results from a fundamental biological difference between this group of cancers and the more common epithelial neoplasms, is still subject of debate [Futreal et al., 2004]. However, two observations plead against fundamental, tissue-specific differences in tumor initiation: (i) although lower in absolute numbers, the proportions of translocated genes among different tumor types are equal, and (ii) even the biological properties of the genes involved do not seem to differ between hematological and solid neoplasms [Mitelman et al., 2004].

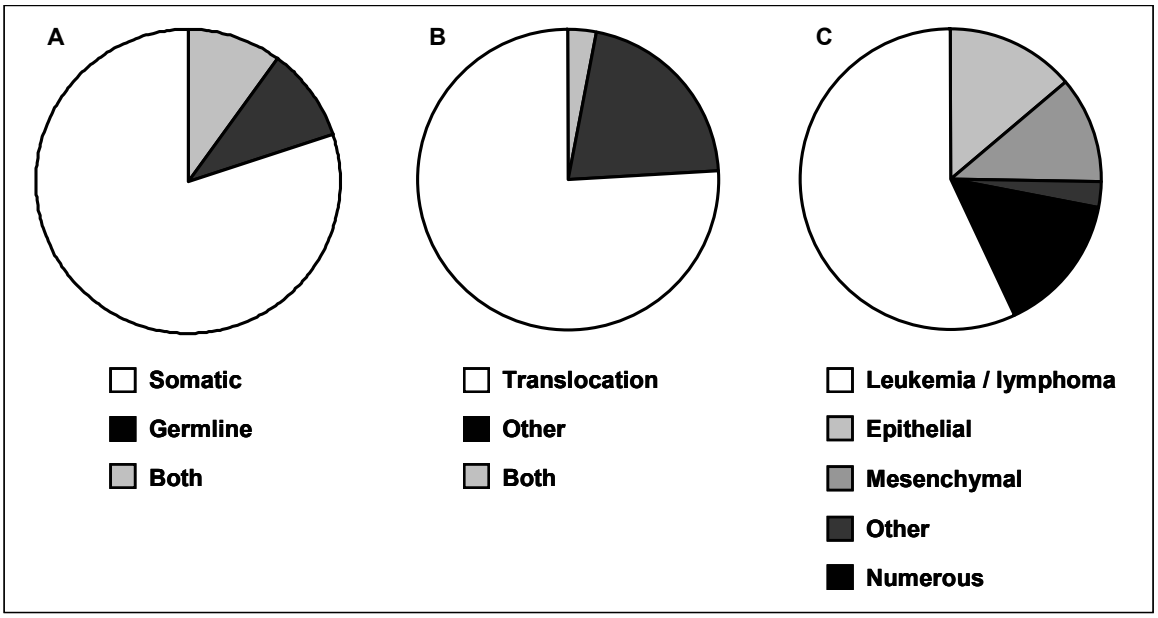

Figure 1 - Human cancer genes. (A) Somatic versus germ line mutations. (B) Proportion of translocations in somatic mutations. (C) Distribution of somatically mutated cancer genes among different tumor types. (Adapted from [Futreal et al., 2004]).

1.1.2 Hematopoiesis

Hematopoiesis is the complex developmental process in which the different types of blood cells are formed in the bone marrow (Greek: *haima,* blood; *poiein,* to make). In contrast to cells of other tissues, blood cells have a very short life time (from a few hours or days for white blood cells to an average of 120 days for red blood cells). This implicates that hematopoiesis is a highly dynamic process, which continues throughout life. The starting point of blood cell development is the pluripotent hematopoietic stem cell (HSC). It is capable of both unlimited self-renewal and differentiation into all mature blood cell types (figure 2). As it is further committed to a specific lineage, its developmental potentials (both proliferative and concerning lineage choice) are gradually restricted.

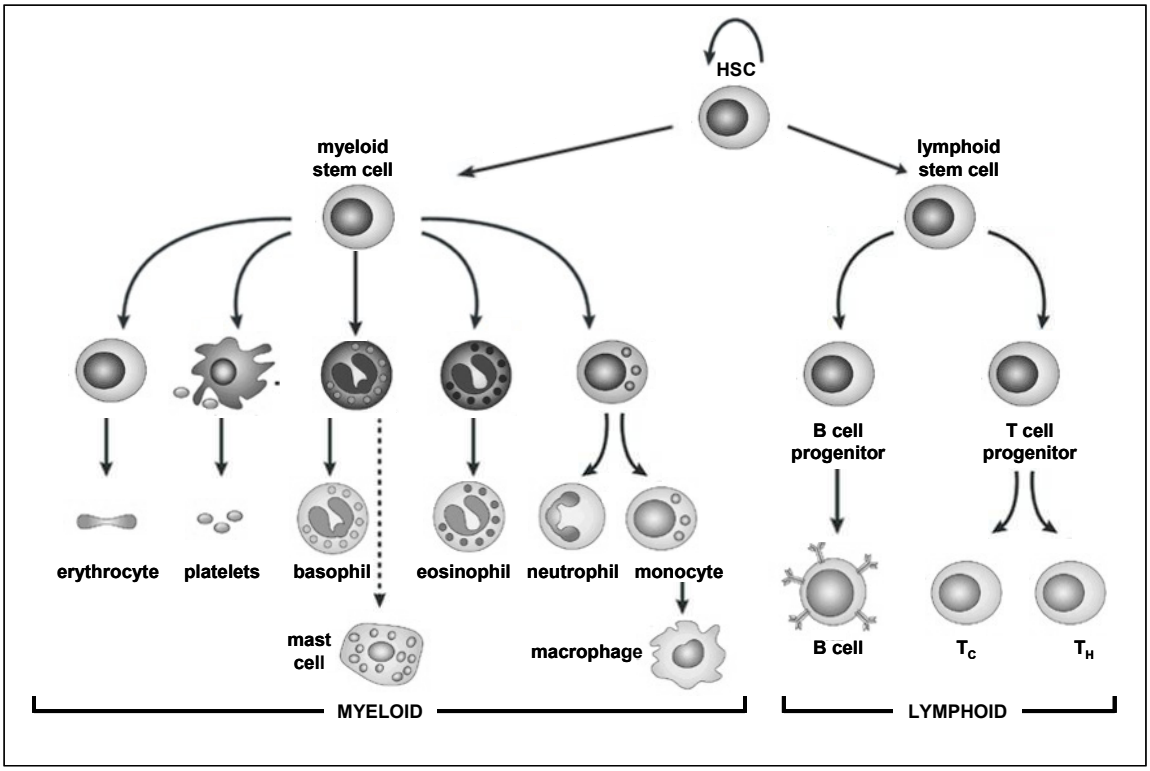

Figure 2 – **Hematopoiesis.** Pluripotent hematopoietic stem cells (HSC) self-renew or differentiate into the different mature blood cell types. T_C: cytotoxic T-cell, T_H: helper T-cell. (Adapted from [Nabel, 2002]).

The cell fate decisions that have to be made at each branching point are dictated by the relative expression levels of lineage-specific transcription factors. Subtle changes in expression levels can have a major impact on cell fate decisions. Knock down experiments of ETS-transcription factor PU.1 for example, illustrate that weak PU.1

expression results in lymphoid commitment of HSCs, while high expression levels dictate myeloid development [DeKoter and Singh, 2000].

Transcription factor expression profiles in turn are governed by extracellular signals, which operate through a variety of intracellular pathways and originate from the cell surroundings. Hematopoietic cells are imbedded in a complex bone marrow micro-environment, which is comprised of stromal cells (a diverse population consisting of fibroblasts, macrophages, endothelial cells, adipocytes and other) and the extracellular matrix (ECM) of collagens, glycoproteins and proteoglycans that is produced by these cells. This stroma functions as a scaffold, but also provides cues concerning survival, quiescence, proliferation, commitment, differentiation, migration, and death of hematopoietic cells (reviewed by [Verfaillie, 1998]). All this information is communicated through cell-cell and cell-ECM interactions and by the exposure of variable concentrations and combinations of soluble factors, like cytokines. Multiple cytokine receptors and cell adhesion molecules on the membranes of hematopoietic precursors are implicated in detection of these external signals.

Cell adhesion molecules on hematopoietic progenitor cells include members of the integrin family [Hynes, 1987]. These are transmembrane α/β glycoprotein heterodimers with an extracellular ligand binding domain and an intracellular tail that lacks enzymatic activity, but induces signal transduction by assembling complexes of other proteins. Engagement of β1 integrins, which are the best characterized, on human B-cells for example, leads to the recruitment of p130CAS [Manie et al., 1997a]. This serves as a docking molecule that assembles other proteins as FAK [Astier et al., 1997] and CRK [Burnham et al., 1996] into a signaling complex, which passes the message on towards the nucleus.

1.1.3 Leukemia

With more than one billion cell divisions each day, the hematopoietic system is at risk for the accumulation of DNA damage. Genetic defects causing abnormalities in the normal developmental program of blood cell formation, result in hematological diseases, such as leukemia. The fundamental biological features of a leukemic cell are (i) the ability to proliferate continuously and (ii) a differentiation block, resulting in its clonal expansion and the accumulation of immature blast cells.

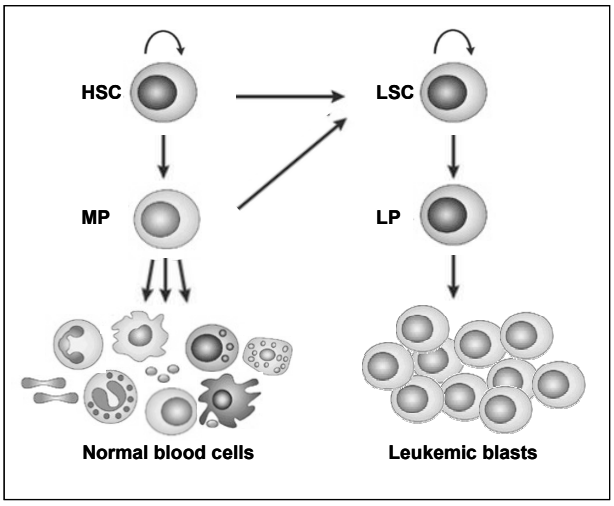

Figure 3 – Schematic representation of a myeloid leukemia. HSC, hematopoietic stem cell; MP, myeloid progenitor; LSC, leukemic stem cell; LP, leukemic progenitor.

Leukemias can be classified according to whether they occur in the myeloid or the lymphoid lineage, and whether they are 'acute' or 'chronic'. Chronic myeloid leukemias are associated with increased proliferation and altered kinase activity. On the contrary, the most prominent feature of acute leukemias is their arrested differentiation, often linked to transcription factor defects [Tenen, 2003]. Taken together, four types of leukemia are distinguished: CML or chronic myeloid leukemia, CLL or chronic lymphocytic leukemia, AML or acute myeloid leukemia and ALL or acute lymphoblastic leukemia. MDS or myelodysplastic syndrome and MPS or myeloproliferative syndrome are pre-leukemic stages, characterized by only differentiation or only proliferation defects, respectively. In the WHO classification, distinct disease entities are further defined based on the combination of morphology, immunological and molecular techniques and clinical features [Ottensmeier, 2001].

Even though many different genetic lesions have been described in hematopoietic malignancies, they frequently target the same signal transduction and transcriptional pathways [Gilliland, 2001]. This is true for both chronic and acute leukemias. Various kinases are involved in CML, but most mutations result in proliferative and survival advantage of the cell via activation of only a limited number of downstream signaling routes, like the MAPKinase and Janus-kinase/signal-transducer-and-activator-of-transcription (JAK/STAT) pathways. Also in acute leukemia a unifying theme emerges. Different transcription factors and transcriptional cofactors are involved in ALL and AML. Figure 4 summarizes the most common translocation-induced aberrations in these diseases. Mutations alter the properties of the wild-type proteins, often resulting in their loss of function, for example by disruption or by dominant negative effects on the residual wild-type allele or on heterologous

transcription factors. As such they ultimately cause hematopoietic differentiation defects. Moreover, there is a sophisticated interplay between transcription factors in hematopoiesis, through which single changes can establish major effects. A second type of mutation may provide the acute leukemic cell with enhanced proliferation potential. Point mutations in FLT3 and c-KIT are recurrently described in this context.

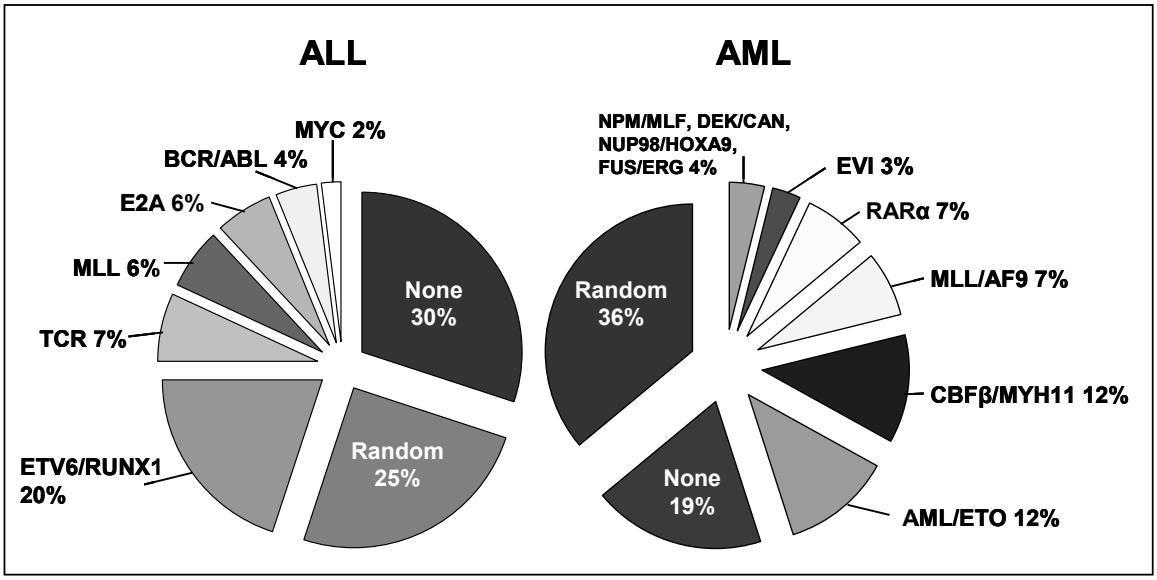

Figure 4 – **Summary of the most common translocations in acute leukemia**. (Based on [Look, 1997] and [Armstrong and Look, 2005]).

Constitutive disturbance of the balance between self-renewal and differentiation is an essential feature of leukemogenesis. Mutations in NOTCH1 and PTEN are useful models of changes in the self-renewal capacity of T-ALL cells. NOTCH1 encodes a transmembrane receptor that is involved in the regulation of stem cell maintenance, normal T-cell development and many other tissues during embryonic development. It exists as a heterodimer at the plasmamembrane and is cleaved upon binding of its ligands. An intracellular fragment is then generated that moves towards the nucleus and initiates transcription. This truncated NOTCH1, which provides signaling without ligand binding, was shown to induce T-ALL in mouse models. Besides via a rare translocation t(7;9), NOTCH1 is involved in more than 50% of T-ALLs by activating mutations in the heterodimerization domain or the C-terminal PEST domain, which has a role in degradation of the intracellular NOTCH1 fragment [Armstrong and Look, 2005], [De Keersmaecker et al., 2005].

Tumor suppressor PTEN (phosphatase and tensin homologue) negatively regulates the phosphatidyl-inositol-3-OH-kinase/Akt pathway, which is crucial for cell

proliferation and survival. Inactivation of PTEN in HSCs initially causes their expansion and mobilization and leads to a myeloproliferative disease in mice [Zhang et al., 2006]. However, the cell cycle deregulation and the decreased retention in the bone marrow niche are to such a degree that PTEN deficient HSCs show an impaired ability to sustain hematopoietic reconstitution in mice and ultimately lead to their decline. Conversely, leukemia initiating stem cells do maintain themselves without PTEN [Yilmaz et al., 2006]. This difference between normal hematopoietic stem cells and cancer stem cells is interesting in the light of selective inhibition of cancer cell growth.

Chromosomal translocations are common genetic lesions in leukemias [Futreal et al., 2004] and several hundred recurring, leukemogenic translocations have been described [Mitelman et al., 2004]. They may represent critical, early events in the genesis of the leukemic clone [Scandura et al., 2002]. A balanced translocation occurs when two chromosomes break and exchange pieces (figure 5A). As a consequence, normal gene expression can be disturbed in either of two ways: a fusion gene is created that encodes a new, chimeric protein (figure 5B), or a gene is placed in a different transcription regulatory context, leading to its ectopic expression (figure 5C) [Rabbitts, 1991], [Look, 1997].

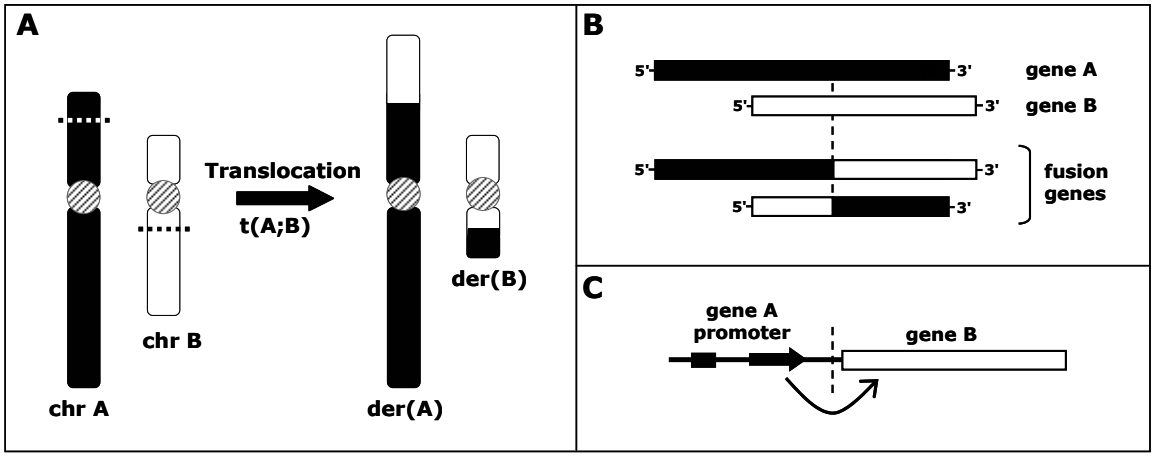

Figure 5 - Translocations. (A) Scheme of a balanced translocation transforming chromosomes A and B (chr A, B) into the corresponding derivative chromosomes der(A) and der(B). (B) Fusion genes encoding chimeric proteins. (C) Altered gene expression due to a different regulatory context.

If both breakpoints are within a gene and an in frame fusion mRNA is created, it encodes a protein in which functional domains of the individual fusion partners are combined. Domains with a role in oligomerization, kinase activity, subcellular localization, DNA-binding and transcriptional activation are often involved in translocations in this way. Combining two or more of these domains creates proteins with new properties, like aberrant transcription factors or ligand-independent receptor kinases, which can activate the signal processing circuitry by mimicking signals that normally arise only after a cell encounters growth signals. BCR/ABL1, generated by the 'Philadelphia translocation' t(9;22)(q34;q11) in CML, is the first identified leukemogenic fusion protein. After tetramerization and auto-activation of its kinase domain, it constitutively activates its downstream targets such as MYC and RAS [Heisterkamp et al., 1985], [Pendergast et al., 1991].
Normal immunoglobulin and T-cell receptor gene rearrangements that occur during B- and T-cell development predispose lymphoid cells to translocations that bring these genes in the vicinity of proto-oncogenes, resulting in inappropriate oncogene expression [Rabbitts, 1994]. This is exemplified by t(8;14)(q24;q32) in Burkitt's B-cell lymphoma. The translocation juxtaposes *MYC* to the immunoglobulin heavy chain enhancer, resulting in its overexpression [Taub et al., 1982], [Leder et al., 1983].

This work further focuses on translocations t(12;17)(p13;q11) and t(12;22)(p13;q12) in acute leukemia.

1.2 Translocations t(12;17)(p13;q11) and t(12;22)(p13;q12)

1.2.1 TAF15/CIZ and EWSR1/CIZ

Translocations t(12;17)(p13;q11) and t(12;22)(p13;q12) are recurrent in acute leukemia. Six patients carrying a translocation t(12;17)(p13;q11) have been reported, while translocation t(12;22)(p13;q12) has been identified twice [Martini et al., 2002]. Table 1 gives an overview of clinical and cytogenetic data of the patients at diagnosis. Of the eight patients, six were diagnosed with acute lymphoblastic leukemia, one with acute myeloid leukemia and one with acute undifferentiated leukemia.

Table 1 - Overview of patients with t(12;17)(p13;q11) or t(12;22)(p13;q12). [La Starza et al., 2005]

Case	Sex/age	Immunophenotype	Diagnosis	Karyotype
1	F/29	MPO$^+$, CD34$^+$, HLA-DR$^+$, CD45$^+$, CD33$^+$, cyCD13$^+$,CD19$^+$, CD22$^+$, CD117$^-$, CD10$^-$, CD24$^-$, CD3$^-$, CD4$^-$, CD7$^-$, CD56$^-$, CD14$^-$, CD11b$^-$	AUL	46,XX,t(12;22)(p13;q12)[21]/ 45,XX,t(12;22) (p13;q12),-21[3]
7	M/4	HLA-DR$^+$, CD34$^+$, TdT$^+$, CD13$^+$, CD33$^+$, CD15$^+$, CD79a$^+$, cCD22$^+$, sCD22$^+$, CD19$^+$, CD10$^+$, clg$^-$, slg$^-$	pro-B ALL	47,XY,+1,der(1;6)(q10;p10), t(12;22)(p13;q11),+21[6]
2	M/29	CD13$^+$, CD33$^+$, CD19$^+$, CD14$^-$, CD41$^-$, CD34$^-$, CD22$^-$, CD24$^-$, CD2$^-$, CD7$^-$	AML-M1	46,XY,t(12;17)(p13;q11)[2]/ 46,XY, t(12;17)(p13;q11),i(8)(q10)[5]/inc[9]
3	F/44	HLA-DR$^+$, CD33$^+$, CD34$^+$, CD13$^+$, CD19$^+$, TdT$^+$, CD22$^+$, CD79a$^+$, CD3$^-$, CD4$^-$, CD10$^-$, CD14$^-$, CD15$^-$, CD56$^-$, CD65$^-$, clg$^-$, slg$^-$	pro-B ALL	46,XX,t(12;17)(p13;q11)[5]/ 46,XX,del(6)(q16;q21),t(12;17)[3]/46,XX[8]
4	M/24	HLA-DR$^+$, CD34$^+$, CD13$^+$, CD19$^+$, CD22$^+$, TdT$^+$, CD2$^-$, CD3$^-$, CD4$^-$, CD7$^-$, CD10$^-$, CD14$^-$, CD15$^-$, CD33$^-$, CD56$^-$, CD65$^-$, clg$^-$, slg$^-$	pro-B ALL	45,XY,t(12;17)(p13;q11)[10]/46,XY[1]
5	F/26	HLA-DR$^+$, CD34$^+$, CD19$^+$, sCD22$^+$,TdT$^+$, CD10$^-$, CD20$^-$, CD7$^-$, CD5$^-$, sCD3$^-$, CD13$^-$, CD117$^-$, CD15$^-$, CD14$^-$, CD11b$^-$, CD11c$^-$	pro-B ALL	46,XX[7]/46,XX,t(12;17)(p13;q11)[2]
6	F/16	HLA-DR$^+$, CD34$^+$, CD19$^+$, CD22$^+$, CD45$^+$,CD13$^+$, CD10$^-$, CD33$^-$, CD2$^-$, CD3$^-$, CD4$^-$, CD8$^-$, CD7$^-$, CD5$^-$, CD20$^-$, CD15$^-$, CD56$^-$, CD41$^-$, CD117$^-$, CD61$^-$, slg$^-$	pro-B ALL	46,XX,t(12;17)(p13;q11)[15/15]
8	M/7	HLA-DR$^+$, CD34$^+$, TdT$^+$, CD19$^+$, CD22$^+$, CD24$^+$, CD33$^+$, CD79A$^+$, CD10$^+$, clg$^-$, slg$^-$	pro-B ALL	46,XY,t(12;17)(p13;q11)[15/15]

The breakpoints of t(12;17)(p13;q11) and t(12;22)(p13;q12) were cloned in our laboratory and are for both translocations on chromosome 12p13 in CIZ, a previously unidentified protein with high similarity to rat Ciz. Ciz is a transcription factor that is involved in bone metabolism and spermatogenesis. On chromosome 17q11, the breakpoint is in TAF15, on 22q12 it is in EWSR1. An overview of the translocations is shown in figure 6.

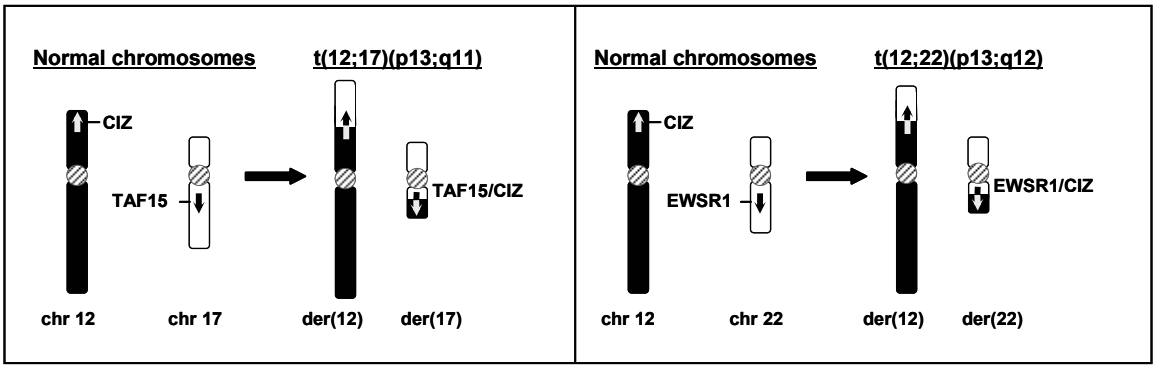

Figure 6 - Translocations t(12;17)(p13;q11) and t(12;22)(p13;q12). Both translocations interrupt the same gene on 12p13: CIZ. On 17q11 the breakpoint is in TAF15, on 22q12 in EWSR1.

Both translocations t(12;17) and t(12;22) generate an in frame fusion protein consisting of the N-terminus of either TAF15 or EWSR1, fused to full-length CIZ. The structure of the fusion proteins is shown in figure 7B. Two alternative forms of t(12;17)(p13;q11) were identified: one fuses exon six of TAF15 to exon three of CIZ, in the other the first nine exons of TAF15 are present. Translocation t(12;22)(p13;q12) fuses the first seven exons of EWSR1 to exon two or three of CIZ.

TAF15 and EWSR1 are members of the TET-family of RNA-binding proteins. TET proteins are recurrently involved in translocations. Fusions of their N-terminal activation domain to a transcription factor or its DNA-binding domain have been described in solid tumors and in rare cases of acute myeloid leukemia [Arvand and Denny, 2001]. The identity of the transcription factor in the fusions determines the phenotype of the tumor. Fusion of CIZ to TAF15 and EWSR1 thus expands the involvement of TET-proteins to acute lymphoblastic leukemia and suggests a role for CIZ in hematopoiesis. In further support of this, Hunger and Zhong characterized an other, cryptic translocation t(12;19)(p13;p13) in ALL, fusing CIZ to the N-terminal activation domain of transcription factor E2A [Hunger and Zhong, 2002].

In what follows, CIZ and its fusion partners TAF15 en EWSR1 will be discussed in further detail.

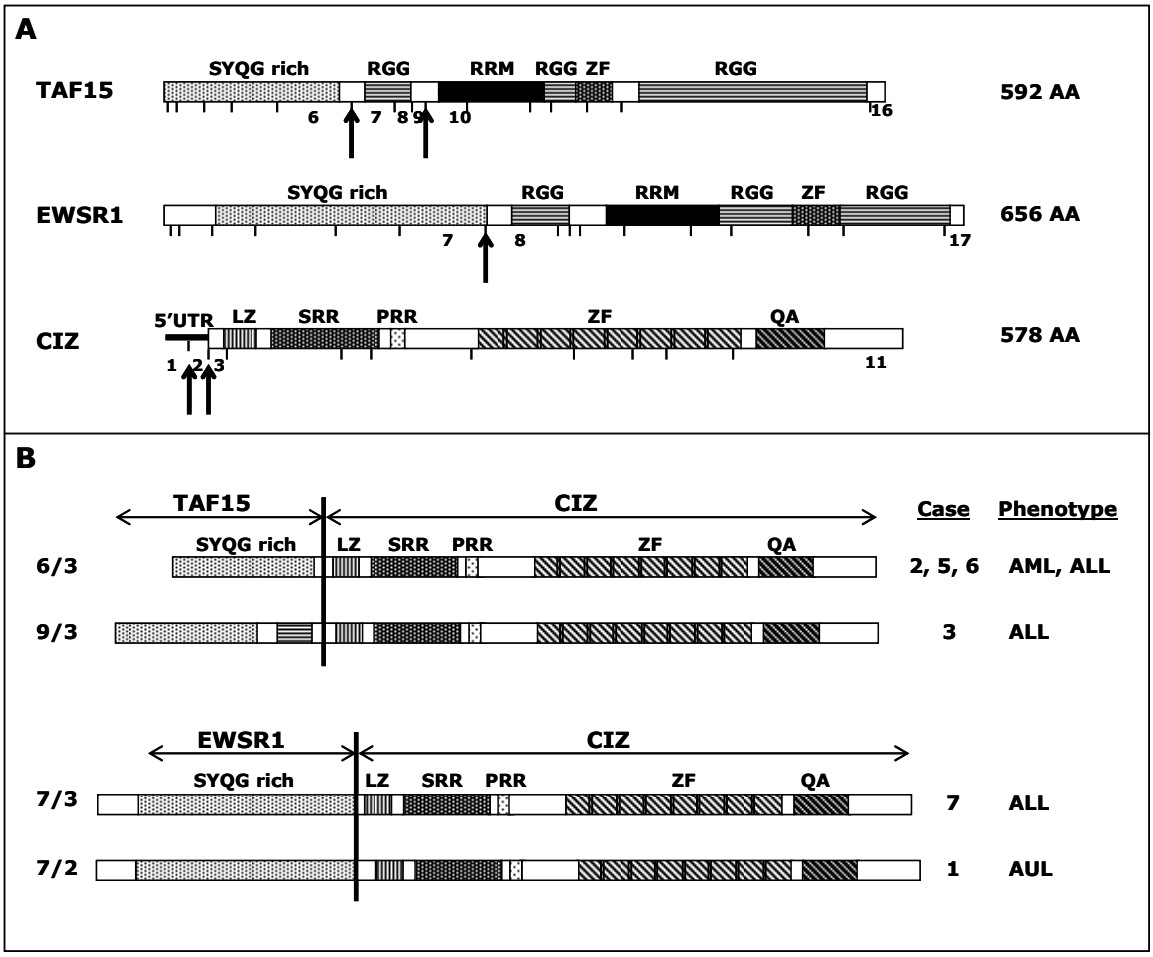

Figure 7 - Structure of CIZ, TAF15, EWSR1 and their fusions TAF15/CIZ and EWSR1/CIZ. (A) Domain structures of TAF15, EWSR1 and CIZ. SYQG: serine-tyrosine-glutamine-glycine, RGG: domain rich in arginine-glycine-glycine, RRM: RNA recognition motif, ZF: zinc finger, LZ: leucine zipper, SRR: serine rich region, PRR: proline rich region, QA: glutamine-alanine rich region. Exon numbers are indicated below the structures. Arrows point at breakpoint positions of t(12;17) or t(12;22). (B) Structure of the fusion proteins TAF15/CIZ and EWSR1/CIZ. Numbers on the left are exons of the fusion partners flanking the breakpoints.

1.2.2 TET proteins

The TET-family

TAF15, EWSR1 and TLS/FUS together constitute the TET-family of RNA-binding proteins [Bertolotti et al., 1996]. The founding member EWSR1 was identified because of its fusion to FLI1 by translocation t(11;22)(q24;q12) in Ewing's sarcoma and related tumors [Delattre et al., 1992]. Then TLS/FUS was cloned based on its location at the chromosome 16 breakpoint of translocation t(12;16)(q13;p11), which fuses TLS to CHOP in myxoid liposarcoma [Crozat et al., 1993]. Finally, TAF15 was discovered in a search for EWSR1 and TLS/FUS homologs [Morohoshi et al., 1996].

Ubiquitously expressed RNA binding proteins

Gene and protein structure of the TET-members is shown in figure 8. They have a similar structure that consists of an N-terminal transcription activation domain which is primarily composed of 31 repeats of a degenerate hexapeptide motif rich in serine, tyrosine, glycine and glutamine, an RNA recognition motif, a C_2C_2 zinc finger and several arginine-glycine-glycine repeat boxes at the C-terminus. While their total genomic sizes differ, the TET-genes share a similar genomic organization with exon/intron junctions at similar sites, indicating that these three genes most likely originate from the same ancestor gene.

TET-proteins are ubiquitously expressed in human tissues and cultured cell lines [Aman et al., 1996]. They have a predominant nuclear localization [Rossow and Janknecht, 2001], although diffuse, cytoplasmic staining also has been reported [Kawano et al., 2001]. Their promoters have no TATA-boxes, but contain G/C-rich stretches [Aman et al., 1996]. Together, these observations suggest a housekeeping role for TET-proteins.

However, data are heterogeneous about the exact cellular function of TET-proteins. They are involved in RNA transcription and splicing and provide a link between both, but they also have a role in DNA recombination and signal transduction.

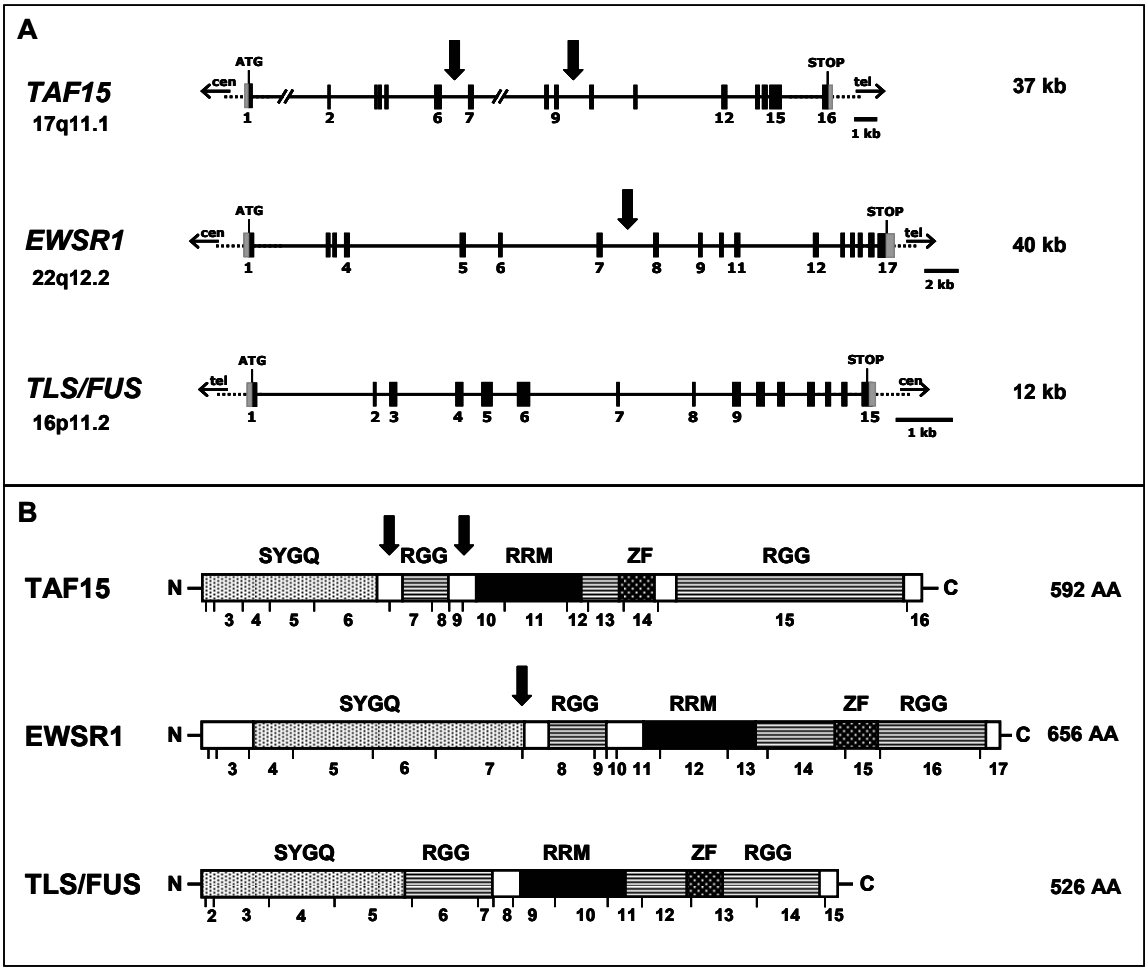

Figure 8 - Gene (A) and protein (B) structures of TET-family members. Chromosome locations are beneath the gene names. Exon numbers are indicated below the structures. Block arrows indicate breakpoints of translocations t(12;17)(p13;q11) and t(12;22)(p13;q12). Tel: telomere, cen: centromere, SYGQ: region rich in serine, tyrosine, glycine and glutamine, RGG: arginine-glycine-glycine repeat region, RRM: RNA recognition motif, ZF: C_2C_2 zinc finger.

Roles of the different TET subdomains

Several roles have been attributed to the subdomains of TET-proteins. The N-terminal SYGQ-rich domain can act autonomously as a transcriptional activation domain [Lessnick et al., 1995]. Its activity is dependent on the conserved tyrosine residues [Feng and Lee, 2001] and is repressed in the context of the full-length protein by the C-terminal RGG-boxes [Rossow and Janknecht, 2001]. Presence of other activation domains however, counteracts this repression [Alex and Lee, 2005]. Therefore, EWSR1 functions as a transcriptional activator in cooperation with CBP/p300. It mediates transcription in a promoter- and cell-specific way and is

suppressed upon interaction of its N-terminus with SF1 [Zhang et al., 1998]. The RNA recognition motif, the zinc finger and the RGG-boxes are all involved in RNA binding. TET-proteins bind pre-mRNA and mRNA as well as ssDNA, with a preference for G/U-rich sequences [Plougastel et al., 1993]. TLS/FUS specifically recognizes a GGUG-motif [Lerga et al., 2001]. The TET RGG-boxes finally, are multifunctional. Besides repressing the activation domain and binding to RNA, they are also responsible for nuclear localization [Araya et al., 2005] and constitute the main site for protein-protein interactions.

Interactions with transcription and splicing machinery

Many protein interaction partners of TAF15, EWSR1 and TLS/FUS have been reported. With their N-terminus, TET-proteins interact with components of the transcription machinery [Petermann et al., 1998], while they recruit several splice factors both on their C- and N-terminus. As such, they are involved in transcription regulation as well as in splicing and they function as adaptors between both. TET interaction partners that belong to the transcriptional machinery are TFIID, a protein complex involved in transcription initiation that consists of a TATA-box binding protein (TBP) and several TBP-associated factors [Bertolotti et al., 1998], and the RNA polymerase II complex [Bertolotti et al., 1996]. Cabeza/SARFH, a *Drosophila* protein with high similarity to human TET-proteins, also associates with RNA polymerase II transcripts and colocalizes with the phosphorylated form of RNA polymerase II [Immanuel et al., 1995]. Proteins involved in splicing that interact with TET-members are hnRNP A1, hnRNP C1/C2 [Zinszner et al., 1994], YB1 [Chansky et al., 2001], splice factors SF1 [Zhang et al., 1998] and U1C [Knoop and Baker, 2000], several members of the serine-arginine (SR) family of proteins (SC35, SRp75) and a novel protein designated TASR for TLS-associated serine-arginine protein [Yang et al., 2000b]. Furthermore, TLS/FUS has been found to be identical to the pre-mRNA associated factor hnRNP P2 [Calvio et al., 1995] and interference of TET-proteins with splicing has been reported in different model systems [Hallier et al., 1998], [Yang et al., 2000b].

Besides transcription and splice factors, other TET interaction partners have been described, suggesting that TAF15, EWSR1 and TLS/FUS are also involved in other cellular processes and signal transduction pathways. EWSR1 interacts with calmodulin and is phosphorylated by protein kinase C in a Ca^{2+}-dependent way [Deloulme et al., 1997]. This phosphorylation inhibits its binding to RNA. Among other EWSR1 interaction partners are BTK (Bruton's tyrosine kinase), which is essential for B-cell differentiation [Guinamard et al., 1997], PYK2, which is a tyrosine

kinase involved in a variety of signal transduction processes [Felsch et al., 1999], transcription factor Brn-3a POU who's activation potential is suppressed by EWSR1 [Thomas and Latchman, 2002] and tumor suppressor BARD1 [Spahn et al., 2002]. TLS/FUS interacts with nuclear receptors of thyroid and steroid hormones [Powers et al., 1998] and TAF15 binds to and is phosphorylated by SRC, which stimulates the activation capacity of the TAF15 N-terminal domain [Lee et al., 2004]. The precise physiological significance of these interactions is still under investigation.

TLS/FUS knock out mice

The role of TLS/FUS was further investigated *in vivo* using a gene targeting strategy in mice. Two different TLS/FUS$^{-/-}$ mice were generated simultaneously. The knock out mouse generated by Hicks and colleagues failed to suckle, died within 16 hours after birth and showed deficient B-lymphocyte development and chromosomal instability [Hicks et al., 2000]. Kuroda and colleagues concluded that TLS/FUS deficiency causes a mild defect in somatic growth, male sterility and enhanced sensitivity to ionizing radiation [Kuroda et al., 2000]. Differences in the phenotypes might be explained by the use of different mouse strains and different targeting strategies, the latter resulting in low level expression of a truncated TLS protein in the model of Kuroda *et al*.

All together, TET-proteins appear to have diverse cellular functions. A latent transcriptional activation domain and interaction with RNA and components of the transcriptional and splicing machinery point to a role in regulating transcription initiation, elongation and RNA-processing. DNA-binding capacity and data from the TLS/FUS knock out mice provide evidence for a role in DNA recombination events and interactions with several cytoplasmic molecules argue for a role in signal transduction.

1.2.3 TET proteins in translocations

Recurrent involvement of TET proteins in translocations

TAF15, EWSR1 and TLS/FUS are repeatedly involved in oncogenic translocations that fuse them to a transcription factor or at least its DNA-binding domain. The most frequent TET fusion protein is EWSR1/FLI1. It is generated by a translocation t(11;22)(q24;q12) and accounts for 85% of Ewing's sarcomas (EWS). EWS is the second most common primary malignant bone tumor in children and adolescents [Arvand and Denny, 2001], and is thought to arise from neural crest progenitor cells. FLI1 is a member of the ETS-family of transcription factors. ETS-proteins share a

common DNA-binding domain and are involved in a variety of cellular processes, such as hematopoiesis and neuronal development. Besides in EWS, they are also known to play a part in leukemias and in prostate cancer, the latter by recurrent fusion of ERG and ETV1 to the androgen responsive elements of the TMPRSS2 promoter [Tomlins et al., 2005].

The structure of the EWSR1/FLI1 chimeric protein is shown in figure 9A. The N-terminal part of EWSR1 is present, but its C-terminal RNA-binding domain is replaced by the ETS DNA-binding domain of FLI1 [Delattre et al., 1992]. This is the common pattern for all TET-fusions.

An overview of all TET-fusions characterized so far, is given in figure 9B. While different types of transcription factors have been involved in TET fusions in various tumor types, fusion to ETS-factors in Ewing's sarcoma is a common theme, with the occurrence of TLS/ERG in acute myeloid leukemia as the only exception. Together with TAF15/CIZ and EWSR1/CIZ, this also is the only TET-fusion that exists in a non-solid tumor.

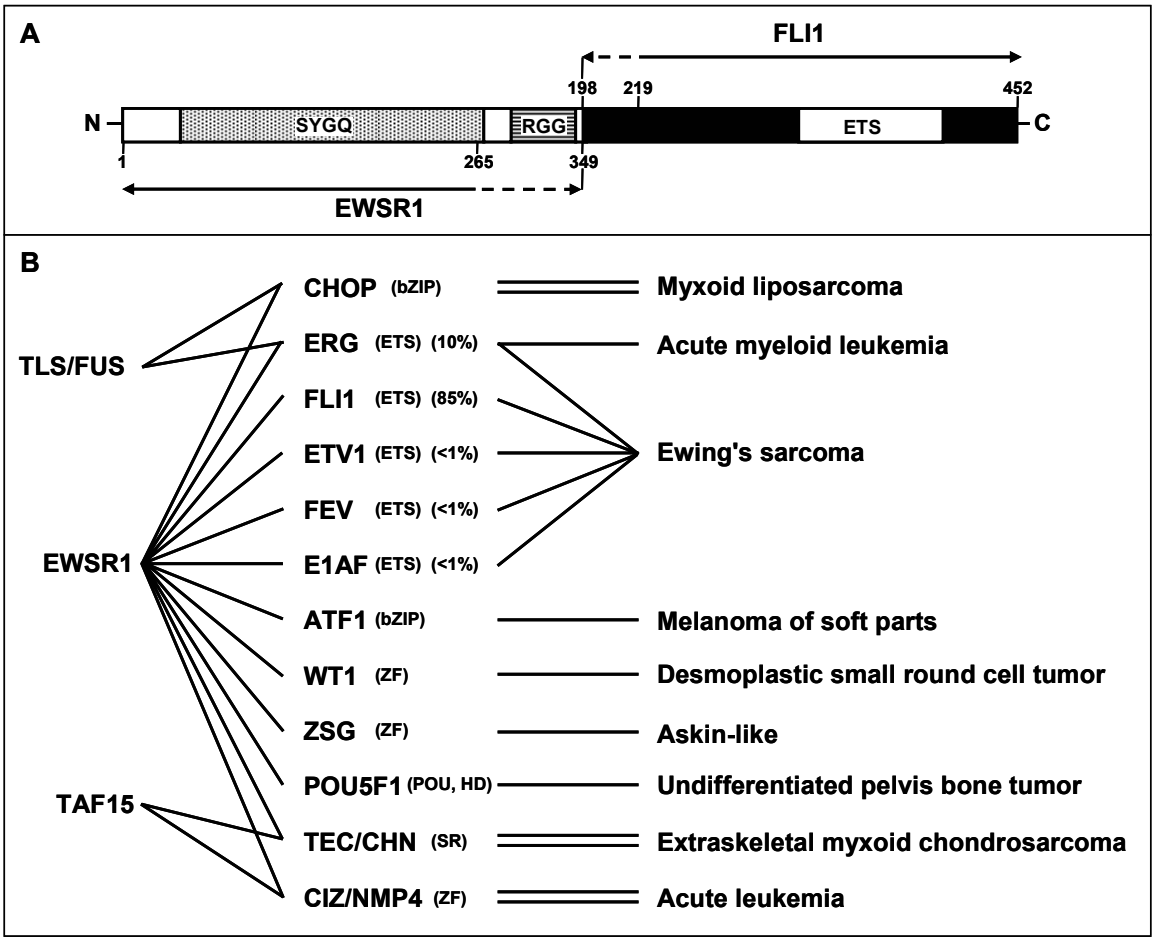

Figure 9 - TET-fusions (A) Structure of EWSR1/FLI1, the most abundant TET-fusion protein. Amino-acid numbers are below the protein for EWSR1, above for FLI1. Dashed parts of the EWSR1 and FLI1-arrows indicate regions with variable presence in the fusion. SYGQ: region rich in serine, tyrosine, glycine and glutamine, RGG: arginine-glycine-glycine repeat region, ETS: ETS DNA-binding domain. (B) Diagram showing the currently known involvement of TET-protein fusions with different transcription factor genes in various types of neoplasia. The type of the transcription factor fusion partners is indicated between brackets (bZIP = basic leucine zipper, ZF = zinc finger, POU = Pit-Oct-Unc DNA-binding domain, HD = homeodomain, SR = steroid receptor). Percentages indicate relative presences of the five ETS transciption factors among EWSR1-ETS fusions.

Transforming properties of TET fusion proteins

Since the different TET-fusions are strictly correlated to the tumors in which they arise, the proteins are thought to be causative for the diseases. Different *in vitro* and *in vivo* model systems have been used to show that TET-fusions indeed have transforming properties and that their specific inhibition reverses these phenotypes.

NIH/3T3 fibroblasts stably expressing EWSR1/FLI1 lose contact inhibition in culture, form colonies in soft agar and enhance tumor formation in SCID mice [May et al., 1993], [Thompson et al., 1999]. They also undergo morphological changes: upon EWSR1/FLI1 expression, the normally spindle-shaped fibroblasts acquire a small round cell appearance that is reminiscent of EWS tumor cells [Teitell et al., 1999]. Other TET/ETS-fusions show similar effects, although not all to the same extent [Thompson et al., 1999]. Finally, the oncogenic potential of EWSR1/ERG was illustrated *in vivo* in a conditional invertor knock-in mouse model, where its lymphocyte-specific expression causes both leukemia and thymoma [Codrington et al., 2005].

Several approaches have been used to illustrate that these transformation phenotypes are specifically caused by the chimeric proteins. Depletion of EWSR1/FLI1 by antisense cDNA, small interfering RNA or a dominant negative FLI1 all resulted in reduced cell proliferation and invasiveness, increased apoptosis and a shift in the cell cycle from S towards G1 [Ouchida et al., 1995], [Kovar et al., 1996], [Dohjima et al., 2003], [Chansky et al., 2004], [Nozawa et al., 2005].

Concerning the molecular mechanism of transformation by TET fusion proteins, two lines of reasoning are explored (figure 10). First, the chimeric proteins can act as aberrant transcription factors and many efforts have been invested in the identification of their target genes. Second, TET-fusions can interfere with normal splicing. The relative contributions of each mechanism to oncogenesis remain to be determined.

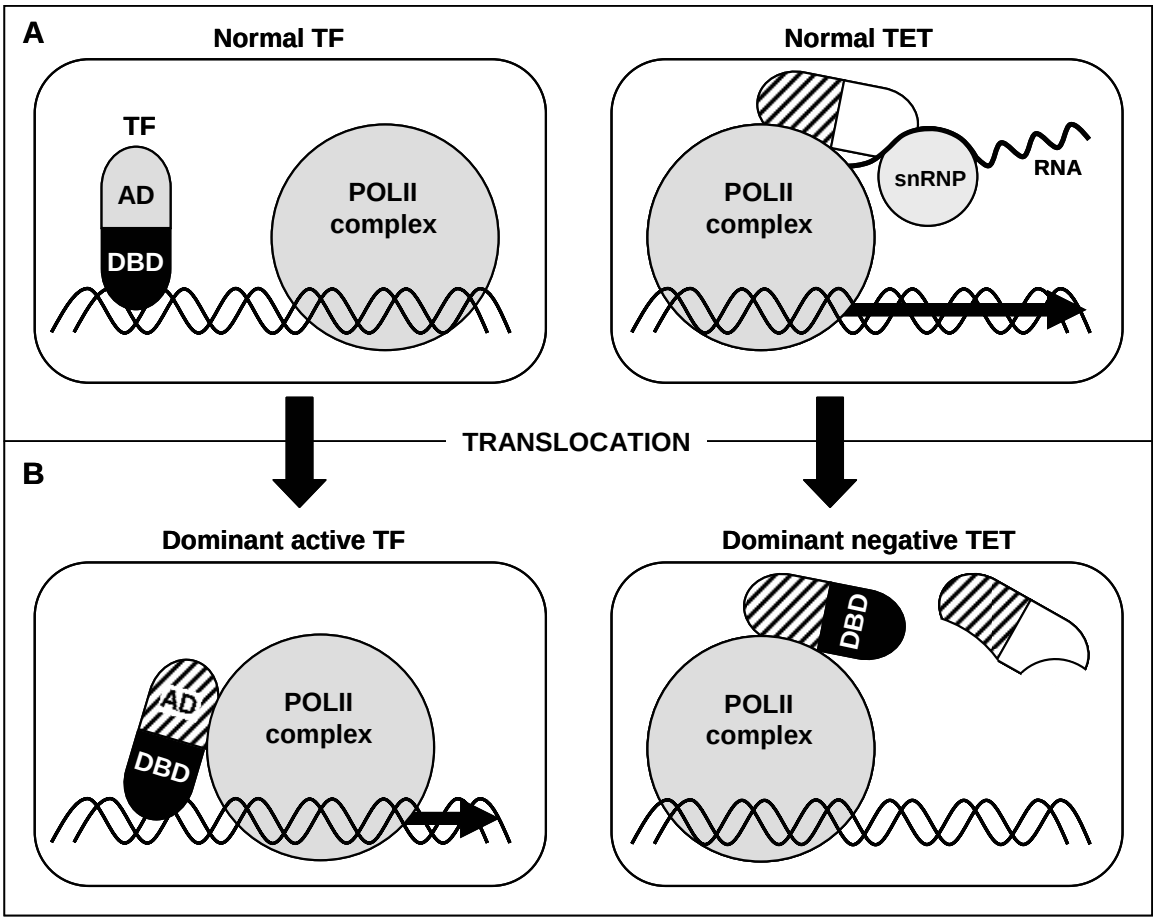

Figure 10 - Potential oncogenic mechanisms of TET/TF-fusions. (Adapted from [Arvand and Denny, 2001]). (A) Before translocation. The transcription factor (TF, left) binds DNA but depends on interaction with other proteins for coupling to the RNA polymerase II complex. The TET protein (right) interacts with both the polII complex, RNA and splice factors, providing a bridge between transcription and RNA processing. The N-terminal activation domain is repressed by the C-terminal RGG-boxes. (B) After translocation. Left: the fusion protein binds DNA with the same sequence-specificity as the original TF and directly interacts with polII. The TET activation domain (AD) is now active. Right: the TET/TF-fusion occupies TET-binding sites on the polII complex with uncoupling of transcription and processing as a consequence.

TET-fusions act as aberrant transcription factors

Several lines of evidence support the hypothesis that TET-fusions act as transcription factors. Many studies in this regard have focused on EWSR1/FLI1 as the prototype of TET fusion proteins. It localizes in the nucleus and has functional DNA-binding (DBD) and transcriptional activation (AD) domains. The FLI1-DBD still binds DNA with the same sequence specificity as normal FLI1 [Mao et al., 1994] and the TET-AD, now separated from to suppressing RGG-boxes, shows greater activation potential than

the AD of wild type FLI1 [Ohno et al., 1993], [Bailly et al., 1994]. Furthermore, both domains are necessary for transformation of NIH/3T3 cells [May et al., 1993]. Deletion or mutation of the FLI1 DBD reduces the transformation potential as well as the ability to modulate transcription of target genes [Jaishankar et al., 1999] and presence of a dominant negative, DNA-binding FLI1 protein abolishes anchorage independent growth [Welford et al., 2001]. Deletion of the TET-AD or exchanging it for the KRAB repressor domain also reduces the oncogenic potency of EWSR1/FLI1 [Chan et al., 2003], while other activation domains in some instances can functionally replace the TET N-terminus [Lessnick et al., 1995].

As new transcription factors with previously unexisting combinations of functional domains, TET/TF-fusions may lead to aberrant expression of target genes. First, the ETS-DBD now cooperates with an other activation domain, resulting in altered activation potential and different protein interaction partners [Arvand and Denny, 2001]. On the other hand, TET/ETS-fusions can also be considered as ectopically expressed ETS transcription factors. While expression of wild type FLI1 for example, is strictly controlled and lineage-specific, after translocation t(11;22) it is under the control of the 5'-regulatory machinery of housekeeping gene EWSR1.

Transcriptional targets of TET-fusions

Many attempts have been undertaken to determine TET/TF target genes and to investigate whether these provide an explanation for the oncogenic properties of TET-fusions. Different strategies have been applied (microarray transcription profiling, representational difference analysis, promoter/reporter-assays) and a number of interesting target genes has been identified. They generally tend to be involved in mitogenic signaling or cell cycle control [Ladanyi, 2002]. A selection of EWSR1/FLI target genes is shown in table 2. Many of them are highly expressed in EWS cell lines and provide a direct link to cell transformation. MFNG and PDGFC for example enhance tumor formation of NIH/3T3 cells in SCID mice [May et al., 1997], [Zwerner and May, 2001], PIM3 promotes anchorage independent growth [Deneen et al., 2003] and overexpression of $p57^{KIP2}$ and IGFBP3 blocks proliferation or induces apoptosis, respectively [Dauphinot et al., 2001], [Prieur et al., 2004]. On the other hand, dominant negative PIM3 and PDGFC proteins as well as reduction of PTPL1 expression levels reduce EWSR1/FLI1 transforming phenotypes [Zwerner and May, 2002], [Abaan et al., 2005], suggesting that aberrant expression of these targets is a key step in EWS tumorigenesis.

Table 2 - Selected EWSR1/FLI1 target genes. Up/down indicates activation or repression of the gene. ES cell lines: Ewing's sarcoma cell lines; nd: not determined.

Gene	Description	Up/ Down	Direct target	Assay	Cell system	Reference
ID2	bHLH protein antagonist	↑	+	microarray	HT-1080	[Nishimori et al., 2002] [Fukuma et al., 2003] [Hu-Lieskovan et al., 2005]
TNC	ECM glycoprotein	↑	+	microarray	HT-1080	[Watanabe et al., 2003]
PIM3	Ser/Thr kinase	↑	nd	microarray	NIH/3T3	[Deneen et al., 2003]
VEGF	endothelial growth	↑	-	immunohistochem	ES-samples	[Fuchs et al., 2004b]
EAT2	EWS/FLI1 activated transcript	↑	+	representational difference analysis	NIH/3T3	[Braun et al., 1995] [Thompson et al., 1996]
MMP3	matrix metallo-proteinase 3	↑	nd	representational difference analysis	NIH/3T3	[Braun et al., 1995]
MFNG	manic fringe, morphogenic	↑	nd	representational difference analysis	NIH/3T3	[May et al., 1997]
TGFβRII	TGFβ type II receptor	↓	+	luciferase	NIH/3T3, ES cell lines	[Hahm et al., 1999] [Im et al., 2000]
$p57^{KIP2}$	cell cycle regulator	↓	-	luciferase	NGP, HeLa, ES cell lines	[Dauphinot et al., 2001]
cMYC	cell cycle regulator	↑	-	luciferase, microarray	NGP, HeLa, ES cell lines	[Bailly et al., 1994] [Dauphinot et al., 2001] [Hu-Lieskovan et al., 2005]
PDGFC	platelet derived growth factor C	↑	nd	phenotypic expression cloning	NIH/3T3	[Hu-Lieskovan et al., 2005]
MMP1	interstitial collagenase	↑	+	luciferase	RK13	[Fuchs et al., 2003]
IGFBP3	regulator of IGFI proliferation and survival signaling	↓	+	microarray after siRNA	A673	[Prieur et al., 2004]
PTPL1	protein tyrosine phosphatase L1	↑	+	luciferase	foreskin fibroblasts	[Lessnick et al., 2002] [Abaan et al., 2005]
COL11A2	collagen constit.	↑	+	luciferase	CADO-E51	[Matsui et al., 2003]

Nevertheless, these target screenings have some limitations. Because of the non-specific histology of Ewing family tumors, the cell type from which these cancers derive is unknown. The afore-mentioned screenings are thus based on overexpression of the fusion genes in a heterologous cell context. This might influence expression regulation. Cell-specific determinants for transcription regulation are for example the expression level of the fusion protein, presence of other, co-regulatory proteins and chromatin accessibility. Recently, two different strategies were applied to circumvent this limitation. In one of the them, differences in the expression profile of a Ewing tumor cell line were evaluated after knocking down the endogenous EWSR1/FLI1 protein by siRNA [Prieur et al., 2004]. The resulting target genes could roughly be grouped into two major functional clusters: one related to ECM structure or remodeling, the other containing regulatory genes of several signal transduction pathways (Wnt, IGFI, EGF, MAPK/STAT and others). A second approach

to avoid artificial model systems was the use of chromatin immunoprecipitation in EWS cells. At least 20% of the candidate targets resulting from this screening were associated with neuronal tissue [Siligan et al., 2005]. Although for both screenings several genes were confirmed to be genuine EWSR1/FLI1-targets by complementary techniques, their overall results differed considerably. Clearly, the issue of TET-fusion transcriptional targets leaves room for further investigation.

TET-fusions interfere with splicing

Several experimental observations indicate that at least part of the oncogenic activity of TET/TF fusions must be caused by a mechanism different from transcription regulation. First, the transforming activity of EWSR1/FLI1 does not strictly depend on DNA-binding. A mutation in the FLI1-DBD that abolishes DNA-binding does not fully disrupt the EWSR1/FLI1 transformation potential [Jaishankar et al., 1999]. Furthermore, the part of the EWSR1 N-terminus that is responsible for transcription activation does not completely coincide with the part required for transformation [Lessnick et al., 1995]. Precisely this TET N-terminus however, is the constant factor in the chimeric proteins.

Disruption of normal splicing could provide an alternative mechanism of transformation. Normal TET-proteins provide a link between RNA transcription and processing: their N-terminus interacts with components of the transcription machinery, while they recruit several splice factors (SR-proteins, YB1) on their C-terminus. When the N- and C-terminal parts of the protein are separated by a translocation, this link is broken. Several TET/TF-fusions indeed have been shown to interfere with splicing mediated by SR-proteins [Yang et al., 2000a], [Yang et al., 2000b] or YB1 [Chansky et al., 2001]. EWSR1/FLI1, in contrast to normal EWSR1, interferes with hnRNPA1-dependent splice site selection in an E1A pre-mRNA splice assay [Knoop and Baker, 2001] and EWSR1/CHN influences distal 5'-splice site usage [Ohkura et al., 2002]. Correct splicing is an important determinant for gene expression and its disruption implies a well acknowledged oncogenic potential [Caballero et al., 2001], [Kalnina et al., 2005].

Altogether, both aberrant transcription regulation and interference with normal splicing contribute to TET-fusion mediated oncogenesis. Perhaps the recurrent generation of chimeric proteins combining functional domains of TET-proteins and transcription factors provides a powerful mechanism to simultaneously disrupt normal transcription and post-transcriptional regulation in tumors.

1.2.4 CIZ/NMP4

Identification of CIZ/NMP4/TNRC1/ZNF384

Ciz or "p130Cas interacting zinc finger protein" was first identified as an interaction partner of adaptor protein p130Cas [Nakamoto et al., 2000]. p130Cas resides at focal adhesions, becomes phosphorylated upon integrin stimulation and serves as a docking site for the assembly of protein complexes that mediate signal transduction towards the nucleus. In a screen for new ligands of the p130Cas SH3 domain, Nakamoto and colleagues isolated *Ciz* from a cDNA library of rat 3Y1 fibroblasts.

Almost simultaneously, the same protein was characterized as Nmp4 or "nuclear matrix protein 4", an architectural transcription factor from rat osteoblasts that binds to the promoter of the *COL1A1* gene [Alvarez et al., 1997], [Thunyakitpisal et al., 2001]. Nuclear matrix proteins constitute a scaffold in the nucleus to which DNA is attached. This matrix provides a physical link with the cell environment via its interconnection with the cytoskeleton. As a member of the nuclear matrix, Nmp4 therefore resides at the endpoint of a mechanical signal transduction pathway that couples changes in cell architecture to differences in gene expression by altering promoter geometry.

The human CIZ ortholog was originally identified in a screen for cDNAs with long CAG repeats from human brain and therefore called TNRC1 (tri-nucleotide repeat containing 1) or CAGH1. According to HUGO nomenclature however, the official name for CIZ is ZNF384 (zinc finger protein 384).

Gene and protein structure

Human *CIZ* is located at chromosome 12p13. It spans 60 kb of genomic DNA and counts 14 exons, of which exons three to eleven contain the open reading frame [Martini et al., 2002]. The protein counts 578 amino acid residues. It consists of an N-terminal putative leucine zipper, a serine rich region, a proline rich region, five to eight Krüppel-type C_2H_2 zinc fingers and a C-terminal glutamine/alanine repeat (figure 11). It is strongly conserved among different species, with 99% amino acid identity between rat and human CIZ outside the polymorphic QA-repeat.

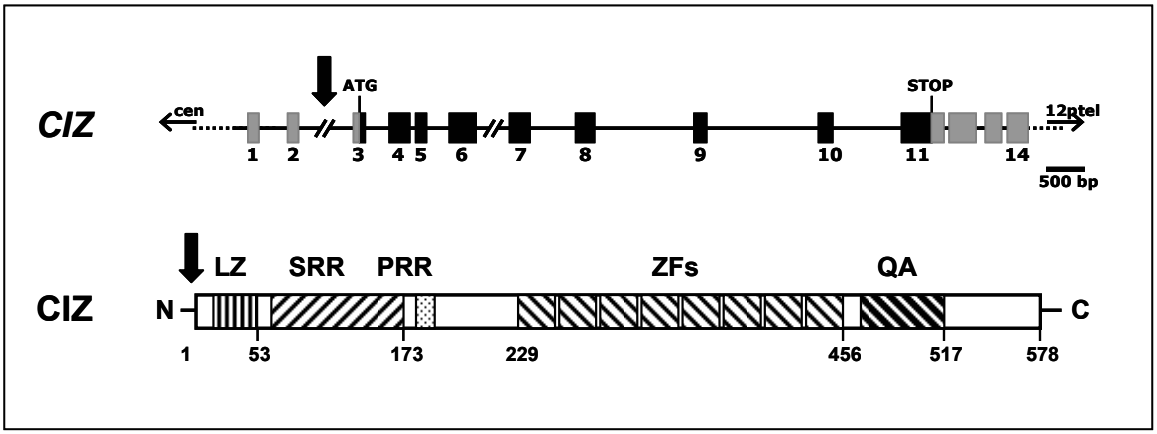

Figure 11 - **Gene and protein structures of human CIZ**. Exon and amino acid numbers are indicated below the structures. Block arrows indicate breakpoints of translocations t(12;17)(p13;q11) and t(12;22)(p13;q12). Tel: telomere, cen: centromere, LZ: leucine zipper, SRR: serine rich region, PRR: proline rich region, ZFs: C_2H_2 zinc fingers, QA: glutamine/alanine rich region.

Different functions have been assigned to several subdomains of CIZ. The proline rich region of rat Ciz serves as an interaction site for the SH3-domain of p130Cas. Interestingly, despite the strong similarity between rat and human CIZ, the human ortholog counts one P-residue less in this region, abolishing the exact match with the consensus sequence for interaction with p130CAS [Kirsch et al., 1998].

The zinc fingers of CIZ constitute a multifunctional domain. They are responsible for both DNA binding and nuclear localization of the protein. Seven different CIZ isoforms have been isolated so far, all splice variants of the same gene [Thunyakitpisal et al., 2001]. They differ in their number of zinc fingers. At least five zinc fingers are necessary but sufficient for CIZ nuclear localization [Feister et al., 2000]. Presence of zinc fingers two, three and six is the minimum requirement for DNA binding [Torrungruang et al., 2002a].

The capacity to mediate a variety of functions for one protein is not exceptional for C_2H_2 zinc fingers. Among transcriptional regulators, those with C_2H_2 zinc fingers as DNA binding domains constitute by far the largest family with several hundred members [Ganss and Jheon, 2004]. Besides DNA binding, these zinc fingers often also mediate protein protein interactions and may contain information for subcellular localization. More than ten different types of zinc finger domains have been described. C_2H_2-type fingers are those using two conserved cysteine and histidine residues to form coordinate bonds with the zinc ion in a tetrahedrical structure, as

such stabilizing the finger-like structure of the domain. They are alternatively referred to as 'Krüppel'-type zinc fingers because of their presence in the Drosophila gene Krüppel, which causes a 'cripple' phenotype when inactivated.

Finally, the glutamine/alanine rich region near the C-terminus of CIZ is involved in transcriptional activation [Torrungruang et al., 2002a]. Glutamine-stretches have been reported to activate transcription when fused to a DNA binding domain [Gerber et al., 1994]. Indeed, deletion of the QA rich region of CIZ reduces its transcriptional activation potential approximately by half. The N-terminal part of CIZ also shows transactivation potential when directly fused to a DNA binding domain. However, this activity is completely masked within the context of the full-length protein.

CIZ is a transcription factor

With its DNA binding and transcriptional activation domains, CIZ has the structure of a transcription factor. Its DNA consensus binding sequence is (G/C)AAAA(A) and was determined by cyclic amplification and selection of targets (CAST) analysis: COS-7 cells were transfected with CIZ-flag and their nuclear extract was incubated with random DNA oligomers. After immunoprecipitation with anti-flag, the precipitated DNA was extracted, amplified by PCR and incubated again with nuclear extract. After five such rounds of 'CASTing', the final PCR-product was cloned and sequenced. The CIZ binding consensus is present in the promoter of *COL1A1* and several matrix metalloproteinases (*MMP1* encoding collagenase 1, *MMP3* or stromelysin, *MMP7* or matrilysin, *MMP13* or collagenase 3). Ciz also induces expression of these genes [Nakamoto et al., 2000], [Shah et al., 2004]. COL1A1 is a building block of collagen type I, which is the major constituent of the ECM in bone tissue. MMP1 and MMP3 on the other hand break down collagen I. This suggests that CIZ is involved in fine-tuning ECM turnover rate, rather than in simple accumulation of the constituent proteins. Ciz transcription regulation has been reported to be parathyroid hormone responsive in the cases of *COL1A1* [Alvarez et al., 1998] and *MMP13* [Shah et al., 2004]. Ciz tempers the transcriptional activation response that is induced by the hormone.

A remarkable characteristic of transcription factor Ciz is its allosteric regulation. The DNA to which Ciz is bound, has an effect on its transcription regulation properties. The C_2H_2 zinc fingers bind to the minor groove of the homopolymeric (dA:dT) recognition motif, which is rather unusual for this type of zinc fingers. They probably receive structural information from the DNA binding site, resulting in site-specific

conformational changes of the protein, which alter its mode of transcription regulation [Torrungruang et al., 2002a].

Ubiquitous CIZ expression

All CIZ orthologs are expressed in all tissues tested so far. Rat Ciz expression was investigated on embryo sections (day 18 post coitus) of bone tissue and both central and peripheral nervous system [Thunyakitpisal et al., 2001]. Adult rat heart, brain, spleen, lung, liver, skeletal muscle, testis and kidney were tested by Northern analysis. Particularly high expression was seen in testis, heart and brain [Nakamoto et al., 2000]. Mouse Ciz was present in all stages of embryonic development with highest expression from day E11 to E15 [Thunyakitpisal et al., 2001]. Human CIZ was present in brain, heart, skeletal muscle, colon, thymus, spleen, kidney, liver, small intestine, placenta, white blood cells and several hematopoietic cell lines.

CIZ expression is driven by two different promoters that initiate transcription at alternative first exons [Alvarez et al., 2005]. Both promoters have housekeeping and tissue-specific characteristics. They lack TATA and CCAAT boxes and have CpG islands, but also contain regulatory sequences for cell- and tissue-specific expression, predominantly supporting bone development, gonadal development and hematopoietic growth. This confirms the broad expression pattern of CIZ and simultaneously suggests a context-specific functionality.

Subcellular localization

Conform its role as a transcription factor, CIZ predominantly localizes in the nucleus. This is the case in all cell types under investigation (primary rat osteoblasts, MC3T3-E1, UMR-106, R0S17/2.8, 3Y1, NIH/3T3, HEK293T) [Nakamoto et al., 2000], [Feister et al., 2000], [Torrungruang et al., 2002b], [Corveleyn et al., 2005]. Nuclear staining is diffuse, with more intense speckles, but absent from nucleoli in NIH/3T3 and HEK293T cells [Corveleyn et al., 2005]. The CIZ zinc finger domain is responsible for this nuclear localization and a minimum of five zinc fingers is required for exclusive nuclear targeting.

Concerning the cytoplasmic localization of CIZ, different results have been reported. Nakamoto and colleagues report focal adhesion staining of Ciz in rat 3Y1 fibroblasts. Moreover, they show nucleo-cytoplasmic shuttling of Ciz using a heterokaryon cell fusion assay [Nakamoto et al., 2000]. Torrungruang and colleagues however, observed Ciz localization at mitochondria, in or close to the Golgi apparatus and faintly throughout the cytoplasm. This differences are probably due to specific

localization patterns of distinct Ciz isoforms [Feister et al., 2000] and the use of antibodies recognizing different isoforms or alternative GFP-fusion proteins.

CIZ functions

CIZ function was investigated *in vitro* and *in vivo*: it is involved in bone development and spermatogenesis and is a novel type inhibitor of BMP2/Smad signaling.

In *in vitro* experiments, mouse osteoblast-like MC3T3-E1 cells showed increased attachment, decreased proliferation and higher *COL1A1* expression levels upon CIZ overexpression [Furuya et al., 2000a]. In the same cell line, Shen established CIZ as a novel type inhibitor of BMP2/Smad signaling. BMPs or bone morphogenetic proteins belong to the TGFβ superfamily of growth factors and are involved in a variety of cellular processes [Massague, 2000]. Upon receptor binding, BMP signals are transduced towards the nucleus by means of Smad proteins (figure 12B). Transduction of BMP signals however, is strictly controlled by several types of inhibitors, acting at all possible stages of the signal transduction pathway. BMP2 is specifically important for bone cell development (figure 12A). Shen and colleagues showed that CIZ suppressed BMP2-induced expression of *Cbfa1, COL1A1, ALP* and *OCN* by blocking Smad1 and Smad5 mediated transcription regulation [Shen et al., 2002]. Since CIZ is different from other BMP-inhibitors because of its subcellular localization and its transcription factor properties, CIZ was denominated a novel type BMP2/Smad inhibitor. Whether CIZ directly interacts with the R-Smads or not and at which stage of signal transduction it acts, has to be investigated.

A Ciz knock out mouse was generated for *in vivo* functional analysis [Nakamoto et al., 2004]. $Ciz^{-/-}$ mice grow to adulthood, but tend to be smaller then their wild type littermates, with slightly reduced body weights. Adult bone mass however, was increased in the knock out mice [Morinobu et al., 2002]. $Ciz^{-/-}$ mice had an increased trabecular bone formation rate and CIZ deficiency enhanced BMP2-induced expression of osteogenic genes (*ALP, COL1A1*) in bone marrow cells. BMP2-injection also enhanced bone formation in the knock out mice about two-fold more than in the wild type, in support of the *in vitro* finding that CIZ acts as an inhibitor of BMP2/SMAD signaling. $Ciz^{-/-}$ mice also showed fertility defects and impaired spermatogenesis with increased apoptosis in spermatogenic cells [Nakamoto et al., 2004]. Although the exact role of Smad1 in spermatogenesis is not know, the protein is expressed in spermatogenic cells and colocalises with Ciz, suggesting that the inhibitory effect of Ciz might again be involved in generation of the phenotype.

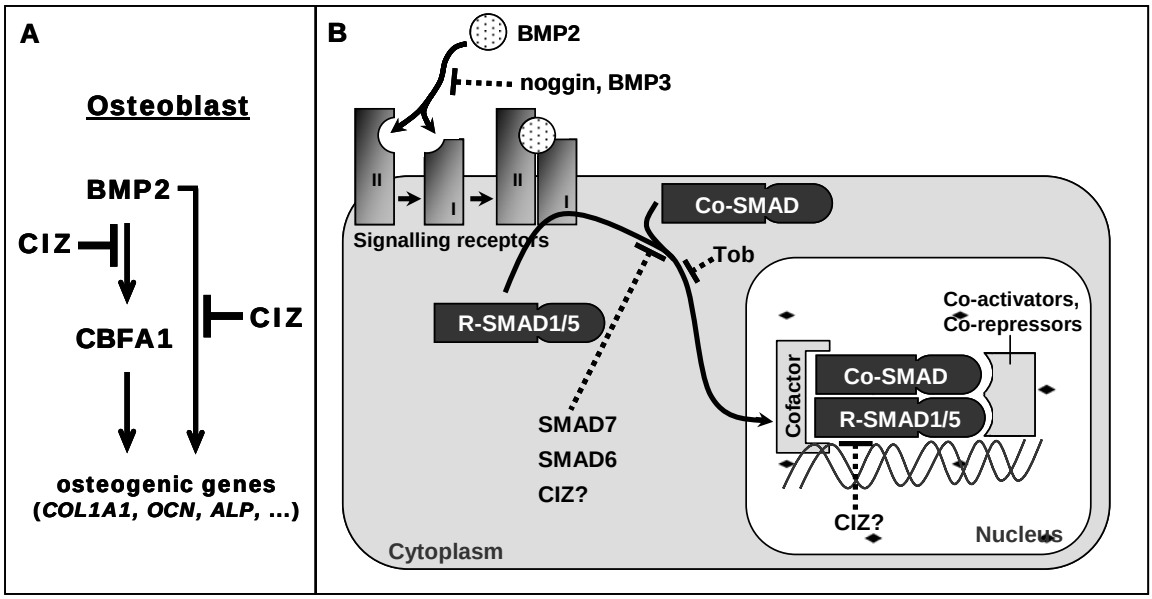

Figure 12 - BMP2/Smad signaling in osteoblasts. (A) BMP2 activates CBFA1 (RUNX2), a transcription factor necessary for osteoblast differentiation. It also induces expression of several osteogenic genes further downstream of CBFA1, like collagen type I (*COL1A1*), alkaline phosphatase (*ALP*) and osteocalcin (*OCN*). CIZ inhibits BMP2 induced expression of these genes. (B) BMP signals are transferred to the nucleus by means of SMAD proteins. Upon receptor binding, BMP2 activates the cytoplasmic tail of its receptor. This leads to phosphorylation of regulatory SMADS (R-SMADS) 1 and 5, which in turn associate with cooperating SMADS (Co-SMADS). The R-SMAD/Co-SMAD complex migrates to the nucleus where it interacts with co-regulatory molecules and directs transcription. BMP/Smad-signaling is strictly regulated by various types of inhibitors, acting at all possible stages of the signaling cascade. Soluble inhibitors as noggin and BMP3 act outside the cell and prevent binding of BMP to its receptor. Inhibitory SMADS (I-SMADS) 6 and 7 prevent signaling by binding to R-Smads. Tob inhibits nuclear transport and/or transcription regulation. CIZ is a novel type inhibitor of BMP2/SMAD signaling. It disturbs R-SMAD1 and 5 induced transcription regulation, but its site of action is not yet known.

CIZ has a variety of functions in multiple tissues. On the one hand, it has a role in spermatogenesis and in bone metabolism and is suggested to be involved in hematopoiesis. On the other hand, it also has housekeeping characteristics. CIZ is adapted to this context-specific functionality in several ways. First, seven different isoforms of the protein have been characterised. Second, CIZ 5' regulatory machinery provides the basis for both ubiquitous expression and tissue-specific regulation. Finally, allosteric regulation allows fine-tuning of CIZ actions based on the specific promoter to which it is bound.

CIZ in translocations

CIZ is recurrently involved in translocations in ALL. Besides its fusion to TAF15 and EWSR1 in translocations t(12;17)(p13;q11) and t(12;22)(p13;q12), it is also involved in a cryptic translocation t(12;19)(p13;p13) in ALL that fuses CIZ to the N-terminus of E2A [Hunger and Zhong, 2002], [La Starza et al., 2005].

1.3 Mouse models for leukemia

1.3.1 The need for an *in vivo* model system

As for all other cancers, leukemia is a complex disease for which interaction with the microenvironment in the host organism is essential. Therefore, it is important to study the disease in animal models. Good models can yield new insights into both normal biology and the molecular pathology of the disease. The discovery of new leukemia genes and pathways in turn provides us with new diagnostic markers and candidate drug-targets. In addition, the models can be used to evaluate these novel therapies or preventive agents and to identify potential leukemogenic risk factors.

1.3.2 The ideal leukemia model

A disease model has to mimic the corresponding human condition as closely as possible. Therefore, the ideal model system for a non-familial leukemia has to meet many criteria. Its genetic lesion should be present only in the tumor forming cell type. Presence of an otherwise wild-type cellular environment is important because it can promote or inhibit cancer development, but also to avoid tumorigenesis outside the tissue of interest. The mutation has to arise at a defined age or developmental stage, since germline mutations may cause developmental defects or embryonic lethality, or they could trigger compensatory factors or pathways that balance the effect of the mutation. The mutation has to be correctly engineered, taking into account the reduction to hemizygosity of the normal allele and the occurrence of a reciprocal translocation when necessary. The expression of the mutated genes should be cell-specific, time-controlled and of relevant expression level. The model system should allow observation of the disease progressing through all stages of tumor development. Finally, it must be possible to study the mutation in the context of the appropriate second hits [Hann and Balmain, 2001], [Ren, 2004].

1.3.3 The mouse is an excellent model organism

Several organisms and different strategies have been used to model hematological malignancies. There are reports of modeling leukemia in zebrafish [Kalev-Zylinska et al., 2002], [Langenau et al., 2003], [Onnebo et al., 2005] and *Drosophila* [Asha et al., 2003]. Because of their availability, relatively low cost and short reproduction time, these model systems are well suited to study cell transformation. However, for analysis of the entire disease process, the mouse is considered the most suitable system because of two major reasons: (i) its striking physiological, anatomical and genomic similarities to humans and the fact that mice develop cancer, and (ii) our

increased understanding of the mouse genome and the development of many different tools to manipulate it as well as to analyze the consequent phenotypes.

Some disadvantages of the mouse as a cancer model are (i) its longer telomere length (40-60 kb compared to 10 kb in humans) [Balmain and Harris, 2000], (ii) other differences in basic cellular processes (like dealing with oxidative stress and the relative contributions of p53 and Rb pathways to cell senescence) [Rangarajan and Weinberg, 2003], (iii) a different drug metabolism and (iv) its shorter lifespan. This implicates that, like for other model systems, extrapolation of results has to be done carefully. In this context, much attention has been devoted to determining how well the mouse leukemias resemble the human diseases. This was originally done by comparison of biological, immunophenotypic and morphological features, but is now also based on molecular profiling techniques (e.g. microarray, comparative genomic hybridization) and non-invasive imaging [Jonkers and Berns, 2002].

1.3.4 Genetically engineered leukemia models

The first leukemia mouse models were xenografts in immunodeficient mice and leukemias induced by chemicals or radiation [Dick, 1996], [Rithidech et al., 1999]. A second generation of models is that of genetically engineered mice, which will be discussed below. Their most prominent advantage over the first generation models, is that they allow analysis of the multiple stages of cancer progression dictated specifically by the genetic lesion of interest.

1. The transgene approach

In the classical transgenetic approach, a DNA construct encoding the oncogene under investigation is injected into one of the pronuclei of a fertilized egg, where it integrates randomly into the genome (figure 13). The injected zygote is implanted in the uterus of a foster mother and its offspring is tested for presence and proper expression of the transgene.

The technique is straightforward, but has some limitations. In contrast to the somatically acquired, sporadic mutations that normally occur in tumorigenesis, the transgene is present in all cells of the body. Moreover the level, tissue and developmental stage of its expression depend on heterologous promoters and/or the site of genomic integration and the copy number.

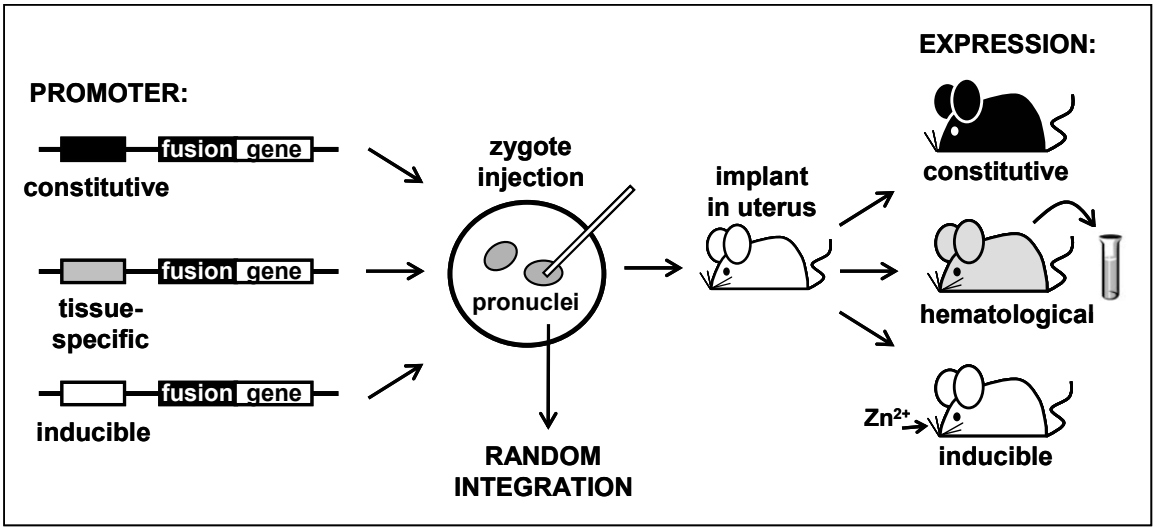

Figure 13 - **The transgenetic approach**. The expression pattern of the transgene can be modified by the use of tissue-specific or inducible promoters. (Adapted from [Bernardi et al., 2002]).

Aberrantly timed oncogene expression can interfere with normal development and lead to embryonic lethality. This was illustrated in an attempt to model ALL by transgenetic expression of BCR/ABL^{p190}, driven by the *bcr* promoter [Heisterkamp et al., 1991]. Initial efforts to model acute promyelocytic leukemia (APL) also failed because the unrestricted expression of the PML/RARα fusion gene under the control of the β-actin promoter resulted in embryonic lethality [He et al., 1997]. However, problems of constitutive transgene expression can at least partly be overcome by the use of inducible promoter systems. When BCR/ABL^{p190} is under the control of the zinc-inducible metallothionein-1 (MT1) promoter, transgenic mice develop normally and die of acute leukemia two to eight weeks after induction of the transgene [Heisterkamp et al., 1990].

Yet if not lethal, aberrant oncogene expression can still cause other unauthentic phenotypes that invalidate the model, like sterility or the formation of tumors outside the tissue of interest. MT1-driven PML/RARα expression for example surprisingly resulted in liver pathologies rather than in APL after zinc induction, stressing the importance of tissue-specific expression [David et al., 1997]. Similarly, the promoter of the tec gene (encoding a cytoplasmic tyrosine kinase preferentially expressed in hematopoietic lineages) has successfully been used to model myeloproliferative disorders caused by BCR/ABL^{210} [Honda et al., 1998], while zinc induced but

ubiquitous MT1-driven expression of the same fusion gene exclusively resulted in B-ALL [Voncken et al., 1995].

Many tissue-specific and inducible promoter systems are available to monitor oncogene expression both temporarily and in terms of tissue distribution. To avoid the leaky and ubiquitous expression of metal-induced promoters, binary systems have been developed in which the induction of expression is dependent on the interaction of two components. The tetracycline-dependent regulatory systems are the most widely used. They control expression at the level of transcription. In the tamoxifen-dependent system, control is on the functionality of the protein [Eilers et al., 1989]. None of these systems however, perfectly matches the expression pattern generated by the endogenous promoter.

2. The knock in approach

The knock in approach differs from the transgenetic strategy in that the integration of the oncogene into the genome is targeted instead of random. This is possible by homologous recombination in embryonic stem cells. As the oncogene replaces the wild type allele at its own genomic locus, it is under the transcription control of the endogenous promoter. Moreover, no other genes are accidentally hit by its integration and the endogenous gene is reduced to heterozygosity as is the case in the human tumors.

Knocking in MLL/AF9 or BCR/ABL^{p190} into their genomic loci resulted in successful models for the corresponding human diseases AML and B-ALL, respectively [Corral et al., 1996], [Castellanos et al., 1997]. However, a knock in model for AML1/ETO, which normally occurs in AML, was embryonically lethal because of a block in foetal hematopoiesis [Yergeau et al., 1997]. Even though the knock in strategy brings the fusion gene under the control of its own promoter, it is still present in the germline and will be expressed not only in hematopoietic precursors, but in all cells and developmental stages in which wild type AML1 is expressed. Combination with the Cre/loxP-system can be used to make this expression conditional.

3. Conditional transgene expression using Cre/loxP

Cre is a DNA-recombinase that specifically recognizes a 34 bp 'loxP' sequence and drives a recombination reaction that results in the excision of a sequence flanked by two loxP-sites ('floxed') in the same orientation or its inversion when they are in opposite directions [Sauer and Henderson, 1988]. When a transgenic animal is generated which carries a floxed 'stop cassette' in between the transgene and its promoter, the intervening sequence prevents transcription of the downstream DNA (figure 14). If the mouse is crossed with a strain expressing Cre in a time and/or tissue-specific or inducible manner, the double transgenic animals will carry a functional oncogene only in those tissues and developmental stages in which Cre is expressed. Except for intercrosses, Cre can also be delivered somatically by viral infection. This strategy allows to limit the number of mutated cells by titrating the virus and thus to mimic the clonal origin of tumorigenesis more closely [Cohen, 1999], [Ren, 2004].

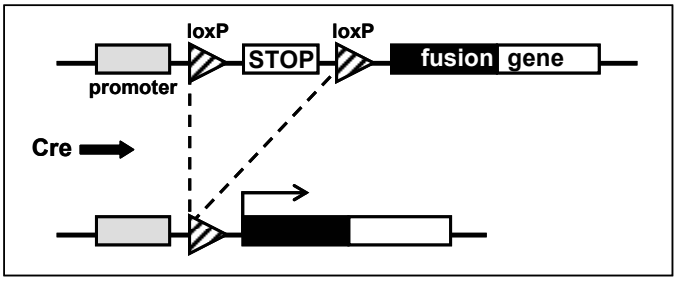

Figure 14 – Conditional gene expression using Cre/loxP. A floxed stop cassette is removed by Cre recombinase, enabling transcription of the transgene.

Disadvantages of the Cre/loxP-system are (i) that the recombinase mediated gene switch is irreversible and (ii) that Cre can induce DNA-damage by its nuclease activity [Loonstra et al., 2001] and might be cytotoxic by catalyzing recombination between pseudo-loxP sites that exist in mammalian genomes [Thyagarajan et al., 2000]. Nevertheless, it remains a versatile tool for conditional transgene expression and furthermore allows mimicking of chromosomal translocations, as is discussed for the translocator and invertor mouse systems.

4. The translocator mouse

For the generation of a translocator mouse, loxP sites are introduced by homologous recombination in ES cells at sites corresponding to the breakpoints of the translocation to be mimicked. The targeted genes in the resulting transgenic mice are fully functional. After crossing with Cre-expressing mice or somatic Cre delivery, a *de novo* translocation takes place generating both derivative chromosomes,

exclusively in the cells that express Cre (figure 15) [Smith et al., 1995], [Van Deursen et al., 1995].

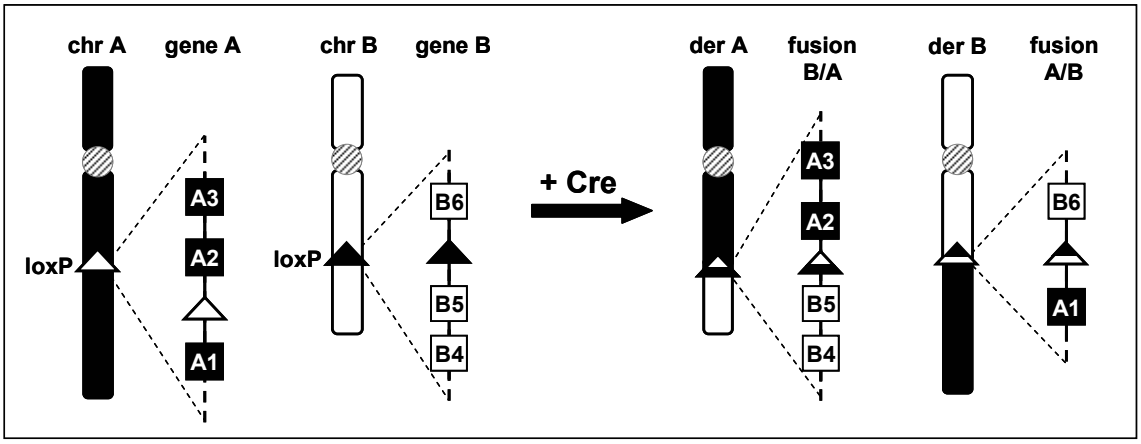

Figure 15 – **The translocator mouse**: Cre/loxP mediated *in vivo* translocation.

This sophisticated model accurately replicates the functional events that lead to the development of the corresponding human cancer: the translocation is somatic and not present throughout development, it happens only at sites of Cre-expression (which can be monitored to be sporadic as discussed above) and both reciprocal fusion genes are present, each possibly contributing to cancer development [Rego and Pandolfi, 2002]. The strategy has been used successfully to create Mll/Af9 [Collins et al., 2000], Mll/Enl [Forster et al., 2003] and Aml1/Eto [Buchholz et al., 2000] fusions *in vivo*.

5. The invertor mouse

The translocator strategy has one real limitation: genes in the mouse genome do not always have the same orientation towards the centromere as their human counterparts. If the genes are misaligned, a translocation would generate dicentric or centromere-less chromosomes. This is avoided by the inversion of one of the fusion genes in the invertor mouse (figure 16). It is a conditional version of the knock-in approach and was first used to mimic generation of the Ews/ERG fusion gene [Forster et al., 2005].

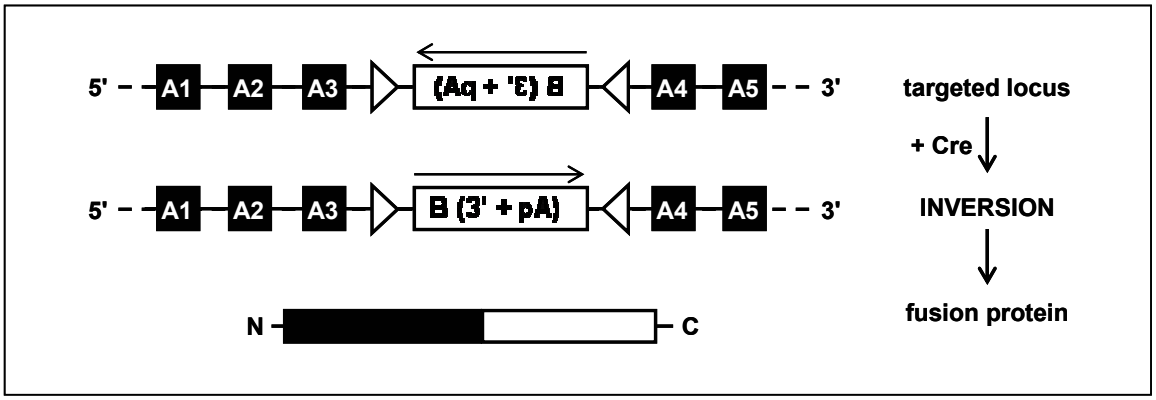

Figure 16 - **The invertor strategy**. A floxed cassette "B(3'+pA)" consisting of the 3' end of a gene B and its poly-adenylation signal, is knocked in to an intron of its translocation fusion partner A, in an orientation opposite to transcription of A. Conditional, Cre-induced inversion of the segment allows expression of the A/B fusion gene.

6. Bone marrow transplant

The bone marrow reconstitution approach basically includes three experimental steps: bone marrow is isolated from femurs and tibia of donor mice, transduced *ex vivo* to introduce an oncogene and transplanted into an acceptor mouse (figure 17). Donor mice are injected with 5'-fluorouracil (5'FU) before bone marrow isolation to stimulate proliferation of immature hematopoietic cells, which is a prerequisite for retroviral transduction [Lerner and Harrison, 1990]. Cells are cultured *in vitro* in the presence of several cytokines (IL3, IL6, SCF) and infected with replication defective retroviruses carrying the oncogene and a selective or fluorescent marker. After transduction, the genetically modified bone marrow is injected into the tail vein of lethally irradiated, syngeneic acceptor mice.

Transplantation of retrovirally transduced bone marrow is a versatile tool for modeling leukemia. The use of adult bone marrow and the ability to sort cells before and/or after transduction enables to work with cell-type specific and somatically acquired instead of germline mutations and this in a far less time-consuming way than for other models with the same characteristics. To bring the level of oncogene expression at pathophysiologically relevant levels, it is important to use optimal retroviral titers. If necessary, cells with correct expression levels can be sorted out before transplantation, or retroviral vectors with transcription or translation regulatory elements can be used [Ren, 2004]. The technique can also be applied to gain insights into the possible contribution of other specific signal transduction

pathways to the disease. This can be monitored using bone marrow from donor mice carrying already one or more mutations. The use of IL3$^{-/-}$ mice as donors in a Bcr/Abl experiment for example, indicated that IL3 is not required for the development of Bcr/Abl dependent CML [Ilaria, Jr., 2004].

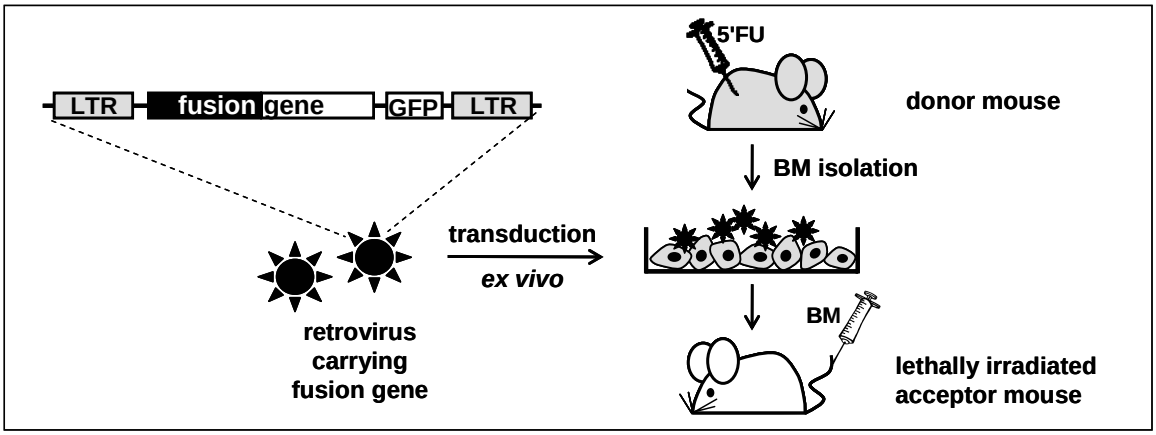

Figure 17 - **Transplantation of retrovirally transduced bone marrow**. Bone marrow is isolated from donor mice, transduced *ex vivo* to introduce an oncogene and finally transplanted into an acceptor mouse. 5'FU: 5'-fluorouracil, LTR: long terminal repeat.

The bone marrow transplant model has two main disadvantages. First, the construct integrates randomly into the genome and can affect more than one hematopoietic target cell. Thus, although modeling clonal oncogenesis more closely than most transgenic and knock-in models, bone marrow transplantation can still lead to oligo- or polyclonal disorders. However, the unique proviral integration site allows to determine the clonality of the disease as well as its transplantability into secondary recipient mice [Ren, 2002]. A second disadvantage of the system is its sensitivity to a number of variables that can have an effect on the latency or the type of the disease. By priming the donors with 5'FU for example, the normal composition of the bone marrow is altered before transduction. This is thought to facilitate the generation of myeloid rather than lymphoid leukemias in the transplanted animals. The use of lentiviruses can provide an alternative, since they are able to infect non-dividing cells and thus allow the use of untreated donor marrow [Vodicka, 2001]. The ratio of transduced versus untransduced cells can also influence disease formation. A selectable marker enables measuring and increasing this ratio. Growth conditions during the *ex vivo* culturing phase of the bone marrow are crucial, since they can trigger differentiation of the cells into specific directions. Finally, different mouse strains exhibit markedly different cancer tendencies, which might be related to

different genetic backgrounds and interactions of the tumor with the microenvironment in the host. It is clear that standardization of the bone marrow transplant approach is important to reduce as many of the experimental variables as possible [Ren, 2004].

Sensitivity to these factors was illustrated in the bone marrow transplant model for CML. Donor marrow of 5'FU-treated BALB/c mice that was infected with Bcr/Abl^{210} generated a myeloproliferative disorder in approximately 25% of acceptor mice [Daley et al., 1990], while other mice developed B-ALL or macrophage/monocytic lineage leukemias. This variability resulted from Bcr/Abl expression in different hematopoietic progenitor cells [Elefanty et al., 1990]. Furthermore, similar experiments in other mouse strains did not form CML-like diseases, reflecting the greater susceptibility of BALB/c mice for Bcr/Abl induced leukemia [Risser et al., 1978], [Ilaria, Jr., 2004]. Refinement of the original protocol resulted in an efficiency shift of generating CML towards 100% [Pear et al., 1998].

Concluding remark

A perfect murine leukemia model should (i) faithfully recapitulate the etiologic, genetic, pathophysiologic and therapeutic characteristics and the natural history of the human condition, and (ii) be an efficient experimental system for the analysis of the molecular mechanisms behind the disease. Although improved technologies have allowed the development of increasingly sophisticated models, it must be recognized that each of them has its specific strengths and weaknesses. No single model will meet all criteria. The most appropriate systems must be selected according to the specific questions that have to be addressed and complementary systems can be used simultaneously to provide an adequate understanding of the disease.

1.3.5 New insights from mouse models: APL as an example

APL or acute promyelocytic leukemia is an AML that is characterized by a differentiation block in the promyelocytic stage of myeloid development. It is associated with balanced, reciprocal translocations that fuse RARα (retinoic acid receptor α) to one of five different fusion partners, indicated together as the X-genes (reviewed by [Merghoub et al., 2001]). In more than 95% of cases, the fusion partner of RARα is PML (promyelocytic leukemia). PLZF (promyelocytic leukemia zinc finger protein) is an other X-gene. Although both X-RARα and RARα-X fusions are present in about 70% to 80% of the patients, it is the X-RARα protein that was

originally thought to cause the disease, because it invariably contains the part of RARα that is responsible for heterodimerization and ligand and DNA-binding. Moreover, the C-terminus of PML, which is present in the reciprocal RARα-PML fusion, has no known function so far. Mouse models however, show that both X-RARα and RARα-X fusions and the reduction of wild type X to a single allele are all involved in the genesis of APL (figure 18).

Different transgenic mouse models for X-RARα induced leukemia were generated using the early myelopoiesis specific promoters of human cathepsin-G (hCG) or human migration inhibitory factor-related protein 8 (hMRP8). In hCG-PML-RARα mice, APL developed in about 10% of mice, with a latency of 12 months [He et al., 1997]. In hMRP8-PML-RARα mice, disease onset was three to nine months [Brown et al., 1997]. Like for the human leukemia, treatment with retinoic acid (RA) was able to induce differentiation of the promyelocytic blasts in both systems. Transgenic hCG-PLZF-RARα mice developed leukemia earlier and with a higher frequency than the PML-mice, but the disease is RA-resistant and its phenotype resembled CML rather than APL since the leukemic cells remained able of terminal differentiation [He et al., 1998]. Altogether, these models confirm the leukemogenic capacities of the X-RARα fusions, but either the low penetrance or the different phenotype of the disease suggest that more genetic hits are necessary for the development of APL.

Therefore, the impact of the reciprocal RARα-X fusion as well as the reduction to heterozygosity of the normal X allele were investigated. While hCG-RARα-PML mice do not develop leukemia, RARα-PML/PML-RARα double transgenics show RA-sensitive APL with a significantly increased penetrance and much earlier than the PML-RARα animals. Thus, while PML-RARα causes the leukemia and determines its phenotype, the reciprocal fusion operates as a 'tumor modifier' that affects disease burden [Pollock et al., 1999]. For PLZF, the situation is different again. RARα-PLZF/PLZF-RARα double transgenics show no accelerated disease onset as for PML, but the disease phenotype shifts from CML in the PLZF-RARα transgenics towards APL, with early myelocytic blasts blocked in their differentiation. Rather than modifying the tumor, RARα-PLZF is involved in determination of the tumor phenotype [He et al., 2000].

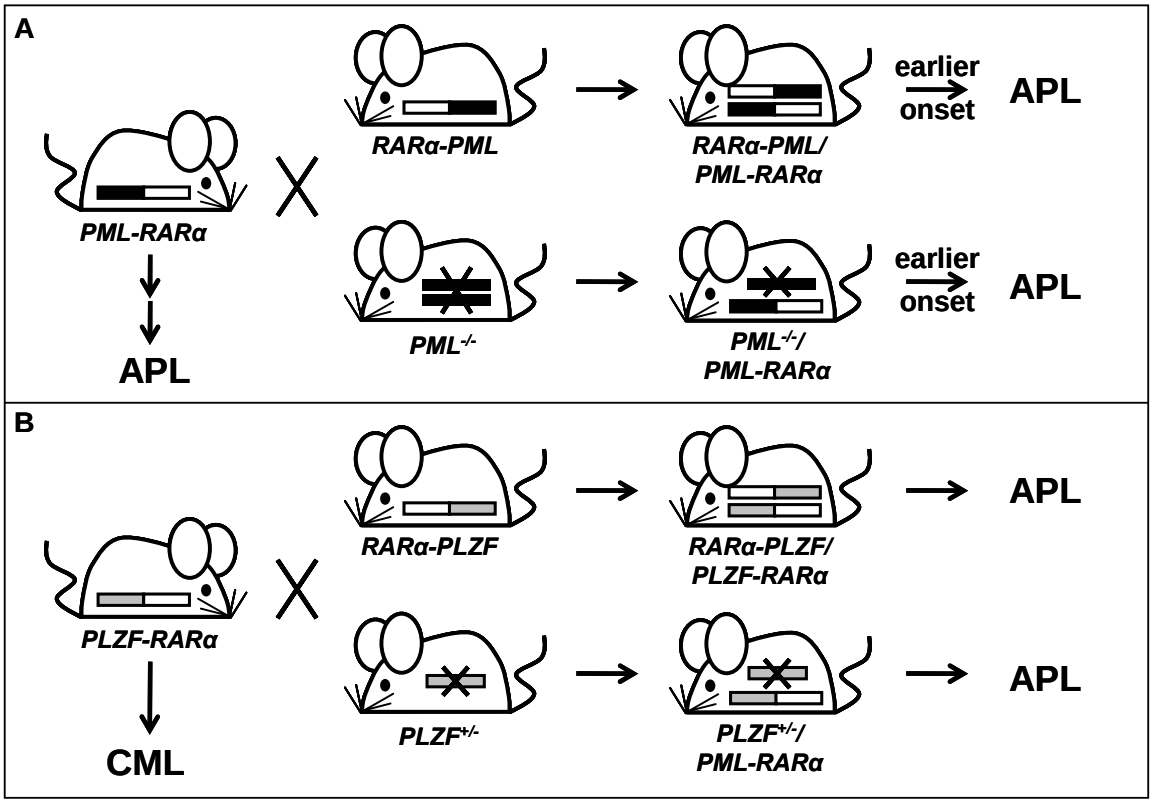

Figure 18 - Impact of reciprocal fusion and X inactivation on X-RARα induced APL. (A) PML- RARα, (B) PLZF- RARα. Adapted from [Merghoub et al., 2001].

Concerning the importance of X-gene reduction, a growth-inhibitory and pro-apoptotic role for both PML and PLZF was observed in knock out mice. As a consequence, disturbance of their normal expression in APL might indeed provide the leukemic cells with a growth and survival advantage, promoting development of the disease. To determine whether inactivation of X would influence leukemogenesis, PML-RARα and PLZF-RARα mice were crossed with $PML^{-/-}$ and $PLZF^{+/-}$ strains, respectively. The effects were similar to those seen in the double transgenics: reduction of PML dramatically increased disease incidence, while decrease of PLZF levels did not affect penetrance, but altered the type of the leukemia from CML towards APL [Rego et al., 2001] [Barna et al., 2000].

Altogether, modeling APL in mice has been essential for the identification of the key players in the generation of APL, which in turn is indispensable for the development of accurate therapeutics.

2 Rationale and aims

It was our general objective to investigate the properties of CIZ and its fusion proteins TAF15/CIZ and EWSR1/CIZ generated by translocations t(12;17)(p13;q11) and t(12;22)(p13;q12) in acute leukemia. The characterization of translocation genes has a double relevance. First, it might yield molecular markers for the diseases that are specifically linked with the translocations. These markers are useful to make an accurate diagnosis, which may in turn allow prediction of remission rates and survival and is critical for selecting the proper treatment. Second, study of the molecular pathology behind the leukemia might result in a better insight into the process of normal hematopoiesis. Moreover, it can provide us with candidate targets for the development of new therapies.

It is well established that TET-proteins are recurrently involved in oncogenic translocations. Fusion proteins generated by these translocations are build up according to a fixed pattern: the TET N-terminal activation domain is fused to a transcription factor or its DNA-binding domain. The identity of this transcription factor determines the type of the subsequent malignancy. Fusion of TAF15 and EWSR1 to CIZ in acute leukemia thus suggests a role for CIZ in hematopoiesis.

It was our specific aim to better understand the oncogenic properties of TET/CIZ-fusions and to learn more about the role of CIZ in normal hematopoiesis. To this aim, three different approaches were developed.
First, we investigated the transforming capacities of TET/CIZ fusions in several *in vitro* and *in vivo* systems. A TET/CIZ transformation model was set up, providing a read-out for further structure/function analysis.
We also searched for interaction partners of CIZ by yeast two-hybrid technology. In contrast to its rat ortholog, human CIZ does not interact with human p130CAS. ZYX, PCBP1 and VIM however, were identified as CIZ interaction partners.
With transcription factor CIZ fused to the TET activation domain, TET/CIZ chimeric proteins have the structure of a transcription factor that might aberrantly regulate gene expression. To investigate this hypothesis, we first confirmed binding of the fusions to the CIZ DNA-binding consensus by electrophoretic mobility shift assays. Then we screened for TET/CIZ transcriptional targets by microarray analysis and identified a number of interesting TET/CIZ-regulated genes.

3 Materials and Methods

3.1 General methods

DNA purification, enzymatic DNA modifications (restriction digests, ligations, radioactive labelings) and other standard procedures were performed according to established protocols [Sambrook et al., 1990].

3.1.1 Polymerase chain reaction (PCR)

PCR was performed using thermostable Taq DNA polymerase (Amersham Biosciences, Uppsala, Sweden) according to standard protocols. For specific purposes, e.g. when proofreading was required, other enzyme systems as Pwo (Roche, Basel, Switzerland) or PfuTurbo (Stratagene, La Jolla, CA) were used. For amplification of products more than 5 kb in length, long-range PCR was performed using the Expand Long Template PCR system (Roche, Basel, Switzerland). Primers are listed in section 3.5.2.

3.1.2 RNA-isolation

Total RNA was isolated from cell lines with Trizol (Invitrogen, Carlsbad, CA) and further purified on RNeasy Mini columns (Qiagen, Hilden, Germany). For RT-PCR, RNA was DNase-treated on column with the RNase-Free DNase Set (Qiagen, Hilden, Germany).

3.1.3 cDNA synthesis and reverse transcriptase PCR (RT-PCR)

RT-PCR experiments were performed on cDNA obtained by randomly primed reverse transcription of 1-2 µg of total RNA using SuperScript II RNase H^- reverse transcriptase and random primers (Invitrogen, Carlsbad, CA).

3.1.4 Quantitative real-time RT-PCR (qRT-PCR)

Gene transcript levels were quantitated with qRT-PCR. The housekeeping genes GAPDH and ACTB were used as normalizers. The amount of PCR product was monitored throughout the PCR procedure using SYBRGreen or fluorescently (JOE, FAM) labeled TaqMan probes. With C_t (threshold cycle) being the PCR cycle at which fluorescence is significantly increased above background level, the fold induction of one gene as compared to an other was calculated based on their differences in C_t-values. Reactions were performed on an ABI 7000 Sequence Detection System

(Applied Biosystems, Foster City, CA) with the qPCR Mastermix Plus for SYBR Green I or the qPCR Core Kit (Eurogentec, Seraing, Belgium). All samples were run in triplicate. Primers are listed in section 3.5.1. All primer pairs were validated to ensure equal amplification efficiencies of target and normalisator amplicons.

3.1.5 Sequencing

Sequencing was performed using the dideoxynucleotide chain termination method [Sanger et al., 1977] with fluorescently labeled dideoxynucleotides, run on an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, CA) and analysed with the VectorNTI suite 8 software package (Informax, Bethesda, Maryland).

3.1.6 In vitro protein synthesis

Expression plasmids were tested by *in vitro* transcription and translation with the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI). Briefly, SP6 or T7 promoter containing plasmids were incubated with a rabbit reticulocyte lysate solution in the presence of ^{35}S-methionine. Protein synthesis was evaluated by SDS-PAGE analysis and autoradiography.

3.1.7 Cell culture and transient transfection

Mouse fibroblast NIH/3T3, rat fibroblast Rat-1 and human embryonic kidney HEK293T cell lines were grown in DMEM-F12 (Gibco, Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FCS) (Hyclone, Logan, UT). The IL3-dependent murine pro-B cell line Ba/F3 [Palacios and Steinmetz, 1985] was cultured in DMEM-F12 supplemented with 10% FCS and 10% conditioned medium of the IL3 producing WEHI-3B cell line. All cells were incubated at 37°C, 5% CO_2. Transient transfections were performed with Fugene 6 (Roche, Basel, Switzerland) or GeneJuice (Novagen, Madison, WI) according to the manufacturers' protocols.

3.1.8 Retrovirus production and transduction

For production of replication-deficient retroviral particles, HEK293T cells were cotransfected with an MSCV plasmid containing the gene of interest and an ecotropic packaging plasmid pIK6.1MCV (provided by Dr. D.G. Gilliland, Boston, MA). Viral titers (10^6-10^7/ml) were determined by transduction of Rat-1 fibroblasts and crystal violet staining of drug-resistant colonies. Cells were transduced with the retroviral supernatant in the presence of 8 µg/ml polybrene (Sigma, St. Louis, MO) at multiplicities of infection varying from 1 to 3, depending on the experiment. Twenty-

four hours after infection, selection was started. Ba/F3 cells were selected in 2 µg/ml puromycin (Clontech BD, Mountain View, CA), 800 µg/ml G418 (Invitrogen, Carlsbad, CA) or 1 mg/ml hygromycin B (Roche, Basel, Switzerland) as appropriate until all mock transduced cells had died. For Rat-1 and NIH/3T3 fibroblasts, 3 µg/ml puromycin was used. To avoid influences of extra mutations or other changes in cell characteristics, culture time between infection and analysis of the transduced cells has to be as short as possible, nevertheless allowing selection of transduced cells first. All experiments with stably transduced cell lines were started within two weeks after infection.

3.1.9 Western blotting and detection

Cells were lysed in NP40 lysis buffer (1% (v/v) NP40; 20 mM Tris.Cl, pH 7.5; 200 mM NaCl) containing protease inhibitors (Complete Cocktail, Roche, Basel, Switzerland). Protein concentrations were determined with the Bradford assay (Biorad, Hercules, CA) and 50-75 µg of protein was separated on SDS-polyacrylamide gels, transferred to PVDF membranes (Amersham Biosciences, Uppsala, Sweden) and detected according to standard protocols. Monoclonal mouse-anti-c-myc 9E10 (1:1000, provided by Dr. J. Creemers, Leuven), monoclonal mouse-anti-flag M2 (1:4000, Sigma, St. Louis, MO) and monoclonal mouse-anti-HA (1:6000, Roche, Basel, Switzerland) were used as primary antibodies. Horseradish-peroxidase labeled sheep-anti-mouse (1:4000, Amersham Biosciences, Uppsala, Sweden) was used as a secondary antibody and detected with Western Lightning Chemoluminiscence Reagent Plus – Enhanced Luminol (Perkin Elmer, Wellesley, MA).

3.1.10 Sequence alignments and database searches

Nucleotide sequence alignments were performed using the BLAST algorithm [Altschul et al., 1990]. Protein sequences were aligned with ClustalW [Chenna et al., 2003]. Information on annotated DNA, RNA and protein sequences and genomes was retrieved predominantly from Entrez, PubMed (Genbank, NCBI) and Ensembl (EMBL - Welcome Trust Sanger Institute) databases.

BLAST:	http://www.ncbi.nlm.nih.gov/BLAST/
ClustalW:	http://www.ebi.ac.uk/clustalw/
Genbank, NCBI:	http://www.ncbi.nlm.nih.gov/
Ensembl:	http://www.ensembl.org/

3.2 Transformation models for CIZ-fusions

3.2.1 Construction of expression plasmids

3.2.1.1 CIZ and CIZ fusions

The open reading frame of human CIZ was amplified by PCR from bone marrow cDNA with primers CIZfl-F1 and CIZfl-R1 [Martini et al., 2002]. DNA fragments encoding the N-terminal parts of TAF15 and EWSR1 were amplified with primer pairs TAF2N-F1/TAF2N-R1 and EWS-F1/EWS-R1b, respectively. The activation domain of VP16 was amplified from the SNATCHII vector with primers VP16-CIZ-F and VP16-CIZ-R [Corveleyn et al., 2005]. All PCR-products were cloned in pGem-T-easy (Promega, Madison, WI) and sequenced. For CIZ, 5'TAF15, 5'EWSR1, TAF15/CIZ, EWSR1/CIZ and VP16/CIZ expression plasmids, the corresponding sequences were first subcloned in pcDNA3.1/Myc-His A (Invitrogen, Carlsbad, CA) using restriction sites provided in the primers. The inserts, myc-his tagged at their 3' end, were then transferred into the retroviral vectors pMSCV-puro and pMSCV-EGFP which contains an IRES-EGFP expression cassette. (Both retroviral vectors were provided by Dr. D.G. Gilliland, Boston, MA.)

3.2.1.2 EWSR1/FLI1

Flag-tagged EWSR1/FLI1 in pSRαMSVtkneo was a gift of Dr. C.T. Denny (Los Angeles, CA). The insert was transferred *Eco*RI/*Hin*dIII to pBluescript-II-SK (Stratagene, La Jolla, CA) and then subcloned by *Xho*I and partial *Bam*HI digestion into *Bgl*II/*Xho*I cut pMSCV-puro.

3.2.1.3 TAF15/CIZ-ΔZN, EWSR1/CIZ-ΔZN

For the generation of a TAF15/CIZ deletion construct lacking the CIZ zinc finger domain, the sequence 5' of the zinc fingers was amplified by PCR on the wild type construct with primers TAF2N-F1 and CIZ-delZN-R. The fragment 3' of the zinc finger domain was amplified with primers CIZ-delZN-R and CIZfl-R1. Both fragments were joined together in pcDNA3.1/Myc-His using the *Eag*I restriction site in the delZN-primers. The insert, myc-his tagged at its 3' end, was then subcloned into pMSCV-puro. A EWSR1/CIZ-ΔZN construct was made following the same procedure.

3.2.2 Focus formation assay

Stably transduced NIH/3T3 cells were seeded at a density of 10^6 cells per 9 cm dish in DMEM-F12 with 10% FCS and incubated at 37°C, 5% CO_2. Medium was refreshed

every 3.5 days. After three weeks, cells were stained with crystal violet (1%) in PBS. Experiments were performed in triplicate with cells of independent transductions.

3.2.3 Growth in soft agar

In 6-well plates (growth area of 9.4 cm^2/well) first a layer of 0.6% agar in DMEM-F12 with 15% FCS was applied. On top of this bottom layer, 10^5 stably transduced NIH/3T3 cells per well were seeded in 0.35% agar in the same medium. After one week, a refresh layer (0.35% agar, same medium) was applied. Colony formation was evaluated until five weeks after seeding. Experiments were performed in duplicate. BCR/ABL-transduced Rat-1 cells were used as a positive control.

3.2.4 Tumour formation in immunodeficient mice

Four million stably transduced NIH/3T3 cells were injected subcutaneously into the right flank of immunodeficient NMRI nu/nu and SCID B17 mice. Six 6-week-old female mice were used per construct. Tumor formation and growth were scored for up to 66 days after injection.

3.2.5 Immunocytochemistry

NIH/3T3, CV1, HEK293T and HeLa cells were grown on cover slips pre-coated with culture medium or poly-L-lysine (Sigma, St. Louis, MO). Twenty-four hours after transfection, cells were fixed in 4% paraformaldehyde, quenched in 50mM NH_4Cl, permeabilised in 0.1-0.4% Triton-X-100, incubated with antibody and mounted in Mowiol 4-88 (Calbiochem, Madison, WI) or Vectashield (Vector Laboratories, Burlingame, CA) containing 0.5 µg/ml DAPI (Roche, Basel, Switzerland). PBS was used for washing steps in between. Primary antibodies were monoclonal mouse-anti-c-myc 9E10 and monoclonal anti-human-vinculin hVIN-1 (Sigma, St. Louis, MO). Goat-anti-mouse AlexaFluor 488 (1/1000, Molecular Probes, Carlsbad, CA) was used as a secondary antibody. F-actin was stained with Texas Red-X phalloidin (Molecular Probes, Carlsbad, CA). CIZ, TAF15/CIZ and EWSR1/CIZ N- and C-terminal GFP fusions were created in pEGFP-C1 and pEGFP-N2 (Clontech BD, Mountain View, CA). Slides were evaluated with an epi-fluorescence microscope (Leica, Wetzlar, Germany).

3.2.6 Bone marrow transplantation and analysis

Female, 6-week-old BALB/c mice were primed with 5-fluorouracil (5'FU, 0.15 mg/g body weight, Sigma, St. Louis, MO) by intraperitoneal injection. 5'FU kills cycling

cells by interfering with pyrimidine synthesis and as a consequence enriches the bone marrow for hematopoietic stem cells. Six days later, mice were sacrificed by cervical dislocation. Femurs and tibiae were flushed with RPMI (Gibco, Invitrogen, Carlsbad, CA) containing 10% FCS to isolate bone marrow. After lysis of red blood cells (in 150 mM NH_4Cl; 0.1 mM EDTA; 10 mM KHCO3, pH 7.4), cells were incubated overnight in transplant medium (RPMI with 10% FCS, 10 ng/ml recombinant murine IL6 and SCF (Peprotech, Rocky Hill, NJ), 6 ng/ml recombinant murine IL3 (Peprotech), penicillin (100 IU, Roche, Basel, Switzerland) and streptomycin (100 µg/ml, Roche, Basel, Switzerland)) at a density of 1-2 x 10^6 cells/ml. For transduction with constructs in pMSCV-EGFP, retroviral supernatant (1 ml per 2 ml of cell suspension), polybrene (8 µg/ml, Sigma, St. Louis, MO) and Hepes buffer (10 mM, Gibco, Carlsbad, CA) were added and cells were centrifuged (30 °C, 90 min, 2500 rpm), incubated for 2 hours at 37 °C, 5% CO_2, washed twice in PBS and resuspended in transplant medium in untreated culture dishes (BD Biosciences, Mountain View, CA). Twenty-four hours later the transduction step was repeated, cells were collected in PBS at a density of 1-2 x 10^6 cells/ml and injected into the tail vein of sub-lethally irradiated (2 x 450 rad) syngeneic recipient mice (500 µl/mouse). Mice were kept in micro-isolator cages with filtered water and autoclaved chow and the animals were sacrificed when they had palpable splenomegaly or were moribund, or, if healthy, at the experimental endpoint.

A. Tumor cell transplantation

Single cell suspensions from spleens of diseased mice were prepared as described below. Cells were counted and diluted to 2 x 10^5 cells/ml. Recipient BALB/c mice were subjected to a single dose of 450 rad and 0.5 ml of the tumor cell suspension was administered by tail vein injection. Animals were sacrificed when they had palpable splenomegaly or were moribund.

B. Assessment and analysis of mice

Animals were monitored several times per week for the development of disease by general inspection and palpation of the spleen and lymph nodes. Mice with clinically evident disease were sacrificed and analyzed. Peripheral blood was collected from the retroorbital cavity using heparinized glass capillary tubes and analyzed by manual and automated complete and differential blood cell counts (ADVIA 120 Hematology system, Bayer, Leverkusen, Germany) and smears (stained with Wright and Giemsa). Mice were anesthetized using methoxyfluorane (Medical Developments, Melbourne, Australia) and then killed by cervical dislocation. Spleen weights and any other pathologic findings at necropsy were recorded. Single cell suspensions of

spleen were prepared by passing tissue through nylon mesh (Falcon, BD, Mountain View, CA) dampened with PBS and were stored in 10% dimethylsulfoxide / 90% FCS, following lysis of red blood cells.

C. Histopathological analysis of murine tissues

Mouse organs were fixed for at least 72 hours in 10% neutral buffered formalin (Sigma, St. Louis, MO), dehydrated in alcohol, cleared in xylene and infiltrated with paraffin on an automated processor (Leica, Wetzlar, CA). Tissue sections of 4 µm were placed on charged slides, deparaffinized in xylene, rehydrated through graded alcohol solutions and stained with hematoxylin and eosin (H&E). Histologic images were obtained on a Nikon Eclipse E400 microscope equipped with a SPOT RT color digital camera model 2.1.1 (Diagnostic Instruments, Sterling Heights, MI).

D. Flow cytometric analysis

Murine spleen and bone marrow single-cell suspensions were prepared by red blood cell lysis (in 150 mM NH4Cl; 0.1 mM EDTA; 10 mM KHCO3, pH 7.4) and washing in PBS containing 0.1% NaN_3 and 0.1% BSA. Cells were preincubated for 20 minutes on ice with supernatant from the 2.4G2 hybridoma cell line to block nonspecific Fc receptor-mediated binding. Aliquots of 0.5×10^6 cells were stained on ice for 20 minutes with monoclonal antibodies specific for B220 (CD45R), CD19, IgM, CD3, CD43, CD41, Gr-1, Mac-1, c-kit, CD31, Thy-1, CD4 or CD8 conjugated with fluorescein isothiocyanate (FITC), phycoerthyrin (PE), allophycocyanin or biotin. Binding of biotinylated primary antibodies was detected using PE-conjugated streptavidin (Immunotech, Marseille, France). Cells were washed once in staining buffer followed by four color flow cytometric analysis with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Cells were gated for viability and GFP positivity, and a minimum of 10,000 events were analyzed.

3.2.7 Murine colony-forming assay

Murine bone marrow cells were harvested and transduced as described for the bone marrow transplantation experiment (without second transduction). Twenty-four hours after transduction, 10^4-10^5 cells were seeded in 1 ml of semisolid medium (Methocult GF M3434, StemCell Technologies, Vancouver, Canada) containing IL3 (10 ng/ml), IL6 (10 ng/ml), SCF (50 ng/ml) and supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and puromycin (2 µg/ml, Clontech BD, Mountain View, CA) in 3.5 cm culture dishes (Nunc), three plates per construct. Cells were serially replated every 10 days, four times in total.

3.3 Interaction partners of human CIZ

3.3.1 Yeast two-hybrid screenings

Yeast two-hybrid (Y2H) screens were performed with the MatchMaker Library Construction and Screening kit (Clontech BD, Mountain View, CA).

3.3.1.1 Bait constructs

For the N-terminal bait, a fragment of human *CIZ* encoding amino acids 1-229 was amplified by PCR using primers CIZ-Y2H-F1 and CIZ-Y2H-R2, digested with *Nco*I/*Sal*I (Roche, Basel, Switzerland) and cloned into the pGBKT7 bait vector. The C-terminal bait (CIZ amino acids 456-578) was generated similarly with primers CIZ-Y2H-F2/R1. Transcriptional activation and toxicity of the clones were tested in *S. cerevisiae* strain AH109.

3.3.1.2 Prey library and screening

First strand cDNA-synthesis was performed on 0.5 µg human bone marrow poly-A^+ RNA (Clontech BD, Mountain View, CA) with MMLV-RT provided in the Matchmaker kit. cDNA was then amplified with the Advantage 2 PCR kit (Clontech BD, Mountain View, CA), size fractionated on ChromaSpin-400 columns (Clontech BD) and inserted into the pGADT7-Rec prey-vector by homologous recombination in yeast. To this end cDNA, *Sma*I-linearised pGADT7-Rec vector and a pGBKT7-bait construct were cotransfected into *S. cerevisiae* reporter strain AH109 using the lithium acetate method [Gietz et al., 1992]. Yeast cells were plated on synthetic dropout medium without leucine and tryptophan (SD/-L/-T) to select for prey and bait plasmids and on medium lacking leucine, tryptophan, adenine and histidine (SD/-L/-T/-A/-H/3AT) to select for interacting proteins. Cells were replated twice on selective medium. Expression of *MEL1* was monitored using indicator plates with 20 mg/l X-α-Gal (Clontech BD) and *lacZ* expression was analysed with a colony-lift filter assay.

3.3.1.3 Analysis of interacting clones

For plasmid isolation, yeast colonies were grown in liquid cultures and lysed with lyticase, SDS and one freeze/thaw-cycle. Then 250 µl of resuspension buffer (50 mM Tris.Cl, pH 8.0; 10 mM EDTA; 100 µg/ml RNase A), 250 µl of lysis buffer (200 mM NaOH; 1% SDS) and 250 µl of neutralisation buffer (3.0 M potassium acetate, pH 5.5) were added to the lysate. Samples were centrifuged and DNA was precipitated with an equal volume of isopropanol, washed en redissolved in water. The DNA was reprecipitated in 0.4 M NaCl; 6.5% PEG8000 and resuspended in

water. Plasmids were then electroporated into *E. coli* JM109 for PCR and sequence analysis with primers pGAD424-for and pGAD424-rev. Interactions were confirmed in yeast with a small scale yeast transformation as described in the Matchmaker manual.

3.3.2 Interaction of CIZ and p130CAS

A fusion of the GAL4 DNA-binding domain (DBD) to the N-terminal 230 amino acids of *Rattus norvegicus* Ciz was created as described for the human homolog with PCR on pSSRαbsr-CIZ8 [Nakamoto et al., 2000] using primers rCIZ-Y2H-F3/R4. The construct was tested for transcriptional activation and toxicity. For fusion of the GAL4 activation domain (AD) to the SH3 domain of p130CAS (amino acids 1-70), RT-PCR with primers CAS-SH3-F/R was performed on poly-A^+ RNA of human placenta (Clontech BD, Mountain View, CA). The fragment was cloned into the *Sma*I-linearised pGADT7-Rec prey vector. The SH3-domain of *R. norvegicus* p130Cas (amino acids 95-165) was cloned similarly using primers rCAS-SH3-F2/R2 on rat liver cDNA (provided by Dr H. De Smedt). Interaction of the proteins was tested in a small scale yeast transformation as described (Matchmaker, Clontech BD, Mountain View, CA).

3.3.3 CIZ deletion constructs

CIZ-ΔN1, missing amino acids 3-72, was generated by PCR with primers CIZ-del1-F and CIZfl-R1, digested with *Bgl*II/*Apa*I and cloned into pMSCV-puro-myc/his. CIZ-ΔN3, missing amino acids 3-229 was generated similarly using primers CIZ-del3-F and CIZfl-R1. For CIZ-ΔN2, which lacks amino acids 158-230, two fragments were generated by PCR with primer pairs CIZfl-F1/CIZ-del2-R and CIZ-del2-F/CIZfl-R1. Both fragments were cloned in pMSCV-puro-myc/his via a *Bgl*II/*Bss*HII/*Apa*I ligation. CIZ-ΔLZ, CIZ-ΔSRR, CIZ-ΔPRR, CIZ-ΔNLS and CIZ-ΔQA, missing the leucine rich region, the serine rich region, the proline rich region, the potential nuclear localisation signal or the glutamine/alanine stretch of CIZ, respectively, were cloned in pMSCV-puro with C-terminal myc/his-tag following the same strategy as described for TAF15/CIZ-ΔZN. All constructs contain a C-terminal myc-tag.

3.3.4 Cloning of full-length ZYX, PCBP1, VIM and CAS

Full-length human vimentin was amplified by PCR on IMAGE clone 2985712 with primers VIM-F1/VIM-R1, digested with *Bam*HI/*Xba*I and cloned into pcDNA3.1-FlagB. For the cloning of PCBP1, the PCR-product of primers PCBP1-F1/PCBP1-R1 on IMAGE clone 6061411 was digested with *Eco*RI/*Xho*I and ligated into the same vector. Full-length human zyxin in pcDNA3.1-FlagB was a gift of Dr M. Petit (Centre for

Human Genetics, Leuven). All three proteins have an N-terminal flag tag. Full-length human *CAS* cDNA was created by PCR with primers BCAR1-F2/R2 on pLXSN-BCAR1 (kindly provided by Dr A. Brinkman [Brinkman et al., 2000]), digested *Bam*HI/*Eco*RI and cloned into pcDNA3.1-HA-B. The protein encoded contains an N-terminal HA-tag.

3.3.5 Co-immunoprecipitation experiments

For co-immunoprecipitation (co-IP) experiments, transient transfections were performed in triplicate in 6-well plates. Cells of three identical wells were harvested together 24 hours after transfection by scraping in ice-cold PBS. One eighth of the cells were lysed in NP40 lysis buffer (1% (v/v) NP40; 20 mM Tris.Cl, pH 7.5; 200 mM NaCl) with protease inhibitors (Complete, Roche, Basel, Switzerland) and analysed by Western to verify construct expression. The remainder of the cells was lysed in non-denaturing lysis buffer (NDLB) (1% (w/v) Triton X-100; 50 mM Tris.Cl, pH 7.4; 300 mM NaCl; 5 mM EDTA; protease inhibitors). This lysate was precleared by incubation with 30 µl NDLB-washed protein-G sepharose beads (Amersham Biosciences, Uppsala, Sweden) for two hours at 4°C. Per sample, 30 µl beads were incubated with mouse-anti-myc or mouse-anti-flag (Sigma, St. Louis, MO) for one hour at 4 °C. The precleared lysate was then combined with the antibody-bound beads for another hour. Beads were washed four times with wash buffer (0.1% (w/v) Triton X-100; 50 mM Tris.Cl, pH 7.4; 300 mM NaCl; 5 mM EDTA; protease inhibitors), resuspended in loading dye and boiled. Samples were run on 4-12% gradient SDS-polyacrylamide gels (Nupage, Invitrogen, Carlsbad, CA). For the CIZ experiments, the sample was divided in two equal parts ($1.40x10^6$ cell equivalents each) for Western analysis with anti-myc and anti-flag. In the experiments with the TET/CIZ fusions, one eighth of the sample ($0.35x10^6$ cell equivalents) was used for Western analysis with anti-flag, the remainder ($2.45x10^6$ cell equivalents) was analyzed with anti-myc. Trueblot antibodies (eBioscience, San Diego, CA), which preferentially recognise non-denatured immunoglobulins, were used as secondary antibodies for the anti-flag IP blots in the experiments with the TET/CIZ fusions. Band intensity of precipitated p130CAS was determined on a Kodak Image Station 440 CF using Kodak 1D 3.5.5B software and normalized for both p130CAS expression levels on the Western blot and the immunoglobulin light chain present on the IP-blot.

3.4 CIZ-fusions as aberrant transcription factors

3.4.1 Electrophoretic mobility shift assay

HEK293T cells were grown in 9 cm culture dishes, transiently transfected with the constructs to be analysed and harvested 24 hours later by scraping in ice-cold PBS. One third of the cells were used to verify expression of transfected constructs by Western analysis. The remainder of the cells was lysed in buffer C (20 mM Hepes.KOH pH 7.9; 420 mM KCl; 1.5 mM $MgCl_2$; 25% (v/v) glycerol) with three freeze-thaw cycles and 10-60 µg of protein was incubated in a 20 µl reaction with $2x10^4$ cpm radioactively labeled, double stranded DNA probe and 1 µg poly(dI-dC) (Amersham Biosciences, Uppsala, Sweden) in 10 mM Hepes; 2.5 mM $MgCl_2$; 0.05 mM EDTA; 8.5% glycerol; 0.05% Triton-X-100 and 1 mM DTT. Samples were separated on a native 4% polyacrylamide gel and detected by autoradiography after drying of the gel. For generation of the double stranded DNA probe, oligonucleotides were annealed in buffer L (Roche, Basel, Switzerland) by slow cooling from 95 °C to room temperature, 5' overhangs were filled in using Klenow DNA polymerase I (USB, Staufen, Germany) and ^{32}P-labeled dCTP and reactions were purified on NAP-5 columns (Amersham Biosciences, Uppsala, Sweden). Oligonucleotides 5'-CGCGTCAA-CCTTTTTCAAAAAGACCAG and 5'-CTAGCTGGTCTTTTTGAAAAAGGTTGA corresponding to the region from -320 to -305 of the human MMP1 promoter were used for the wild type probe. Competition experiments were performed by adding a 1000-fold molar excess of mutant probe oligonucleotides 5'-CCTGTGTCAGAGAGA and 5'-TCTCTCTGACACAGG). Monoclonal mouse-anti-myc was used to supershift the DNA-protein complex.

3.4.2 TRAP assay

The telomerase repeat amplification protocol or 'TRAP' assay to detect telomerase activity was performed with the *T*elo*TAGGG* Telomerase PCR ELISA^{plus} kit (Roche, Basel, Switzerland). In brief: if a cell lysate is telomerase positive, telomeric repeats are added to a biotin-labeled primer. Elongation products are then amplified by PCR and hybridised to a digoxigenin labeled detection probe. Products are immobilised via the biotin label and detected via ELISA with a horseradish peroxidase conjugated anti-digoxigenin antibody. An internal standard is co-amplified to exclude false-negatives because of PCR inhibitors possibly present in the cell lysate. Heat inactivated samples are included as a specificity control. Experiments were performed with stably transduced NIH/3T3 cells.

3.4.3 Microarray analysis

3.4.3.1 Cell system and RNA isolation

Ba/F3 cell lines with inducible EWSR1/CIZ expression were established using the TetOff Gene Expression System (Clontech BD, Mountain View, CA). First, the pTet-Off regulatory plasmid was transduced into the cells and by limiting dilution two monoclonal Ba/F3-TetOff lines (Ba/F3-3C3, Ba/F3-3D2) were generated. EWSR1/CIZ was digested *Bgl*II/*Pme*I from pcDNA3.1/Myc-His, cloned into *Bam*HI/*Hpa*I cut pRevTRE and the construct was transduced into both Ba/F3-TetOff lines. The Ba/F3-TetOff-EWSR1/CIZ cells were grown in the presence of tetracycline (2 µg/ml, Sigma, St. Louis, MO). To induce EWSR1/CIZ expression, tetracycline (Tc) was removed by washing three times in PBS. Cells were resuspended in Tc-free medium and washed once more after three hours. Tet-System Approved fetal bovine serum (Clontech BD, Mountain View, CA) was used in the Tc-free medium. Fifteen to 24 hours after expression induction, cells were harvested. Equal amounts of control cells without EWSR1/CIZ induction were collected just before Tc removal. Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA) and further purified on RNeasy Mini columns (Qiagen, Hilden, Germany).

3.4.3.2 Microarray platform, hybridisation and data analysis

The GeneChip® Mouse Genome 430A 2.0 Arrays (Affymetrix, Santa Clara, CA) was used. It is a single array representing approximately 14,000 well-characterized mouse genes. Hybridisation was performed at the VIB Microarray Facility, Leuven. Total RNA was controlled for its integrity and purity using Agilent Bioanalyzer and the Nanodrop spectrophotometer, respectively. Probes were prepared from 5 µg total RNA, showing no signs of degradation or impurities (260/280 and 260/230 >1.8), according to Affymetrix's guidelines. Briefly, from total RNA, poly-A^+ RNA was reverse transcribed using a poly dT-T7 primer and labeled during a T7 *in vitro* transcription reaction using the IVT Labeling Kit (Affymetrix, Santa Clara, CA). The probes were purified (GeneChip Sample Cleanup Module, Affymetrix) and analysed again for yield (30-120 µg) and purity (260/280 and 260/230 >1.8). Twenty µg of the resulting amplified RNA (aRNA) was fragmented with alkaline hydrolysis and resuspended with control spikes in 300 µl hybridisation buffer (Eukaryotic Hybridisation Control Kit, Affymetrix). The Genechips were hybridised in a rotisserie oven at 45 °C and washed and stained in the GeneChip Fluidics Station 400 (Affymetrix) using the EukGE-WS2v4 protocol. Scanning was done with the GeneChip Scanner 3000 (Affymetrix) and image analysis was performed in Affymetrix

GeneChip® Operating Software (GCOS). GCOS automates the control of GeneChip Fluidics Stations and Scanners, it acquires data, manages sample and experimental information and performs gene expression data analysis. For every gene, the GeneChip® Mouse Genome 430A 2.0 array provides a probe set of 10 to 16 probe pairs, each consisting of one probe cell containing a 25 bp oligonucleotide matching perfectly to the gene, and another probe cell containing the same oligonucleotide except for one mismatch at position 13. In the GCOS-analysis, differences between the perfect match and the mismatch of each probe pair are calculated and compared between two arrays. Next, two algorithms are used to generate change significance and change quantity metrics for every probe set. The first algorithm calculates a change p-value, which indicates an increase, a decrease, or no change in gene expression. The second algorithm generates a quantitative estimate of the change in the form of a 'signal log ratio', which is translated into a 'fold change' value.
Onto-Express was used for functional data implementation (http://vortex.cs.wayne.edu/Projects.html). Toucan2 was used for analysis of CIZ binding sites in gene promoters [Aerts et al., 2005].

3.4.4 Luciferase reporter assay

For the generation of the luciferase reporter construct, 4.5 kb of the human SPIC proximal promoter (position -4493 to +1) was amplified by PCR on BAC clone RP11-482I4 using primers SPIC-F15/R8. The PCR-product was digested *Kpn*I/*Xho*I and cloned into the pGL3-promoter vector (Promega, Madison, WI).
HEK293T cells were seeded in 6-well plates (250000 cells/well, 1 well/sample) and transiently cotransfected 24 hours later with 250 ng of the SPIC luciferase reporter construct, one of the expression vectors (CIZ: 750 ng; EWSR1/CIZ-ΔZN: 100 ng; TAF15/CIZ, EWSR1/CIZ, VP16/CIZ or empty pMSCV vector: 1 µg) and 40 ng of a β-galactosidase plasmid. The total amount of DNA was equal in all samples by the use of extra empty vector DNA when necessary. Cells were harvested 24 hours later by scraping in PBS. Half of the cells was lysed in NP40 lysis buffer and used for Western analysis. The remainder of the cells was lysed in 1x Passive Lysis Buffer (Promega, Madison, WI). One fourth of the lysate was used to measure luciferase activity using the Luciferase Assay System (Promega) on a FluoStar Galaxy luminometer (BMG LabTechnologies, Offenburg, Germany). Beta-galactosidase activity was measured with the Galacton-Plus Substrate System (Applied Biosystems, Foster City, CA) and used for normalization of luciferase values. Experiments were performed in triplicate. Differences between samples were analysed with a one-tailed Student's t-test. The cut-off p-value was set at 0.01.

3.5 List of oligonucleotides

3.5.1 Oligonucleotides for qRT-PCR

Primers and probes were developed with PrimerExpress 2.0.0 (Applied Biosystems, Foster City, CA).

Atp6V0a1-F1	5'-CCCAGCAGTTTTTCGATGAG
Atp6V0a1-R1	5'-GCTCCAAGAGTGATGAGGACTCT
B3galt2-F1	5'-CCAAAAGTATTTTCAGTAGACATAATCC
B3galt2-R1	5'-ATGTCCCTCGATGTCATTCTTTC
Calml4-F1	5'-AGCGGCACCTGCAGACT
Calml4-R1	5'-TGGTCAGGAAGGTGGAGAAG
Emp1-F1	5'-CCACTGCCATTATGCTGTTTGT
Emp1-R1	5'-CATTTGCGTAATCTGCAACCA
gapdh-TMf	5'-ACTGGCATGGCCTTCCG
gapdh-TMr	5'-CAGGCGGCACGTCAGATC
Igfbp4-F1	5'-GCACGGAGCTGTCGGAAA
Igfbp4-R1	5'-TGTTGGGATGTTCGCTCTCA
Il6-F1	5'-GGAAATCGTGGAAATGAGAAAA
Il6-R1	5'-AGTGCATCATCGTTGTTCATACA
Itgb3-F1	5'-TGCCGGGATGACATCGA
Itgb3-R1	5'-GGTACAATTCACGGCGTTTTT
Mmp8-F1	5'-AGAAACGTGGACTCAAGATTCC
Mmp8-R1	5'-GAGTCCCAAAGAATGTCCAAATT
mTERT-F3	5'-CCTCCAGACAGTCTGCATCAATA
mTERT-R3	5'-TCCTAACACGCTGGTCAAAGG
MuACT_TMf	5'-ACCCACACTGTGCCCATCTAC
MuACT_TMp	5'-AGGGCTATGCTCTCCCTCACGCCA
MuACT_TMr	5'-AGCCAAGTCCAGACGCAGG
MuGAPDH-TMp	5'-ACGACGGACACATTGGGGGTAGGA
PU1-F1	5'-CGTGCAAAATGGAAGGGTTT
PU1-R1	5'-TCTGAATCGTAAGTAACCAAGTCATC
Socs3-F1	5'-GCGGGCACCTTTCTTATCC
Socs3-R1	5'-TGACGCTCAACGTGAAGAAG
SpiB-F1	5'-CGACCTGGACAGCTGCAA
SpiB-R1	5'-CCAGCCCCATGTAGAGTCAA
SpiC-F1_TM	5'-CTGGTACAACCCCCCTTCT
SpiC-P1_TM	5'-AAAGGGAGGAAGAGGCAGGAGA
SpiC-R_TM	5'-GGTATTCAAACAGCCGAAGCT
Stam2-F1	5'-CAACGAGCTCACCTTTAAACATG
Stam2-R1	5'-TTTTCTCCTTGCCACCAGTT
Tm4sf3-F1	5'-CCAGAAGGCCATGATTGTGT
Tm4sf3-R1	5'-AGTCGGCAGCTCCATTCTC
Tnfrsf9-F1	5'-GACAGCCGAACTGTAACATCTG
Tnfrsf9-R1	5'-GGGTAGAGGAGCAAAACTTCTTG

3.5.2 Other oligonucleotides

Primer3 software at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi (Whitehead Institute for Biomedical Research) was used for primer design.

BCAR1-F2	5’-ACAGGATCCTATGAACCACCTGAACGTGCT
BCAR1-R2	5’-AAAGAATTCTCAGGCGGCTGCCAGCTGGC
CAS-SH3-F	5'-GACCATGGATATGAACCACCTGAACGTGCTG
CAS-SH3-R	5'-ACGTCGACGCTTCTTATCATACATGCCCAC
CIZab1F	5'-CCCATTTCGGCTCCCATGATT
CIZab1R	5'-GGTCTCGGTGTGTGACTTGGA
CIZ-del1-F	5'-TTAAGATCTATGGAATCAGACCAGCTGACCCCA
CIZ-del2-F	5'-TTTGCGCGCATGTGCTCACTGACATTC
CIZ-del2-R	5'-AAAGCGCGCTTGTGAGCCAGGGGGA
CIZ-del3-F	5'-TTAAGATCTATGGAATGCCGGATGTGCTCACTG
CIZ-delZN-F	5'-ACCTACCGGCCGCCTGATCTTCAGCAACAG
CIZ-delZN-R	5'-AGGCGGCCGGTAGGTCTTGCCGTCTTTCTG
CIZex11b	5'-CTGCCCCAGGAGACTGGA
ciz-Ex4R2	5'-TCTGGCAACAGCTGATCCTTC
CIZex6b	5'-CCCGAGATGAATGACCCTTA
CIZfl-F1	5'-GAGATCTAATGCCGGCAGAATGGAAGAATCTCACTTC
CIZfl-R1	5'-AGGGCCCAGAGCTGGCCAGGTGCTCCAC
CIZ-Y2H-F1	5'-GACCATGGCGAGAATGGAAGAATCTCACTTC
CIZ-Y2H-F2	5'-TACCATGGCGCCTGATCTTCAGCAACAGGTG
CIZ-Y2H-R1	5'-TAGTCGACATAGAGCTGGCCAGGTGCTCCAC
CIZ-Y2H-R2	5'-AGTAGTCGACATCTGTAGGTCTTGCCGTCT
EWS-F1	5'-GAGATCTGAGAAAATGGCGTCCACGGATTAC
EWSR1b	5'-TGCCGGCCTGCCCGTAGCTGCTGCTCTGT
EWSR1bf	5'-CCCAAACTGGATCCTACAGC
GAPD247f	5'-AATCCCATCACCATCTTCCA
GAPD596r	5'-ACAGTCTTCTGGGTGGCAGT
mEAT2f	5'-CTGACCAAGCGAGAGTGTGA
mEAT2r	5'-TCCCTCTTTGGCATAGGTTG
MFNGf	5'-CCCTCAGCTACGGTGTCT
MFNGr_m	5'-TCGTAGCCTTTCCTGTCA
mMMP3-F1	5'-GAGGAGCTAGCAGGTTATCCTAAA
mMMP3-R1	5'-AGCTACACAGTGCTTCTGAACATC
mMMP7-F1	5'-AAGGAGAGATCATGGAGACAGCTT
mMMP7-R1	5'-GATGTCTCGCAACTTCATGTTACC
PCBP1-F1	5'-AAGAATTCCGATGCCGGTGTGACTGAAAGT
PCBP1-R1	5'-AACTCGAGACTAGCTGCACCCCATGCCCTT
PCBP1-R3	5'-TGATCTTACACCCGCCTTTC
pGAD424-for	5'-TTGGACGGACCAAACTGCGTATA
pGAD424-rev	5'-TGAAGTGAACTTGCGGGGTTTT
rCAS-SH3-F2	5'-TACCCGGGAGCCGCGACAAGAACGTGCTG
rCAS-SH3-R2	5'-TACCCGGGCTTCTTGTCATACATGCCAAC

rCIZ-Y2H-F3	5'-GACCATGGCGAGAATGGAAGAGTCTCACTTC
rCIZ-Y2H-R4	5'-AGTCGACATCTGTAGGTCTTGCCATCT
SPIC-F15	5’-ATGGTACCGCGTGTAGGGCCAGTTTAGT
SpiC-R2	5'-GGAGAACAGCCTCGCTGAA
SPIC-R8	5’-GCGCTCGAGTTCCTTAGCAATTGTTGCTTGA
TAF2Ncf	5'-CAGGATCAAGTGGTGGTGAC
TAF2N-F1	5'-GAGATCTGCCACCATGTCGGATTCTGGAAGT
TAF2N-R1	5'-AGCCGGCATCTGTTCTGGGTCCATAATC
VIM-F1	5'-TTGGATCCATCCACCAGGTCCGTGTCCTCG
VIM-F4	5'-CAATGTTAAGATGGCCCTTGA
VIM-R1	5'-CCTCTAGACTTATTCAAGGTCATCGTGATG
VP16-CIZ-F	5'-CGAGATCTGCCACCATGGCCCCCCCGACCGATGTC
VP16-CIZ-R	5'-CATTCTGCCGGCCCCACCGTACTCGTCAAT
ZYX-F1	5'-CTCAGGTCCAACTCCATGTC
ZYX-R1	5'-ACATTCTGCCTCTGAGGATGC

4 Results

This chapter is divided into three parts:

4.1 Transforming properties of TET/CIZ-fusions

This first part of the results was described in [Martini et al., 2002]. Our contribution to this work was the investigation of TET/CIZ transforming capacities and the set-up of a TET/CIZ transformation model that was subsequently used in further structure/function analysis [Corveleyn et al., 2005]. Here we also developed a bone marrow transplant model.

4.2 Interaction partners of human CIZ,
based on [Janssen and Marynen, 2006].

4.3 TET/CIZ-fusions as aberrant transcription factors

This part describes the investigation of TET/CIZ DNA-binding characteristics by EMSA analysis (our contribution to [Martini et al., 2002]) and the transcription profiling of EWSR1/CIZ-expressing Ba/F3 cells.

4.1 Transforming properties of TET/CIZ-fusions

4.1.1 Introduction

Oncogenic mutations endow cancer cells with altered properties such as unlimited replicative potential, the ability to invade other tissues, self-sufficiency in growth signals and others. Many cell and animal models have been developed to determine to what extent specific mutations contribute to cell transformation. The oncogenic properties of TET/CIZ-fusions were investigated by several *in vitro* and *in vivo* transformation assays.

4.1.2 *In vitro* transformation models

A. Loss of contact inhibition

Contact-inhibition is defined as cell-cell contact mediated inhibition of growth and plays a fundamental role in regulating cell growth *in vitro* and *in vivo* [Dietrich, 1998]. In cell culture, normal fibroblasts grow until they contact neighboring cells in a confluent monolayer, and then exit the cell cycle. In contrast, tumor cells are characterized by loss of contact-inhibition. They grow criss-cross over one another and pile up in multilayered 'foci'.

In a focus formation assay, NIH3T3-fibroblasts stably expressing TET/CIZ-fusions displayed a clearly transformed phenotype. In contrast to control cells expressing CIZ or the N-terminal activation domain of TAF15 or EWSR1 alone, cells expressing TAF15/CIZ or EWSR1/CIZ fusions grew beyond confluency to a higher cell density and formed multiple foci (figure 19A,B). Moreover, expression of the chimeric proteins also altered cell morphology. Islands of small round cells emerged already five days after plating (figure 19C), resulting in the presence of two distinct cell populations in the final plates: small, round cells among more spindle-like cells with the 'herring-bone' appearance that is characteristic of fibrosarcoma (figure 19A). Finally, TET/CIZ fusions also influenced cell growth before confluency was reached: while control NIH/3T3 cells grow 'around holes' in a honeycomb-like pattern, cells expressing the fusions immediately spread out and grew scattered all over the culture dish (figure 19D).

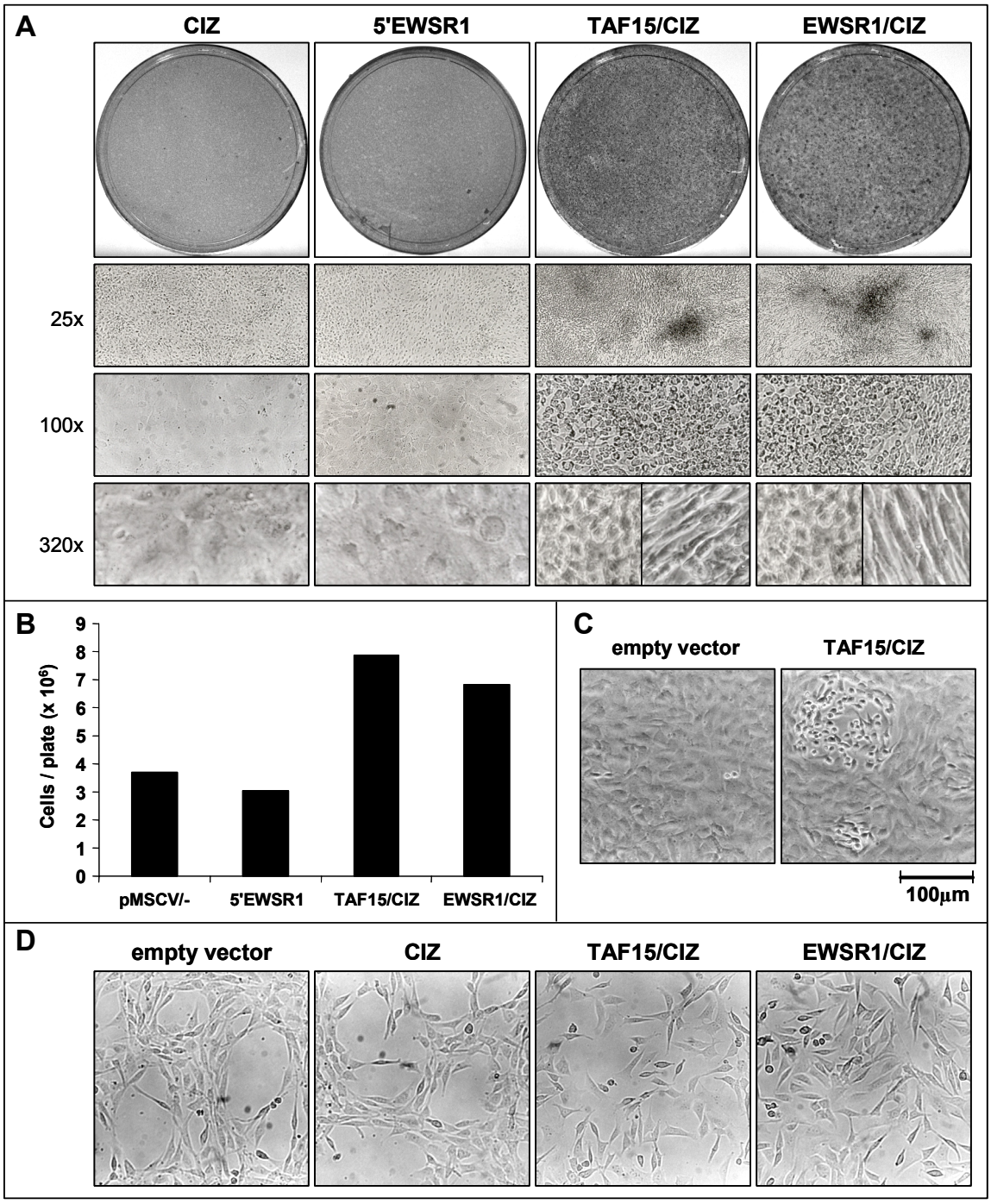

Figure 19 – Focus formation assay. (A) Culture dishes were stained after 27 days. Microscopic pictures were taken at three different magnifications as indicated. For the TET/CIZ fusions, two different areas of the same dish are shown at highest magnification to illustrate both the small round cell (left) and the spindle shape (right) morphology. (B) Dishes identical to the ones in A were trypsinized and cells were counted. (C) Islands of small round cells appear at day five after plating. (D) TET/CIZ-expressing cells grow scattered over the dish (pictures at day 3).

B. TET/CIZ fusions do not confer anchorage independent growth

Most mammalian cell types are anchorage-dependent for growth: they require a surface on which to flatten out and divide. Transformed cells might loose this requirement and become able to grow in a semi-solid medium. NIH/3T3 cells stably expressing TET/CIZ-fusions did not grow substrate-independently in soft agar (figure 20).

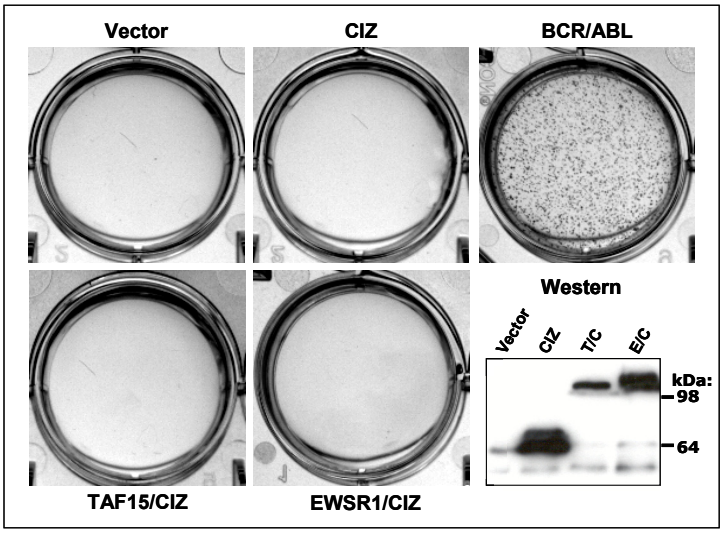

Figure 20 – Colony formation in soft agar. Stably transduced NIH/3T3 cells (10^5/well) were seeded in soft agar. Pictures were taken after five weeks.

C. Hematopoietic immortalization assays

In the adult mouse bone marrow, hematopoietic progenitors proliferate and differentiate to generate mature blood cells. When cultured in an appropriate viscous medium, the progeny of an individual precursor becomes visible as a discrete cell colony. However, cells of a normal mouse bone marrow population no longer form colonies after two serial replatings because proliferation capacities decrease when cells are committed further along a specific lineage. Nevertheless, when an oncogene blocks the cells in an early, undifferentiated and proliferating stage, colonies will still be visible after three or more platings.

Bone marrow of 5'FU-treated BALB/c mice was transduced to stably express different TET/CIZ-fusion constructs, but did not show a transformed phenotype in this assay. From the second plating onwards, only diffuse cell groups were present. However, when bone marrow was transduced with VP16/CIZ, an artificial fusion construct consisting of CIZ and the strong *Herpes simplex* VP16 activation domain, dense colonies were microscopically detectable from the second plating onwards (figure 21B) and became macroscopically visible in the fourth plating (figure 21A).

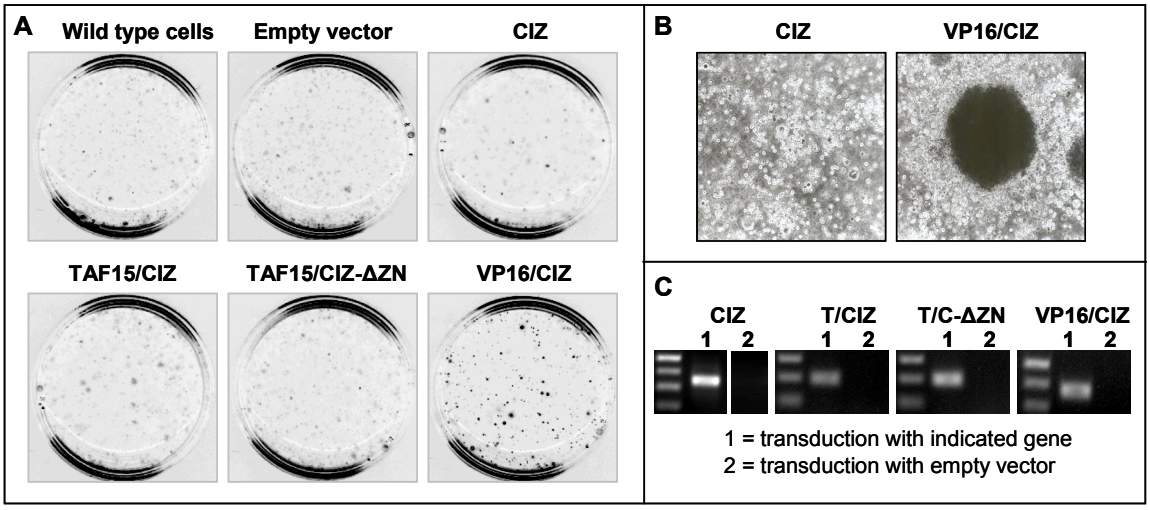

Figure 21 – **Colony assay of murine bone marrow cells**. (A) Only bone marrow cells transduced with VP16/CIZ form dense colonies which are macroscopically visible in the fourth plating. (B) VP16/CIZ colonies are already microscopically detectable in the second plating. (C) RT-PCR to verify construct expression.

4.1.3 *In vivo* transformation models

A. Assay of tumorigenicity in immunodeficient mice

Tumorigenicity is the ability of cultured cells to give rise to progressively growing tumors *in vivo*. The tumorigenic effect of the TET/CIZ fusions was tested by transduction of NIH/3T3 cells and subsequent inoculation of the cells on nude or SCID mice, which are immunodeficient to rule out interference of the immune defense by the recipient animal or rejection of the transplant.

Nude mice are homozygous for a mutation at the *nu* locus on chromosome 11, which causes them to be hairless and to completely lack a thymus [Gershwin et al., 1975]. Absence of the thymus leads to many defects in the immune system, including a much reduced lymphocyte population composed almost entirely of B-cells and failure to reject skin and tumor grafts. No tumor formation by TET/CIZ fusions was observed in these mice (figure 22).

SCID (severely combined immunodeficient) mice carry a mutation at the *scid* locus on chromosome 16, which specifically impairs differentiation of stem cells into both mature T- and B-lymphocytes [Bosma et al., 1983]. Therefore, the immune system of these mice is further impaired when compared to nude mice, and some reports say that SCID mice will allow tumor growth of lines which are not tumorigenic in

nude mice [Xie et al., 1992]. However, also in SCID mice the TET/CIZ-fusions were not tumorigenic.

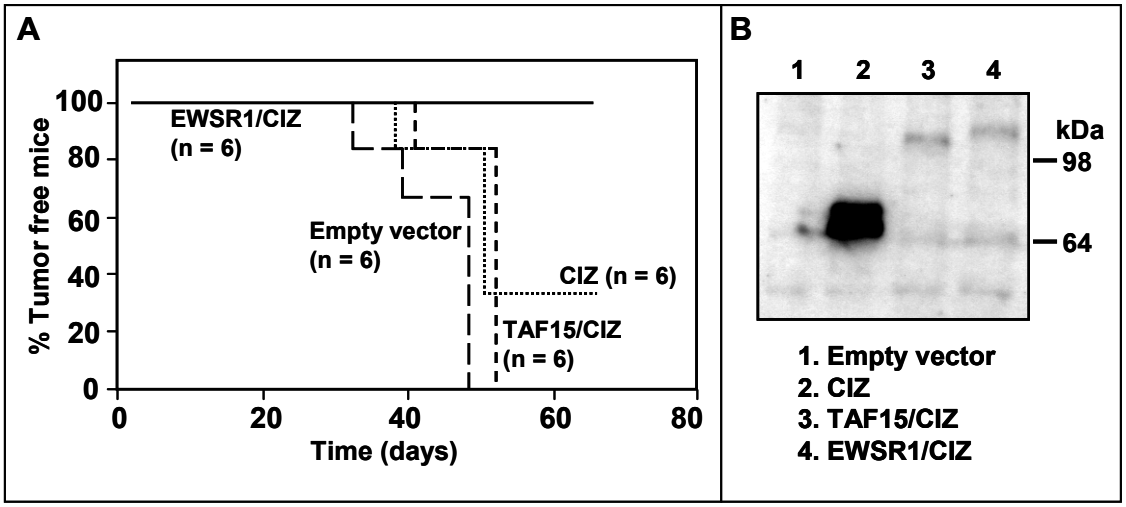

Figure 22 – **Tumor formation in immunodeficient mice.** (A) Tumor formation rates after subcutaneous injection of stably transduced NIH/3T3 cells in NMRI nu/nu mice. Tumors of 0.5 cm in diameter were considered. (B) Western to verify construct expression.

B. Mouse bone marrow transplantation

We sought to examine the *in vivo* consequences of expressing the TET fusion oncoproteins TAF15/CIZ and EWSR1/CIZ in primary hematopoietic cells using a murine bone marrow transplantation (BMT) model. Briefly, whole bone marrow harvested from 5-fluorouracil-primed mice was transduced with retrovirus containing either CIZ or the EWSR1/CIZ or TAF15/CIZ fusions. The retroviral vector contained a cassette with an internal ribosomal entry site (IRES) and EGFP. Transduced bone marrow cells were transferred by injection into the lateral tail vein of lethally irradiated syngeneic recipient mice. Expression of the constructs in bone marrow and spleen of transplanted animals was verified by RT-PCR (figure 23).

In control experiments, mice receiving cells transduced with full-length CIZ engrafted normally and had a normal survival with no evidence of disease after a follow-up of more than 300 days (figure 23, table 3). In contrast, all animals transplanted with cells containing EWSR1/CIZ developed a lethal hematopoietic disease with a median latency of 108 days. In addition, 6/8 animals receiving bone marrow transduced with TAF15/CIZ also developed a lethal hematopoietic neoplasm, but with a longer

median latency of 236 days and a reduced penetrance (75% versus 100%) compared to the EWSR1/CIZ group.

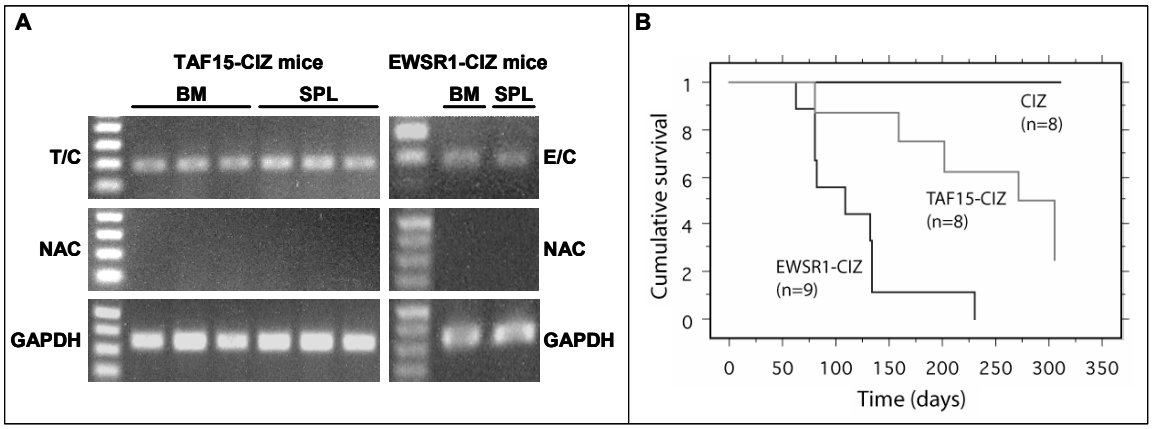

Figure 23 – **Construct expression in and survival of bone marrow transplanted mice**. (A) RT-PCR analysis of TAF15/CIZ (T/C) EWSR1/CIZ (E/C) in bone marrow (BM) and spleen (SPL) of transplanted mice. NAC: 'non amplification control' for genomic contamination. (B) Kaplan-Meier survival plot of mice receiving bone marrow transplants transduced with CIZ, TAF15/CIZ or EWSR1/CIZ.

Table 3 – Analysis of EWSR1/CIZ, TAF15/CIZ and CIZ BMT animals

	#Diseased/ # Transplanted	Latency (d) (median)	Spleen wt (mg) (median)	WBC (10^6/ml) (median)	Platelets (k/mm^3) (median)
EWSR1/CIZ	9/9	62-250 (108)	300-954 (592)	4.9-26.1 (6.8)	66-348 (89)
TAF15/CIZ	6/8	79-304 (236)	230-998 (428)	3.2-161.4 (51.3)	53-591 (221)
CIZ	0/8	n.a. > 300	92-124 (108)	3.9-7.4 (4.9)	402-1112 (538)

Although white blood cell (WBC) counts were on average higher in the TAF15/CIZ group, both TAF15/CIZ and EWSR1/CIZ animals exhibited variable WBC counts, with some animals demonstrating a moderate to marked leukocytosis and other animals displaying more normal WBC counts (table 3, figure 24). Examination of peripheral blood smears from TAF15/CIZ animals revealed that the leukocyte population was comprised of a mixture of mature and immature myeloid cells as well as a significant population of leukemic blast forms which were also seen in peripheral blood smears of the EWSR1/CIZ animals, although to a less significant degree (figure 24). Of note, all animals in both the TAF15/CIZ and EWSR1/CIZ groups displayed significant

thrombocytopenia when compared with the CIZ control group as determined by automated complete blood counts (CBC) (table 3) that was confirmed upon peripheral blood smear examination.

Gross pathologic analysis of diseased TAF15/CIZ and EWSR1/CIZ animals at the time of sacrifice demonstrated emaciation, ruffled unkempt fur and moderate to marked splenomegaly, with spleen weights ranging from 300-954 mg for EWSR1/CIZ animals and 230-998 mg for TAF15/CIZ animals compared to 92-124 mg in CIZ animals (table 3).

Histopathologic examination (figure 24) of all spleens from diseased animals demonstrated effacement of normal splenic architecture in both EWSR1/CIZ and TAF15/CIZ groups by a marked expansion of red pulp, with sheets of immature myeloid cells mixed with frequent apoptotic bodies and mitotic figures in comparison to CIZ spleen sections. Bone marrow images from CIZ animals display preserved marrow architecture with normal ratios of myeloid to erythroid elements. In contrast, bone marrow sections of both TAF15/CIZ and EWSR1/CIZ groups again revealed infiltration and effacement of normal hematopoietic elements by sheets of immature myeloid elements and blast forms. Morphologically identical leukemic infiltrates were also noted in the livers from these diseased animals in both a perivascular and sinusoidal distribution that was absent in CIZ transduced animals.

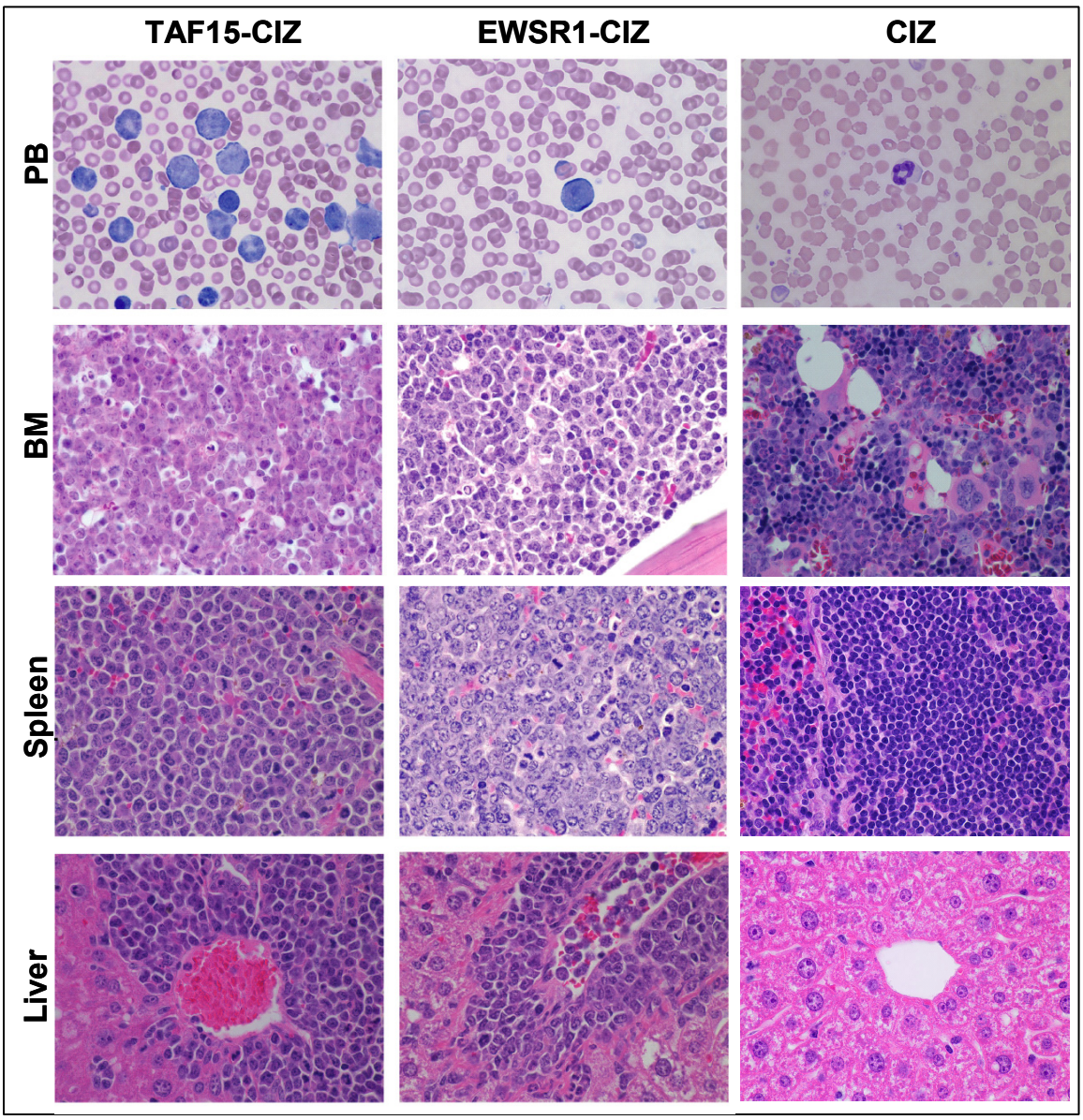

Figure 24 – **Histopathology of TAF15/CIZ, EWSR1/CIZ and CIZ BMT model**. Images of peripheral blood (PB) smears and pathology (H&E, 600x) from representative sections of bone marrow (BM), spleen and liver of transplanted animals.

Flow cytometric analysis for lineage-specific antigens and EGFP was performed on single cell suspensions from spleen of the diseased TAF15/CIZ or EWSR1/CIZ animals (figure 25). A large percentage of spleen cells were positive for EGFP compared with CIZ only animals. When gated for EGFP, the vast majority of TAF15/CIZ cells were positive for Mac-1, confirming the myeloid lineage of these cells. Although only a small percentage of TAF15/CIZ diseased cells were reactive for

c-kit (a marker associated with primitive hematopoietic cells), nearly all Mac-1 positive cells lacked expression of the mature granulocytic marker Gr-1.

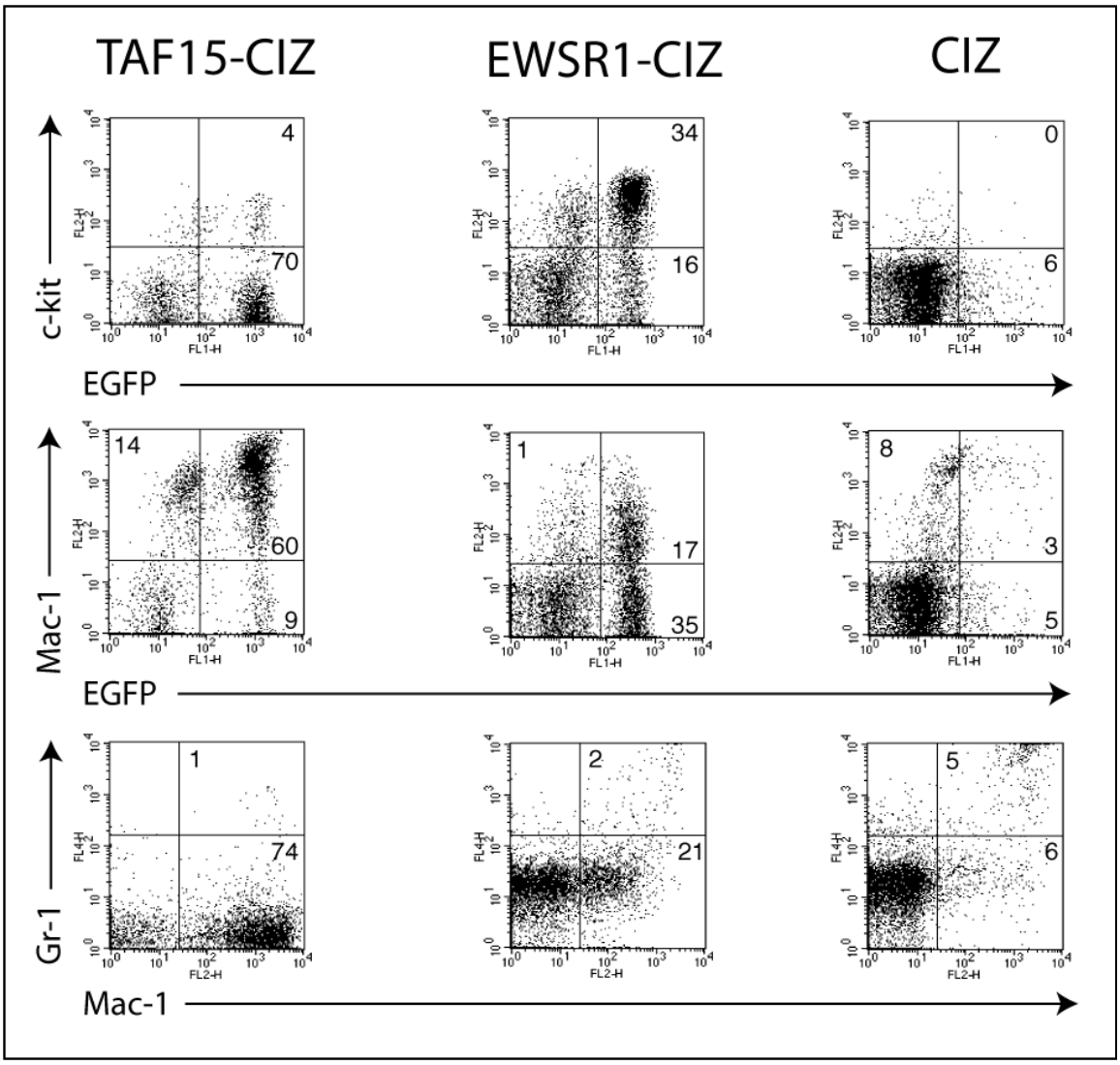

Figure 25 - **Immunophenotype of cells from spleen of BMT mice**. Flow cytometric analysis was performed on spleen cells of transplanted mice. Transplanted bone marrow was transduced with TAF15/CIZ, EWSR1/CIZ or CIZ and EGFP. Splenocytes were stained with a combination of antibodies to Mac-1, Gr-1, or c-kit. Dot plots were gated for live cells based on forward and side scatter and 7-AAD staining and demonstrate a predominant population of Mac-1^{lo}, c-kit(+), Gr-1(-) cells in EWSR1/CIZ spleens. A predominant Mac-1+, Gr-1(-) population was observed in TAF15/CIZ animals with only a small number of cells positive for c-kit.

Among EWSR1/CIZ diseased cells, a significant portion of EGFP positive cells exhibited expression of the myeloid lineage marker Mac-1, although to more variable

and lower levels than seen with TAF15/CIZ (figure 25). Comparable to TAF15/CIZ, EWSR1/CIZ cells also lacked expression of Gr-1. However, unlike for TAF15/CIZ, EWSR1/CIZ cells did exhibit strong expression of the immature hematopoietic marker c-kit. No abnormalities were observed in T-cell or B-cell populations and comparable results were obtained from immunophenotypic analysis of cells from the bone marrows from both TAF15/CIZ and EWSR1/CIZ animals (data not shown).

To assess if the TAF15/CIZ and/or EWSR1/CIZ-induced disease was transplantable, we injected $1x10^6$ spleen cells from diseased mice from each group into sublethally irradiated secondary recipient BALB/C mice by lateral tail vein injection. All secondary recipient animals of both TAF15/CIZ and EWSR1/CIZ diseased spleen cells developed a comparable fatal hematopoietic disease as observed in the primary animals characterized by splenomegaly, thrombocytopenia, and a variable degree of leukocytosis (table 4). Interestingly, the median latency of disease onset of the TAF15/CIZ secondary recipients was significantly shorter (33 days) than those of the EWSR1/CIZ secondary recipients (124 days).

Table 4 – Analysis of TAF15/CIZ and EWSR1/CIZ secondary transplant animals

	#Diseased/ # Transplanted	Latency (d) (median)	Spleen wt (mg) (median)	WBC (10^6/ml) (median)	Platelets (k/mm^3) (median)
EWSR1/CIZ	8/8	87-137 (124)	503-1627 (592)	2.6-19.1 (5.4)	28-178 (45)
TAF15/CIZ	8/8	31-49 (33)	441-827 (671)	4.8-51.0 (32.7)	32-216 (98)

These data indicate that both EWSR1/CIZ and TAF15/CIZ are capable of inducing an acute myeloid leukemia in a murine bone marrow transplant model, whereas transduction of the full-length CIZ transcription factor cannot induce leukemic disease on its own.

4.1.4 Discussion

The transformation properties of the TET/CIZ-fusion oncoproteins were investigated in several experimental systems. *In vitro* analysis allowed the establishment of a read-out for further structure-function analysis [Corveleyn et al., 2005]. *In vivo* BMT studies resulted in a mouse model that could be used for analysis of the disease.

In an *in vitro* focus formation assay, NIH/3T3 fibroblasts overexpressing TAF15/CIZ or EWSR1/CIZ acquire a transformed phenotype by exhibiting contact independent growth. Moreover, TET/CIZ fusions also alter cell morphology in this system. A similar effect was seen upon EWSR1/FLI1 and EWSR1/ETV1 induced NIH/3T3 tumor formation in SCID mice. While in identical conditions, other oncogenes induce formation of solid, spindle cell tumors consistent with fibrosarcomas, tumors arising from NIH/3T3 cells expressing EWSR1/FLI1 and EWSR1/ETV1 consist of sheets of small round cells, reminiscent of Ewing's sarcoma [Thompson et al., 1999]. This histology has not previously been found in fibroblasts. Similarly, overexpression of EWSR1/FLI1 in the murine myoblast cell line C2C12 causes an alteration in cellular morphology, characterized by a more cuboidal appearance, again resembling Ewing's sarcoma tumor cell morphology [Eliazer et al., 2003]. The molecular mechanisms involved in these alterations and their role in tumor manifestation have to be further investigated.

The transforming capacity of TAF15/CIZ and EWSR1/CIZ is also clear from the *in vivo* bone marrow transplantation experiments, where they induce an AML characterized by splenomegaly, thrombocytopenia and a variable degree of leukocytosis that is transplantable into secondary recipient animals. It is remarkable however, that the BMT mice develop AML, while six of the eight patients carrying translocation t(12;17) or t(12;22) suffer from ALL. This difference between the genetically engineered mouse model and the genuine human cancer can be caused by several experimental parameters [Wong and Witte, 2001], [Maddison and Clarke, 2005]. First, priming of the mouse bone marrow with 5'-fluorouracil to enrich for hematopoietic stem cells as well as the culture conditions during the *ex vivo* transduction step, influence the target cell population that will carry the fusion oncogenes. Secondly, identical genetic lesions do sometimes produce very different pathologies in different mouse strains, because of the importance of host/cancer interactions after transplantation. The expression level of the fusion proteins can also have an impact on the type and efficiency of disease formation, and is dependent on the integration site in the host genome. Nevertheless, the bone marrow transplant

data demonstrate the transforming properties of TET/CIZ fusions *in vivo* in a whole animal system. This model further confirms their pathogenic role in leukemogenesis and also expands the number of transcription factor fusion proteins that are capable of inducing an acute leukemia phenotype in mice.

However, in other assay systems, the transforming capacity of the TET-fusion proteins was less apparent. Indeed, expression of TAF15/CIZ or EWSR1/CIZ cannot induce substrate-independent growth in soft-agar assays, nor are there any differences in tumor-forming kinetics in nu/nu or SCID mice. Finally, contradictory to our expectations, in hematopoietic immortalization assays the expression of the fusions in primary bone marrow cells failed to confer self-renewal and serial replating capabilities to the cells. Interestingly, an artificial VP16/CIZ fusion construct was able to confer serial replating properties to primary murine bone marrow cells, suggesting that the TET transactivation domains are not sufficient to confer self-renewal properties to their CIZ fusion proteins in the context of this assay.

First, this overall picture of TET/CIZ transformation capacities fits in the large variability that has been described for the oncogenic potential of TET-fusion proteins in general. With the N-terminal TET-activation domain fused to a complete transcription factor or its DNA-binding domain, all oncogenic TET-fusions share a common structural pattern. This also suggests a common mechanism of transformation. The strength of the oncogenic potential however, varies substantially among the different TET-fusions and is determined by the identity of the transcription factor. While both EWSR1/FLI1 and EWSR1/ETV1 accelerate tumorigenesis of NIH/3T3 cells in SCID mice for example, only EWSR1/FLI1 enables fibroblast growth in semi-solid medium [Thompson et al., 1999]. In contrast to TET/CIZ, TLS/ERG causes increased proliferation and self renewal capacities of hematopoietic progenitors *in vitro* [Pereira et al., 1998], and while both TLS/ERG and EWSR1/ERG induce anchorage independent growth of NIH/3T3 cells, TLS/ERG differs from EWSR1/ERG in its ability to inhibit the differentiation into neutrophils of the LG mouse myeloid precursor cell line and to induce its growth factor independent growth [Ichikawa et al., 1999]. Clearly, not all transformed phenotypes are shared by all TET-fusions and distinct fusions behave differently in various experimental systems. The results obtained for TAF15/CIZ and EWSR1/CIZ suggest that CIZ endows the fusions with a moderate transformation potential. Secondary genomic hits might be necessary for manifestation of the disease.

Secondly, it is striking that TET/CIZ fusions support precisely those transformation phenotypes for which the cellular microenvironment is important. For loss of contact inhibition, it is thought that defects in the membrane of the cells interfere with the ability to correctly sense the cellular environment. In contrast, anchorage independence mostly points in the direction of changes in growth factor activity. Most fibroblast cells require a surface on which to flatten out before they can divide. This hypothesis is based on the idea that growth factor receptors are exposed maximally on the cell surface when it is flat, while they are hidden in the membrane folds of a cell in suspension. Transformed cells might overcome this by expressing more or constitutively active receptors. The results of an anchorage independence test usually also correspond very well to tumorigenicity assays in immunodeficient mice [Freedman and Shin, 1974]. Subcutaneous injection of transduced cells into the mice fails to incorporate the input of tissue-specific stromal cells [Boehm and Hahn, 2005]. Finally, while both TAF15/CIZ and EWSR1/CIZ display a transforming potential in an *in vivo* bone marrow transplant model, the stronger VP16 activation domain is necessary for transformation of the same cells in an *in vitro* context. Taken together, these data suggest that the oncogenic properties of the TET-fusion genes may be dependent upon the cellular and microenvironment.

These results not only lead to a better insight into the oncogenic capacities of TAF15/CIZ and EWSR1/CIZ, but also provide a model system for TET/CIZ induced transformation which can be used as a read-out in further structure-function analysis. In this way, the focus formation phenotype was used to show that the CIZ zinc finger domain, which mediates DNA-binding, is necessary for cell transformation, while deletion of other functional domains has no effect, and that the 5'-TET domain in the fusion is functionally interchangeable with other, independent activation domains (E2A, VP16). Together, this need for DNA-binding and for fusion to an extra transcriptional activation domain, strongly suggest that TET/CIZ fusion proteins exert their oncogenic potential at least in part by deregulating the expression of specific target genes.

4.2 Interaction partners of human CIZ

4.2.1 Introduction

To gain more insight into the molecular mechanisms behind the potential functions of CIZ and its role in leukemogenesis, we explored CIZ protein interactions using yeast two-hybrid technology. CIZ is highly conserved among different species. Rat and human CIZ are 96% identical. There is one remarkable difference however: one P-residue in the proline rich, p130Cas interacting region of rat Ciz is absent in the human protein. Since CIZ is extremely conserved and because rat and human CIZ nevertheless differ in their p130Cas interacting domains, we investigated whether human CIZ also interacts with p130CAS. In contrast to its rat homolog, human CIZ does not interact with p130CAS. Zyxin, PCBP1 and vimentin however, were identified as CIZ binding partners. Given the interaction between human zyxin and p130CAS, these results suggest that zyxin indirectly enables the interaction of CIZ with p130CAS which is described in rat.

4.2.2 Human CIZ does not interact with p130CAS

Domain structures of CIZ and p130CAS are depicted in figure 26A and are virtually identical for human and rat proteins [Martini et al., 2002], [Nasertorabi et al., 2004]. Rat Ciz interacts via its proline rich region with the SH3 domain of p130Cas [Nakamoto et al., 2000]. With exception of the polymorphic QA-rich region at the C-terminus, rat and human CIZ show 99% identity on the amino acid level. There are only six non-conserved residues. Interestingly, P189, which is part of the PXKP motif of rat Ciz that is recognized by the p130Cas SH3 domain [Kirsch et al., 1998], is absent in the human sequence (figure 26B). We thus investigated whether the lack of this P-residue affects the interaction of human CIZ with human p130CAS in a yeast two-hybrid screen. The N-terminal parts of rat and human CIZ were cloned as baits; the SH3 domains of rat and human p130CAS were used as preys (figure 26A). The interaction of rat Ciz with rat p130Cas was confirmed in the experiment (figure 26C). Furthermore, rat Ciz also interacts with the SH3 domain of human p130CAS. Human CIZ however, does not interact with the SH3 domain of either human or rat p130Cas. This result indicates that the absence of the P-residue indeed affects p130CAS recognition by the proline rich region of human CIZ.

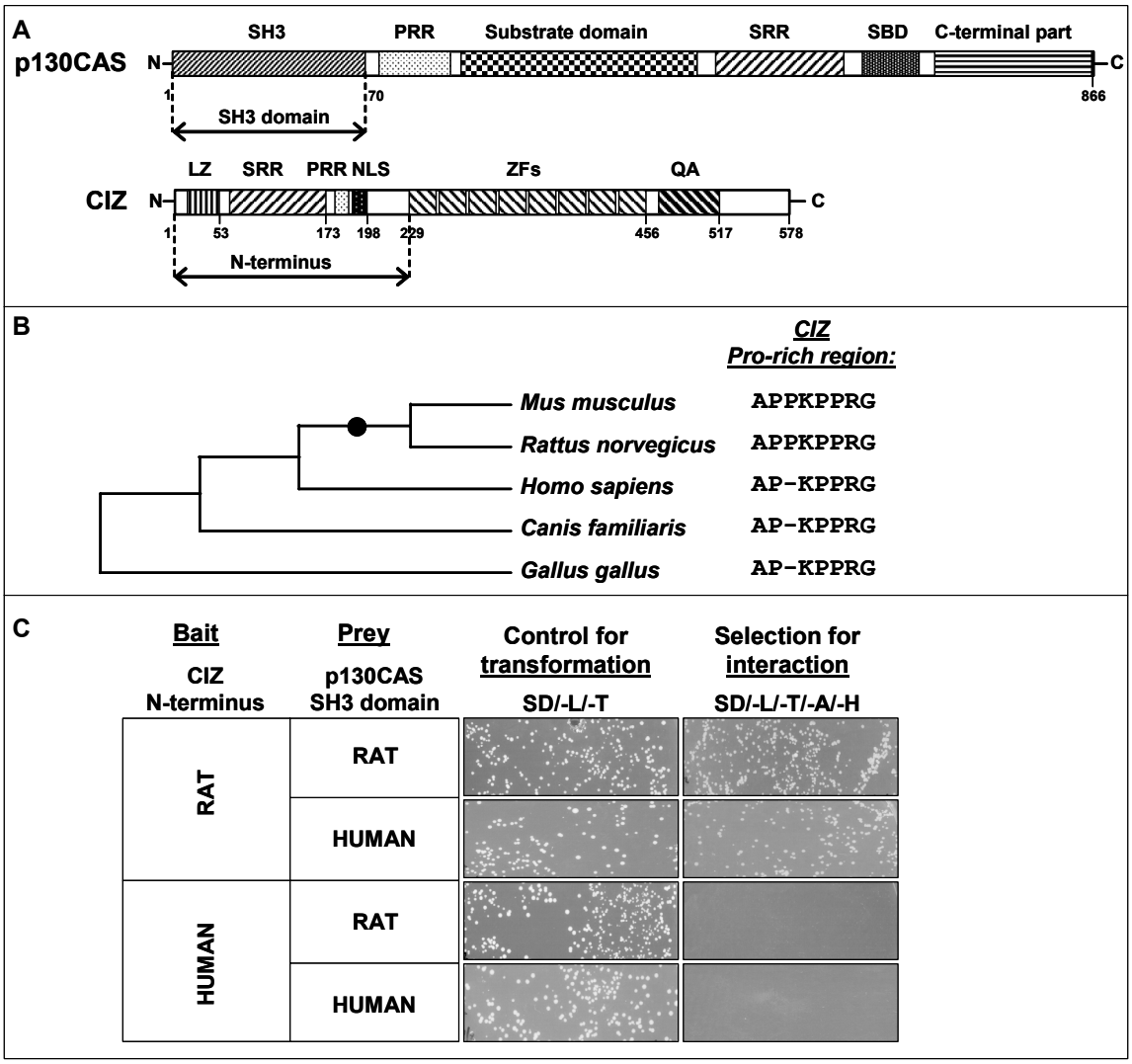

Figure 26 - **Human CIZ does not interact with human p130CAS.** (A) Structure of human p130CAS and CIZ proteins. p130CAS contains an SH3 domain (SH3), a proline rich region (PRR), a substrate domain, a serine rich region (SRR) and a Src binding domain (SBD). CIZ has a putative leucine zipper (LZ), a serine rich region (SRR), a proline rich region (PRR) and a putative nuclear localization signal (NLS) at the N-terminus, followed by eight Krüppel-type C_2H_2 zinc fingers (ZFs) and a glutamine-alanine stretch at the C-terminus. Amino acid numbers are indicated below the protein structures. Arrows indicate the domains that were investigated. (B) Proline rich region of CIZ orthologs. Phylogenetic tree for human, dog, rat, chicken and mouse (length of branches is not to scale). Sequence of the proline rich region is indicated on the right. A black dot indicates the appearance of an extra P-residue in the proline rich region of Ciz. (C) Interaction of the proline rich region of CIZ with the SH3 domain of p130CAS. Yeast is cotransfected with bait and prey constructs as indicated on the left and grown on different selective media to check for transformation efficiency and for interaction of bait and prey. The N-terminal part of rat Ciz interacts with the SH3 domain of both rat and human p130CAS. The N-terminus of human CIZ does not interact with either rat or human p130CAS.

4.2.3 Zyxin, PCBP1 and vimentin as novel binding partners for CIZ

To identify other CIZ interaction partners, we performed two independent yeast two-hybrid screenings; one with the CIZ N-terminus containing the variant proline rich region as a bait, the other with the C-terminal part of CIZ (figure 27A), both against a prey library of human bone marrow cDNA. Yeast transformation yielded approximately 10^6 and $0.2x10^6$ colonies for the experiments with the N-terminal and the C-terminal bait, respectively. Colonies with positive interaction phenotypes were selected and inserts of their prey vectors were identified. The interaction of clones encoding an in frame fusion with the GAL4 activation domain with the CIZ bait was confirmed in yeast. This reduced the number of interacting clones to nine for the N-terminal bait and one for the C-terminus. The inserts of the selected prey vectors are depicted in figure 27B. Five of the peptides interacting with the CIZ N-terminus are derived from the C-terminal part of zyxin, all of them encoding the three zyxin LIM domains. The remaining four colonies from the screening with the N-terminal part of CIZ all encode parts of the poly-(rC) binding protein 1 (PCBP1), covering the second and part of the first K-homology (KH) domain. No p130CAS clone was isolated with the N-terminal bait. The clone interacting with the C-terminal bait encoded a fragment of vimentin.

Zyxin is a nucleo-cytoplasmic shuttling protein with multiple protein-protein interaction domains [Macalma et al., 1996] and possible roles in both cytoskeletal organization and intracellular communication between the plasma membrane and the nucleus [Nix and Beckerle, 1997], [Nix et al., 2001]. The LIM domain is a cysteine rich zinc finger domain named after the first three proteins in which it was identified (Lin-11, Isl-1 and Mec-3). It functions as a specific protein-binding interface and mediates interaction of zyxin with p130CAS [Yi et al., 2002] and other proteins [Schmeichel and Beckerle, 1994]. PCBP1 is an RNA-binding protein [Kiledjian et al., 1995] with a role in mRNA stabilization [Leffers et al., 1995] and control of translation initiation [Makeyev and Liebhaber, 2002]. Its KH domains are involved in RNA binding. Vimentin is a building block of intermediate filaments [Herrmann et al., 1996].

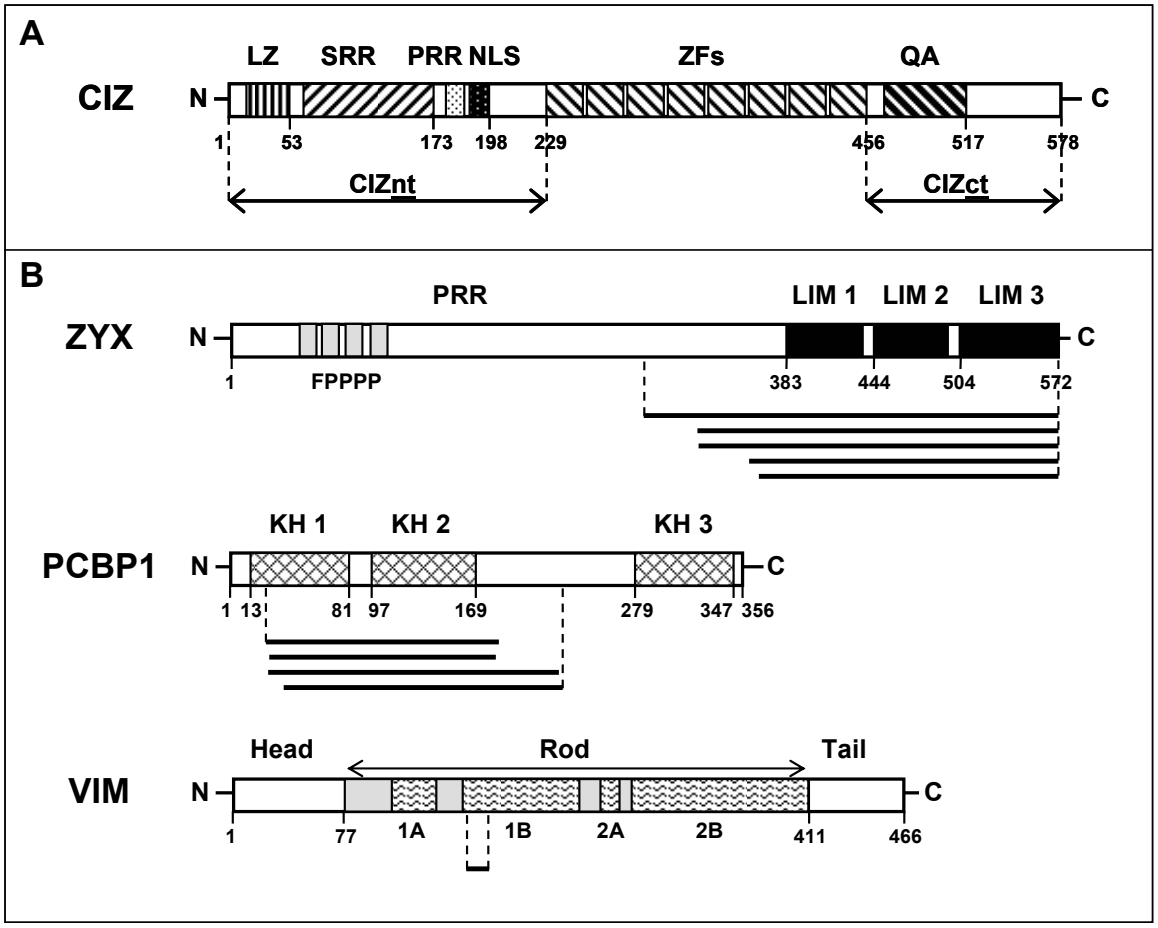

Figure 27 - **Bait proteins and interacting clones.** (A) Arrows indicate the N-terminal (CIZnt) and C-terminal (CIZct) parts of CIZ used as baits in the yeast two-hybrid screenings. (B) Protein structures of zyxin, PCBP1 and VIM. Zyxin has a proline rich region (PRR) and three C-terminal LIM domains (LIM). FPPPP: proline rich repeat. PCBP1 has three K homology domains (KH). Vimentin has a non-helical N-terminal head domain, a central α-helical rod and a non-helical C-terminal tail. 1A, 1B, 2A and 2B indicate the helices in the rod domain. Amino acid numbers are shown below the proteins. Lines below the proteins indicate clones picked up in the yeast two-hybrid screening.

4.2.4 CIZ interacts with zyxin, PCBP1 and vimentin in mammalian cells

To investigate the *in vitro* association of full length CIZ with full length zyxin, PCBP1 and vimentin, co-immunoprecipitation experiments were performed. Plasmids containing myc-tagged CIZ were transfected into HEK293T cells together with one of the flag-tagged, full-length interaction partners. When zyxin, PCBP1 or vimentin is precipitated from whole cell lysates with an anti-flag antibody, CIZ is detected in the precipitate (figure 28A). Alternatively, when CIZ is pulled down with an anti-myc

antibody, zyxin, PCBP1 and vimentin are co-precipitated (figure 28B), suggesting that the interactions can take place in mammalian cells.

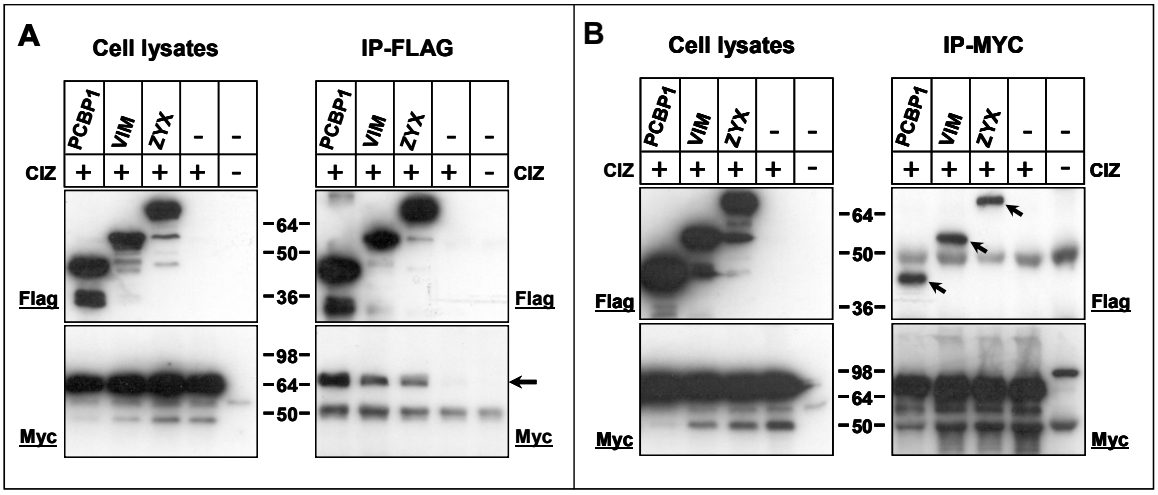

Figure 28 - Co-immunoprecipitation of CIZ with PCBP1, zyxin and VIM. HEK293T cells were transfected with DNA encoding CIZ and a candidate interaction partner or with CIZ alone as indicated above the blots. (A) Flag-tagged proteins (PCBP1, VIM, zyxin) were precipitated and the coprecipitated proteins were detected by Western analysis with anti-myc. Left-hand blots: Western analysis of cell lysates showing expression of transfected constructs. Right-hand blots: proteins present in the immunoprecipitate. Antibodies used for detection are indicated next to each blot. Molecular weight markers are given in kDa. Arrows indicate the co-precipitating proteins. (B) Reverse experiment: myc-tagged CIZ is precipitated; co-precipitating proteins are visualized with anti-flag.

4.2.5 Domains of CIZ involved in the interaction

To identify the domains involved in the interaction with zyxin, we constructed a number of CIZ deletion mutants (figure 29A) and performed co-immunoprecipitation experiments to assess the binding capacity of the truncated proteins (figure 29B). Loss of interaction of zyxin with the CIZ-ΔN1 mutant shows that the first 72 amino acids of CIZ are necessary for interaction with zyxin (figure 29B). Deletion of the proline rich region does not affect interaction, whereas as expected no interaction occurs with the C-terminal zinc-finger domain.

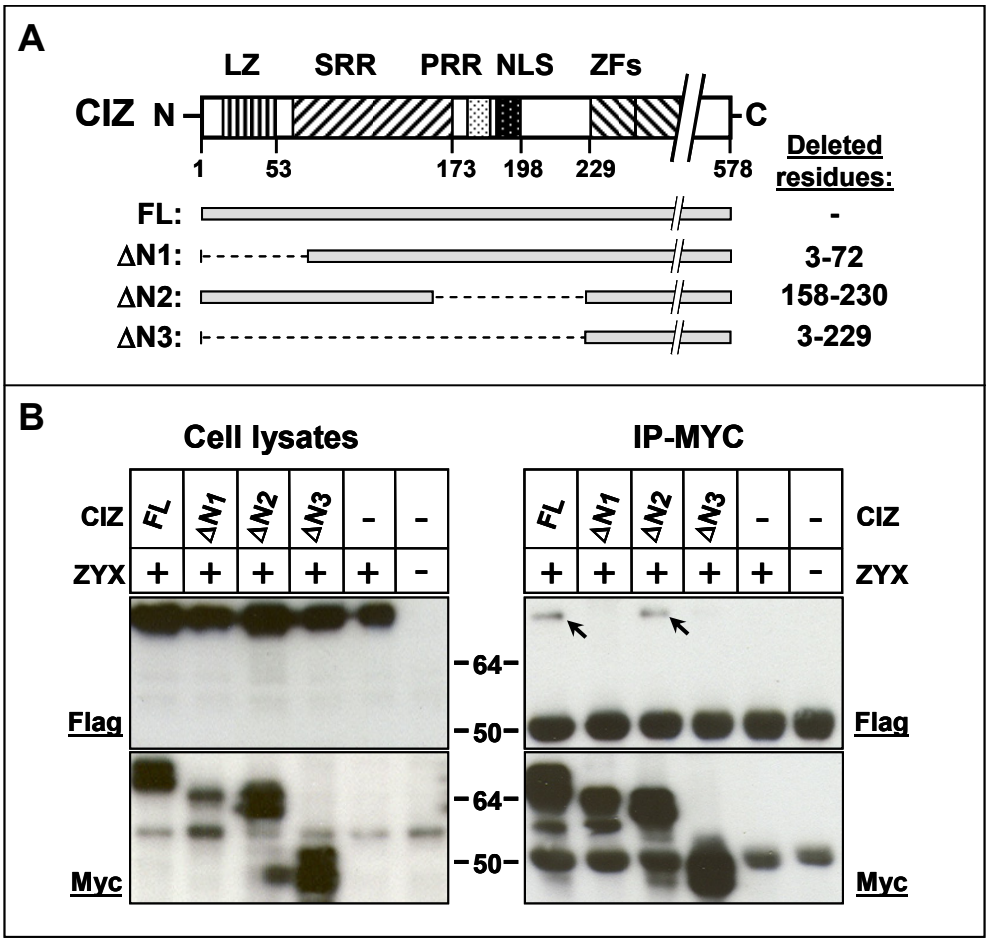

Figure 29 - **Identification of zyxin interacting domains in CIZ.** (A) Schematic representation of various CIZ deletion mutants, with dotted lines indicating the deleted parts. Corresponding amino acid numbers are indicated on the right. (B) The first 72 amino acids of CIZ are necessary for interaction with zyxin. Co-immunoprecipitation experiment of zyxin and various CIZ deletion mutants; Western blots of cell lysates (left side) and myc-immunoprecipitates (right side) are shown. Transfected constructs are indicated above the blots, antibodies used for detection next to each blot. Arrows indicate zyxin that co-precipitates with CIZ.

4.2.6 Zyxin mediates the interaction between human CIZ and p130CAS

It is of particular interest that zyxin is a known p130CAS interaction partner [Yi et al., 2002]. This raises the question whether in human cells, zyxin might indirectly mediate the interaction between CIZ and p130CAS. To test this hypothesis, we investigated whether all three proteins could be coprecipitated as a ternary complex. To this end, HEK293T cells were cotransfected with CIZ, zyxin and p130CAS and CIZ was precipitated from the cell lysates using an anti-myc antibody (figure 30). In the presence of zyxin, p130CAS clearly co-precipitates with CIZ (figure 30A, lane 1 of the IP blot). However, when zyxin is not present, a weak p130CAS signal is also

present (lane 2). This is partly due to aspecific binding of p130CAS to the beads, as can also be seen in the negative control lanes 5 and 6. Secondly, we suggest that the presence of endogenous zyxin is most likely responsible for the weaker signal in lane 2. After quantification of the intensity of the p130CAS bands (figure 30B) and correction for the aspecific binding, the amount of p130CAS co-precipitating in the presence of zyxin is 4.6 times the amount present without zyxin. These data support the hypothesis of zyxin as a mediator of p130CAS signaling towards human CIZ.

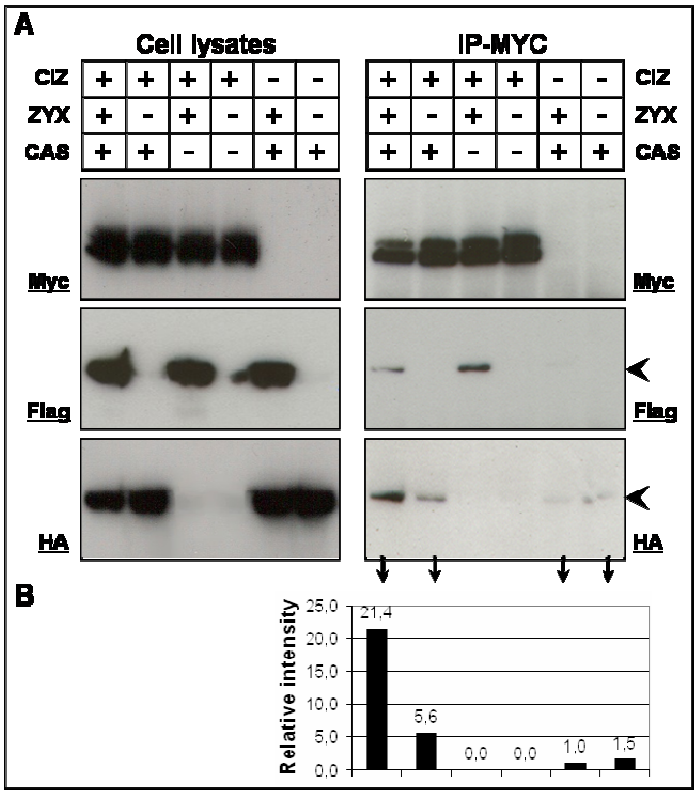

Figure 30 - Zyxin mediates the interaction of human CIZ and p130CAS. HEK293T cells were transfected with CIZ (myc-tag), zyxin (flag-tag) and/or p130CAS (HA-tag) as indicated above the blots. (A) CIZ was precipitated with anti-myc and the co-precipitated proteins were detected by Western with anti-flag and anti-HA. Left-hand blots: Western of cell lysates showing transfected constructs. Right-hand blots: proteins present in the precipitates. Antibodies used for detection are indicated. Arrowheads indicate coprecipitating zyxin and p130CAS. (B) Band intensity quantification of co-precipitating p130CAS.

4.2.7 TAF15/CIZ and EWSR1/CIZ do not interact with PCBP1, VIM and ZYX

Differences in protein interactions between CIZ and the TET/CIZ-fusions might be relevant for the transforming properties of the oncogenic fusion proteins. Therefore, we first investigated the expression of zyxin, PCBP1 and VIM in bone marrow of the patients. Zyxin and PCBP1 mRNA were detected by RT-PCR in the bone marrow of all patients tested (figure 31A). Vimentin expression could not be detected in one patient.

Next, co-immunoprecipitation experiments were performed in HEK293T cells whereby PCBP1, vimentin or zyxin was precipitated with anti-flag. A weak TET/CIZ signal was observed in the precipitates (figure 31B,C). This signal however, was also present in the precipitate from cells without flag-tagged PCBP1, vimentin or zyxin, suggesting an aspecific interaction of TET/CIZ with the G-sepharose beads. Various exposure times resulted in the same relative intensities of the four TET/CIZ bands, indicating that observation was in the linear detection range. Furthermore, the lower IP-blots (detected with anti-myc) in figure 31B and C contain seven times more protein ($2.45x10^6$ cell equivalents) than the upper blots (detected with anti-flag, $0.35x10^6$ cell equivalents). Thus, if part of the TET/CIZ signal would nevertheless be due to specific interaction with PCBP1, vimentin or zyxin, it will be much weaker than in the case of CIZ (figure 28, equal amounts of protein loaded on both gels). In conclusion, fusion of CIZ to the N-terminus of TAF15 or EWSR1 disturbs its interaction with PCBP1, vimentin and zyxin.

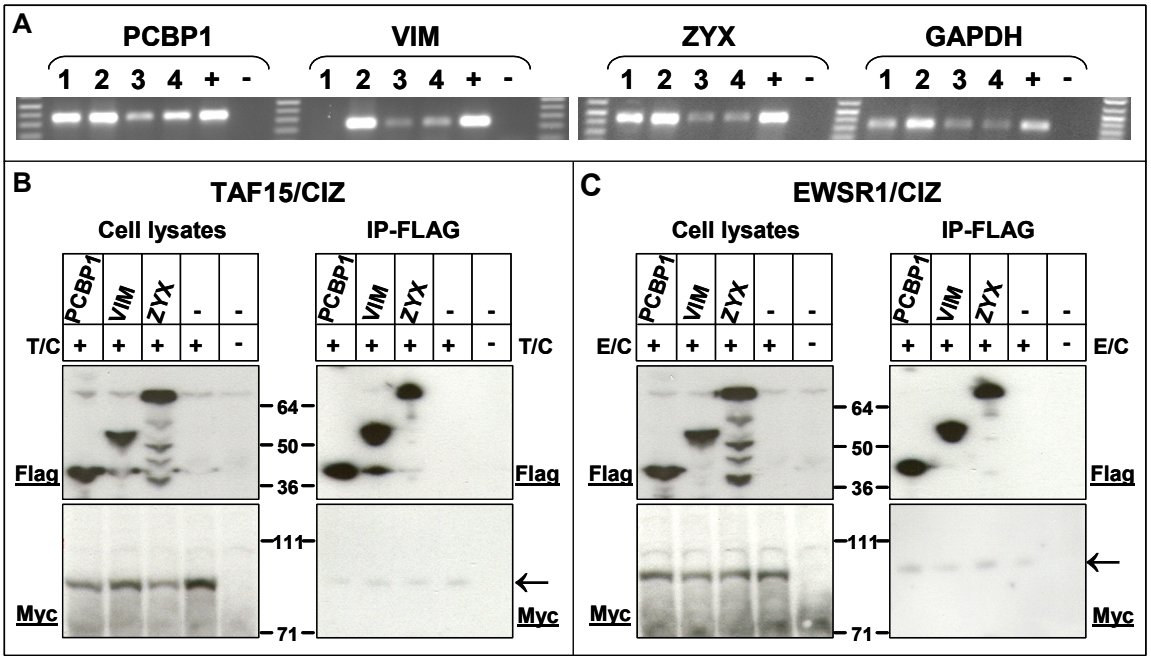

Figure 31 - **No interaction of TET/CIZ-fusions with PCBP1, VIM and zyxin.** (A) RT-PCR to check expression of PCBP1, VIM and zyxin in bone marrow of patients carrying translocations t(12;17)(p13;q11) (lanes 2,3,4) or t(12;22)(p13;q12) (lane 1). PCR on human placenta cDNA is performed as a positive control (+), without template as a negative control (-). (B) and (C), Co-immunoprecipitation with anti-flag to investigate interaction of TAF15/CIZ (B) and EWSR1/CIZ (C) with PCBP1, VIM and zyxin. Western blots of cell lysates (left sides) and flag-immunoprecipitates (right sides) are shown. Transfected constructs are indicated above the blots (T/C: TAF15/CIZ, E/C: EWSR1/CIZ), antibodies used for detection next to each blot. Arrows indicate TET/CIZ precipitates.

4.2.8 Discussion

A. Human CIZ does not interact with p130CAS

Integrins form the major class of receptors used by cells to contact with the extracellular matrix (ECM) [Longhurst and Jennings, 1998]. They transfer ECM signals across the plasma membrane into the cell, but lack further catalytic signal transducing activity. A protein complex is assembled at their cytoplasmic tails, initiating signal transduction through one or more downstream pathways. Adaptor protein p130CAS is phosphorylated upon engagement of integrins [Bouton et al., 2001], enables assembly of protein complexes at their cytoplasmic domains in focal adhesions and is involved in ECM signal transduction. In lymphocytes, β1-integrin signaling involving p130CAS phosphorylation is crucial for cell development [Manie et al., 1997b]. Correct localization, survival, differentiation and proliferation of B- en T-cells are indeed critically dependent on interactions of the hematopoietic precursor cells with their microenvironment in the bone marrow [Coulombel et al., 1997]. As a consequence, defects in integrin signaling are recurrently involved in leukemogenesis [Cacciola et al., 1997]. In B-lineage acute lymphoblastic leukemia for example, a correlation has been established between defective β1-integrin signaling and high levels of leukemic cells that prematurely egress from the bone marrow [Geijtenbeek et al., 1999].

Rat Ciz is involved in processes for which interaction with the ECM is crucial, it interacts with p130Cas at focal adhesions and shuttles between focal adhesions and the nucleus, which makes it a good candidate ECM signal transduction and effector molecule. Our experiments however indicate that human CIZ, lacking one proline residue in the p130Cas interacting domain as present in rat Ciz, does no longer interact with p130CAS. This is in agreement with Kirsch et al., who determined PXKP as the consensus for interaction with the SH3 domain of p130CAS [Kirsch et al., 1998]. The corresponding human sequence PKP lacks one residue. The proline rich regions of dog and chicken Ciz are identical to that of human CIZ, while both rat and mouse proteins show the additional P-residue (figure 26B). This suggests that during evolution, the extra residue appeared somewhere in the rodent branch of the phylogenetic tree.

In a screen to identify interaction partners for the N-terminal and C-terminal domains of CIZ, we consequently did not find p130CAS, but we found zyxin, PCBP1 and vimentin to bind CIZ.

B. Zyxin as a mediator of p130CAS signaling towards CIZ

Zyxin is an adaptor protein that interacts with p130CAS at focal adhesions [Yi et al., 2002] and communicates ECM signals by shuttling between focal adhesions and the nucleus [Nix and Beckerle, 1997]. Like CIZ, zyxin is ubiquitously expressed [Macalma et al., 1996]. It colocalizes with integrins at sites of cell adhesions and serves as a docking site for the assembling of protein complexes involved in downstream signaling. But zyxin also shuttles towards the nucleus and is thus postulated to be an integrin signal transducer itself [Nix and Beckerle, 1997]. Zyxin consists of a tandem array of protein interaction domains and has many other interaction partners. Zyxin might thus mediate interaction of CIZ with p130CAS in focal adhesion complexes. Interestingly, during evolution, rat and mouse Ciz gained an extra P-residue allowing a direct interaction of Ciz with p130Cas at focal adhesions.

For its hypothesized role as a communicator between p130CAS and CIZ, its C-terminal LIM-domains are crucial. Not only do they constitute an interaction site for both CIZ and p130CAS [Yi et al., 2002], they are also responsible for targeting zyxin to focal adhesions [Nix et al., 2001]. Two other binding partners for the LIM domains of zyxin have been identified: cysteine rich protein 1 (CRP1) [Sadler et al., 1992] and tumor suppressor protein H-warts/LATS1 [Hirota et al., 2000]. Via the proline regions in its N-terminus, zyxin also interacts with the proto-oncogene VAV [Hobert et al., 1996] and with proteins involved in actin filament dynamics, like α-actinin [Reinhard et al., 1999] and members of the Ena/Vasp family [Drees et al., 2000].

C. Interaction with PCBP1

PCBP1 or hnRNP E1 (heterogeneous nuclear ribonuclear protein E1), together with PCBP2 and hnRNP K, constitute a subfamily of KH-domain RNA-binding proteins [Ostareck-Lederer and Ostareck, 2004]. They have a diverse role in regulation of gene expression at the posttranscriptional level, through effects on mRNA transport, stability and translational control [Ostareck-Lederer et al., 1998]. Like CIZ, PCBP1 is broadly expressed in human and mouse tissues. It shuttles between the cytoplasm and the nucleus, where it is enriched in nuclear speckles [Chkheidze and Liebhaber, 2003].

Members of the PCBP1 subfamily have three KH domains of which two are located N-terminal and one is more to the C-terminus. All four PCBP1 clones interacting with CIZ contain the second and almost the entire first KH-domain of PCBP1. The KH domain was first identified in hnRNP K and is a 65-70 AA RNA binding domain. If not phosphorylated, PCBP1 binds to C-rich pyrimidine stretches in the 3'UTR of several

genes by means of its KH-domains [Makeyev and Liebhaber, 2002]. KH domains can also function as protein binding interfaces [Ostareck-Lederer and Ostareck, 2004].
The PCBP1 subfamily of hnRNPs is especially involved in the control of mRNA translation and stability in hematopoietic cell differentiation [Ostareck-Lederer and Ostareck, 2004]. HnRNP K and another hnRNP that might be PCBP1 [Bustelo et al., 1995] specifically interact with VAV, a proto-oncogene that is exclusively expressed in hematopoietic cells and plays a crucial role in development of B- and T-cells [Bustelo, 2000]. It becomes phosphorylated upon engagement of a variety of cell surface receptors. Via its interaction with hnRNPs, VAV transmits signals from the plasma membrane towards specific RNA targets, possibly regulating their turnover and translation [Ostareck-Lederer et al., 1998]. Interestingly, VAV also interacts with zyxin [Hobert et al., 1996]. This suggests the existence of a protein complex involved in signal transduction from the plasma membrane to the nucleus, with CIZ and PCBP1 as effector molecules regulating gene expression on the transcriptional and posttranscriptional level, respectively.
One target gene of such a complex might be *COL1A1*, encoding type I collagen. CIZ binds to a consensus sequence in the *COL1A1* promoter [Alvarez et al., 1997] and activates its transcription [Furuya et al., 2000b]. PCBP1 interacts with the 3'UTR of the transcript and, by stabilizing it, further enhances collagen type I expression [Thiele et al., 2004]. Therefore, interaction of CIZ and PCBP1 might allow them to cooperate in the regulation of gene expression, each acting in turn on a different stage of the expression process.

D. Interaction with vimentin

Vimentin is the major compound of intermediate filaments (IF) in cells of mesenchymal origin. It consists of a central α-helical rod domain that is flanked by non-helical head and tail domains. For the formation of filaments, vimentin monomers anneal longitudinally to each other.
In this study, we report the interaction between a region at the most N-terminal site of the second α-helix in the vimentin rod domain and CIZ. Many proteins are known to interact with vimentin. Some of them crosslink the protein with other elements of the cytoskeleton [Svitkina et al., 1996], which is consistent with its structural function. But also non-structural proteins, like stress response factors or protein kinases, bind to vimentin [Evans, 1998]. These second type of interactions take part in the role of the IF network as a signal communicator. Considering the interaction of CIZ with vimentin as an interaction of this second kind, it fits again in the hypothesis of CIZ as a transcription factor responding to signals which originate at the cell periphery.

E. CIZ interactions and leukemogenesis

With the N-terminal TET activation domain fused to a full-length transcription factor, chimeric TET/CIZ-proteins are new transcription factors with possibly altered activation potential. It was shown before that the transforming capacities of these fusions are indeed at least partly due to their action as aberrant transcription factors [Corveleyn et al., 2005]. With CIZ as an effector molecule for integrin signaling and since defects in integrin function are related to acute lymphoblastic leukemia [Geijtenbeek et al., 1999], changes in CIZ transcription regulation provide a link to leukemogenesis. With zyxin as a communicator of integrin signals to CIZ and with the cooperative role of PCBP1 in the expression control of CIZ induced transcripts at the posttranscriptional level, changes in the interaction with these proteins might as well influence CIZ expression regulation, and as such change the answer of the cell to ECM signals.

On the other hand, DNA-binding independent transformation by TET-fusions has been reported [Jaishankar et al., 1999] and an additional oncogenic effect on RNA-processing has been suggested [Yang et al., 2000a]. TET-proteins bind RNA polymerase II with the N-terminus and recruit several SR-splice factors on the C-terminus, providing a link between transcription and transcript processing. When the two sites of the protein are separated by a translocation, this link is broken. This possibly influences posttranscriptional processing, which is a determinant for gene expression. In the case of the TET-fusions to CIZ, this uncoupling of transcriptional and posttranscriptional expression regulation is even twofold, since not only the two halves of the TET-protein are separated, but the interaction of CIZ with PCBP1 is lost too.

Upon fusion of CIZ to TAF15 or EWSR1, the interaction with all three interaction partners is different. This is not due to different subcellular localization of CIZ and the fusions, since they show the same nuclear staining patterns. A mechanism is suggested whereby presence of the N-terminal TET moiety may occlude the normal site of interaction or may impair the proper folding of the hybrid protein, and thus interfere with the ability of normal CIZ to interact with zyxin, PCBP1 and vimentin.

4.3 TET/CIZ-fusions as aberrant transcription factors

4.3.1 Introduction

The molecular mechanism of transformation by TET-fusions is currently explained in either of two ways: first, the chimeric proteins can act as aberrant transcription factors; second, TET-fusions can interfere with normal splicing. While an effect of TAF15/CIZ or EWSR1/CIZ on splice site selection could not be demonstrated [Corveleyn et al., 2005], this chapter reports on our investigations of TET/CIZ-fusions as aberrant transcription factors.

First we demonstrated that TAF15/CIZ and EWSR1/CIZ still bind to the DNA binding consensus site of CIZ by an electrophoretic mobility shift assay. Then we searched for TET/CIZ transcriptional targets.

4.3.2 DNA binding capacity of TET/CIZ fusions

CIZ is a transcription factor that specifically binds to the DNA consensus (G/C)AAAAA(A), which is present in the promoter of several matrix metalloproteinases (MMPs). Its overexpression induces transcriptional upregulation of MMP-1 (collagenase), MMP-3 (stromelysin) and MMP-7 (matrilysin) [Nakamoto et al., 2000]. To determine whether the TET/CIZ fusions still bind to the same DNA sequence, an electrophoretic mobility shift assay (EMSA) was performed. Therefore, HEK293T cells were transiently transfected with expression plasmids encoding myc-tagged TAF15/CIZ or EWSR1/CIZ. Total cell lysates were incubated with a ^{32}P-labeled DNA probe corresponding to a region in the MMP-1 promoter (position -320 to -305) containing the CIZ DNA binding consensus. For both CIZ-fusions, a gel shift appears that is supershifted with an anti-myc antibody and which disappears upon competition with a 1000-fold excess of cold, wild-type probe (figure 32). In conclusion, TAF15/CIZ and EWSR1/CIZ specifically bind to the CIZ DNA binding consensus.

A subsequent promoter-reporter assay however, showed no enhancement of MMP-7 transcription upon overexpression of TAF15/CIZ or EWSR1/CIZ, suggesting that MMPs might not be the most important target genes of the fusions.

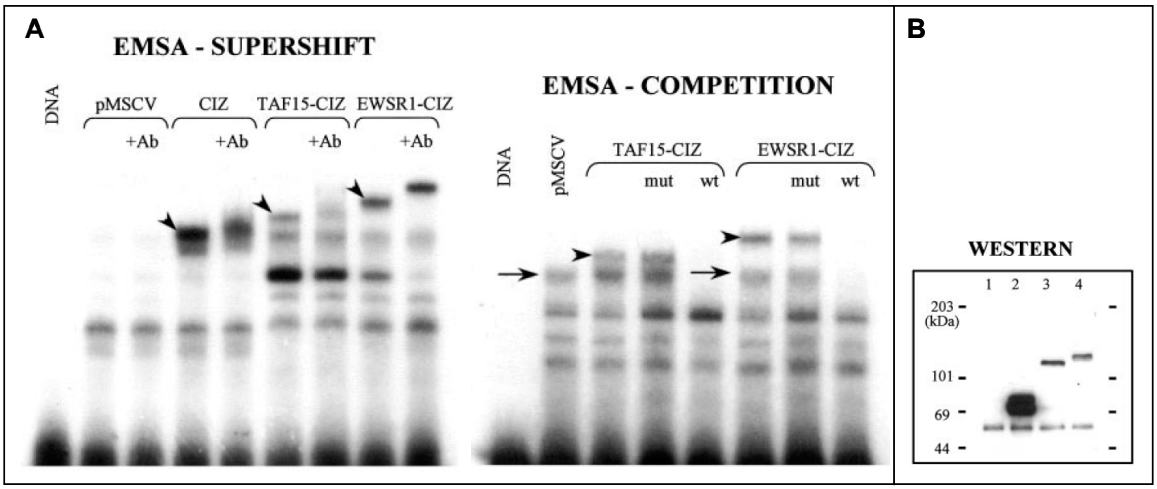

Figure 32 – DNA binding of TET/CIZ fusion proteins. (A) Total lysate of HEK293T cells transiently transfected with myc-tagged CIZ, TAF15/CIZ or EWSR1/CIZ was capable of forming a gel shift complex with a ^{32}P-labeled oligonucleotide containing the CIZ DNA binding consensus sequence. This complex was supershifted with a monoclonal anti-myc antibody (Ab) and disappeared in the presence of a 1000-fold excess of cold wild-type probe (wt), whereas the same amount of cold mutant probe (mut) did not compete for the binding. Arrowheads indicate shifted complexes. Arrows in the competition panel indicate signals resulting from endogenous CIZ expression. (B) Western blot analysis of the cell lysates. Lane 1: empty pMSCV vector; lane 2: CIZ; lane 3: TAF15/CIZ; lane 4: EWSR1/CIZ. Proteins were detected using the anti-myc monoclonal antibody.

4.3.3 Identification of EWSR1/CIZ transcriptional targets

To identify TET/CIZ transcriptional target genes, we first focused on a number of known CIZ and EWS/FLI1 target genes. No overexpression of *Mmp7*, *Mmp3*, *Eat2* (EWS/FLI1 activated transcript 2) [Thompson et al., 1996] or *Mfng* (manic fringe) [May et al., 1997] could be observed by RT-PCR in NIH/3T3 or Ba/F3 cells stably expressing the TET/CIZ fusions.

Also for TERT (telomerase reverse transcriptase, the enzymatic component of the telomerase complex), which is transcriptionally activated by several EWSR1/ETS-fusions [Takahashi et al., 2003], [Fuchs et al., 2004a], no overexpression could be detected in transiently transfected HEK293T or in stably transduced NIH/3T3 cells by a telomeric repeat amplification protocol (TRAP assay).

To identify novel TET/CIZ transcriptional target genes, we then performed a general expression profiling of Ba/F3 cells overexpressing EWSR1/CIZ.

A. *Experimental setup: expression profiling of a BaF3-TetOff-EWSR1/CIZ cell line*

For microarray analysis, a Ba/F3 cell line with stable but inducible EWSR1/CIZ expression was generated. The murine pro-B lymphoblastic Ba/F3 cell line was chosen because of its similarity with the t(12;17) and t(12;22) leukemia samples. An inducible expression system was used to circumvent the apparent cytotoxic effect of EWSR1/CIZ and to minimize differences between the samples with and without EWSR1/CIZ expression.

The Tet-Off system (Clontech BD) was used for inducible transgene expression. First two monoclonal Ba/F3-TetOff cell lines (Ba/F3-3C3 and Ba/F3-3D2) were generated which constitutively express a tetracycline-controlled transcriptional activator. (Ba/F3-3C3 was used for microarray analysis, Ba/F3-3D2 for a biologically independent confirmation by RT-PCR). Next, both Tet-Off cell lines were transduced with either the empty pRevTRE vector (V) or EWSR1/CIZ (EC), preceded by a minimal CMV promoter and a Tet responsive element. Cell lines were cultured in the presence of tetracycline to suppress transgene expression (figure 33A).

For induction of EWSR1/CIZ expression, tetracycline was withdrawn from the medium. As shown by Western analysis (figure 33B), a strong EWSR1/CIZ induction was observed 15 hours after tetracyclin removal. Leaky EWSR1/CIZ expression was visible at time point zero, just before removal of tetracyclin. Samples EC-t0, EC-t15 and V-t15 were used for hybridization on the GeneChip® Mouse Genome 430A 2.0 Array (Affymetrix) as described in the materials and methods chapter.

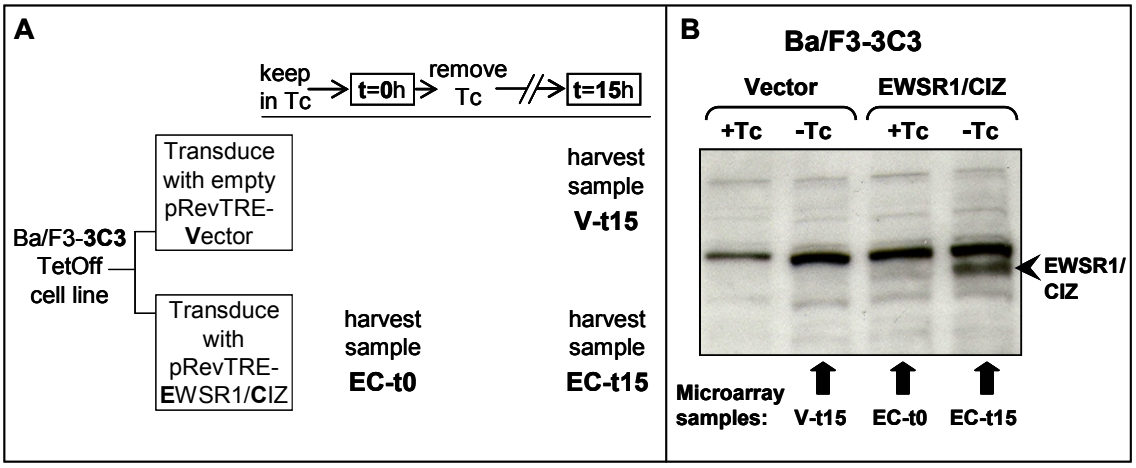

Figure 33 – Inducible EWSR1/CIZ expression in the Ba/F3-3C3 TetOff cell line. (A) Sample overview (Tc = tetracyclin). (B) Western analysis of EWSR1/CIZ. In the EWSR1/CIZ transduced cell line, leaky EWSR1/CIZ expression is visible in the presence of Tc and strong induction 15 hours after its removal.

B. Micro-array outcome summary

Microarray data analysis was performed with the Affymetrix GeneChip® Operating Software (GCOS). Each RNA sample was hybridized to an individual chip. First, experimental variation between the hybridizations was minimized by scaling the signal intensity of each array to the same target value. Next, samples were compared two by two to detect and quantify changes in gene expression. Figure 34 shows the numbers of genes with at least two-fold changes in expression levels. In table 5 to table 8, the top 20 of genes with highest differences in expression levels are listed for the comparisons of EC-t15 and EC-t0 with V-t15.

A

	EC-t15 versus V-t15	EC-t0 versus V-t15	EC-t15 versus EC-t0
Increase (≥ 2-fold)	90	301	260
Decrease (≥ 2-fold)	33	254	289

B

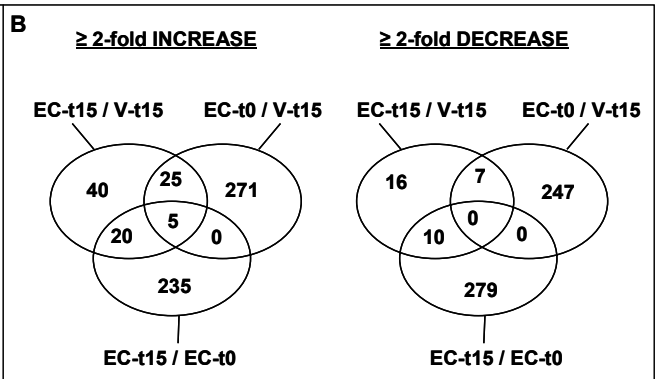

Figure 34 – Expression profile of Ba/F3-3C3-EC cells: outcome overview. (A) Numbers of genes with an at least two-fold change in expression level. (B) Distribution of at least two-fold changes among the different comparisons between the three samples EC-t15, EC-t0 and V-t15.

Table 5 – Genes with highest increase in expression level in Ba/F3-3C3 cells 15 hours after EWSR1/CIZ induction when compared to vector-transduced cells. (Changes of genes indicated with an arrow were tested by qRT-PCR).

Gene Symbol	Gene Title	Fold Change	RefSeq ID
Spic ←	Spi-C transcription factor (Spi-1/PU.1 related)	891	NM_011461
Tm4sf3 ←	transmembrane 4 superfamily member 3	274	NM_146010
Itgb3 ←	integrin beta 3	34	NM_016780
Calml4 ←	calmodulin-like 4	13	NM_138304
BC003324	cDNA sequence BC003324	11	NM_030259
Mmp8 ←	matrix metalloproteinase 8	10	NM_008611
Atp6v0a1 ←	ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	9	NM_016920
Il2ra	interleukin 2 receptor, alpha chain	8	NM_008367
Mc1r	melanocortin 1 receptor	8	NM_008559
Sfrp1	secreted frizzled-related sequence protein 1	7	NM_013834
Pcdh15	protocadherin 15	6	NM_023115
Itpkb	inositol 1,4,5-trisphosphate 3-kinase B	6	---
Tcf7l2	transcription factor 7-like 2, T-cell specific, HMG-box	5	NM_009333
Eif2ak3	eukaryotic translation initiation factor 2 alpha kinase 3	5	NM_010121
Gzmb	granzyme B	5	NM_013542
Tnfrsf9 ←	tumor necrosis factor receptor superfamily, member 9	5	NM_011612
Papss2	3'-phosphoadenosine 5'-phosphosulfate synthase 2	5	NM_011864
Usp18	ubiquitin specific protease 18	5	NM_011909
Tnfsf4	tumor necrosis factor (ligand) superfamily, member 4	5	NM_009452
Rrad	Ras-related associated with diabetes	4	NM_019662

Table 6 - Genes with highest decrease in expression level in Ba/F3-3C3 cells 15 hours after EWSR1/CIZ induction when compared to vector-transduced cells. (Changes of genes indicated with an arrow were tested by qRT-PCR).

Gene Symbol	Gene Title	Fold Change	RefSeq ID
B3galt2 ←	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	-26	NM_020025
Ccl9	chemokine (C-C motif) ligand 9	-5	NM_011338
C1s	complement component 1, s subcomponent	-5	NM_144938
Stam2 ←	signal transducing adaptor molecule (SH3 domain and ITAM motif)	-5	NM_019667
Ccr1	chemokine (C-C motif) receptor 1	-3	NM_009912
Aldh1a7	aldehyde dehydrogenase family 1, subfamily A7	-3	NM_011921
Olr1	oxidized low density lipoprotein (lectin-like) receptor 1	-3	NM_138648
S100a6	S100 calcium binding protein A6 (calcyclin)	-3	NM_011313
0610009D07Rik	RIKEN cDNA 0610009D07 gene	-3	NM_025323
Brunol4	bruno-like 4, RNA binding protein (Drosophila)	-2	NM_133195
Nebl	nebulette	-2	---
Socs3 ←	suppressor of cytokine signaling 3	-2	NM_007707
Mmp19	matrix metalloproteinase 19	-2	NM_021412
Prg2	proteoglycan 2, bone marrow	-2	NM_008920
Mylk	myosin, light polypeptide kinase	-2	NM_139300
Igfbp4 ←	insulin-like growth factor binding protein 4	-2	NM_010517
Ssx2ip	synovial sarcoma, X breakpoint 2 interacting protein	-2	NM_138744
F13a	coagulation factor XIII, alpha subunit	-2	NM_028784
Ly75	lymphocyte antigen 75	-2	NM_013825
Bace	beta-site APP cleaving enzyme	-2	NM_011792

Table 7 - Genes with highest increase in expression level in Ba/F3-3C3 cells with leaky EWSR1/CIZ expression when compared to vector-transduced cells. (Changes of genes indicated with an arrow were tested by qRT-PCR).

Gene Symbol	Gene Title	Fold Change	RefSeq ID
Spic ←	Spi-C transcription factor (Spi-1/PU.1 related)	630	NM_011461
Tm4sf3 ←	transmembrane 4 superfamily member 3	256	NM_146010
Acvr1	activin A receptor, type 1	23	NM_007394
Mmp8 ←	matrix metalloproteinase 8	20	NM_008611
B230208H17Rik	RIKEN cDNA B230208H17 gene	20	---
Spata6	spermatogenesis associated 6	12	NM_026470
Gzmb	granzyme B	9	NM_013542
Tgm2	transglutaminase 2, C polypeptide	8	NM_009373
Hbb-y	hemoglobin Y, beta-like embryonic chain	8	NM_008221
Lgals3	lectin, galactose binding, soluble 3	7	NM_010705
Hist1h1c	histone 1, H1c	6	NM_015786
Rnf128	ring finger protein 128	6	NM_023270
Greb1	gene regulated by estrogen in breast cancer protein 1	6	NM_015764
Hist1h2bc	histone 1, H2bc	5	NM_023422
H2-Ob	histocompatibility 2, O region beta locus	5	---
Cd14	CD14 antigen	5	NM_009841
BC003324	cDNA sequence BC003324	5	NM_030259
Cxcl4	chemokine (C-X-C motif) ligand 4	5	NM_019932
Pcyt1a	phosphate cytidylyltransferase 1, choline, alpha isoform	5	NM_009981
Atp6v0a1 ←	ATPase, H+ transporting, lysosomal V0 subunit a isoform 1	5	NM_016920

Table 8 - Genes with highest decrease in expression level in Ba/F3-3C3 cells with leaky EWSR1/CIZ expression when compared to vector-transduced cells. (Changes of genes indicated with an arrow were tested by qRT-PCR).

Gene Symbol	Gene Title	Fold Change	RefSeq ID
Emp1 ←	epithelial membrane protein 1	-119	NM_010128
Nupr1	nuclear protein 1	-23	NM_019738
Lmnb2	lamin B2	-8	NM_010722
---	Mm transcript with similarity to Hs protein enhancer of invasion 10	-6	---
Rbmx	RNA binding motif protein, X chromosome	-6	NM_011252
C1s	complement component 1, s subcomponent	-6	NM_144938
Brunol4	bruno-like 4, RNA binding protein (Drosophila)	-5	NM_133195
Zmynd19	RIKEN cDNA 2700064H14 gene	-4	NM_026021
Serpina3g	serine (or cysteine) proteinase inhibitor, clade A, member 3G	-4	---
Ung	uracil-DNA glycosylase	-4	NM_011677
Nek6	NIMA (never in mitosis gene a)-related expressed kinase 6	-4	NM_021606
Serpinb1a	serine (or cysteine) proteinase inhibitor, clade B, member 1a	-4	NM_025429
Dhfr	dihydrofolate reductase	-3	NM_010049
Srm	spermidine synthase	-3	NM_009272
Cdc6	cell division cycle 6 homolog (S. cerevisiae)	-3	NM_011799
Shmt1	serine hydroxymethyl transferase 1 (soluble)	-3	NM_009171
Ncl	nucleolin	-3	NM_010880
Gart	phosphoribosylglycinamide formyltransferase	-3	NM_010256
Socs3 ←	suppressor of cytokine signaling 3	-3	NM_007707
Pim2	proviral integration site 2	-3	NM_138606

C. qRT-PCR validation

Thirteen genes (nine upregulated, four downregulated) were selected for PCR validation of the microarray result. They are listed in table 9. Selection was based on high changes in expression level or because of involvement in hematopoiesis, cancer or special relevance in the context of CIZ investigations.

The Ba/F3-3D2 TetOff cell line, transduced with either EWSR1/CIZ (EC) or the empty vector (V), was used for the generation of biologically independent samples; cells were harvested just before removal of tetracycline (EC-t0) or 24 hours thereafter (EC-t24, V-t24).

For 8/9 of the upregulated and 2/4 of the downregulated genes, the change in expression was confirmed by qRT-PCR. Figure 35 shows EWSR1/CIZ expression in Ba/F3-3D2 cells and qRT-PCR results for seven of these genes.

Table 9 – Genes selected for PCR-validation of the microarray result. The fold change obtained by qRT-PCR in a biologically independent sample (Ba/F3-3D2-EC-t24 compared to V-t24) is shown.

Gene symbol	Gene name	Change	Fold ECt15/Vt15	Fold ECt0/Vt15	qRT-PCR
SpiC	Spi-family member C	UP	891	630	10644
Tm4sf3	Transmembrane 4 superfamily 3	UP	274	256	-
Itgb3	Integrin beta 3	UP	34	-	21
Calml4	Calmodulin-like 4	UP	13	2	50
Mmp8	Matrix metalloproteinase 8	UP	10	20	18
Atp6v0a1	ATPase, H+ transporting, lysosomal V0, subunit a, isoform 1	UP	9	5	9
Tnfrsf9	Tumor necrosis factor receptor superfamily, member 9	UP	5	2	13
Emp1	Epithelial membrane protein 1	UP	3	-119	8
IL6	Interleukin 6	UP	2	4	2
Igfbp4	Insulin-like growth factor binding protein 4	DOWN	-2	-2	-5
Socs3	Suppressor of cytokine signaling 3	DOWN	-2	-3	-2
Stam2	Signal transducing adaptor molecule 2	DOWN	-5	-	-
B3galt2	UDP-Gal:bGlcNAc b1,3-galactosyltransferase polypeptide 2	DOWN	-26	-	-

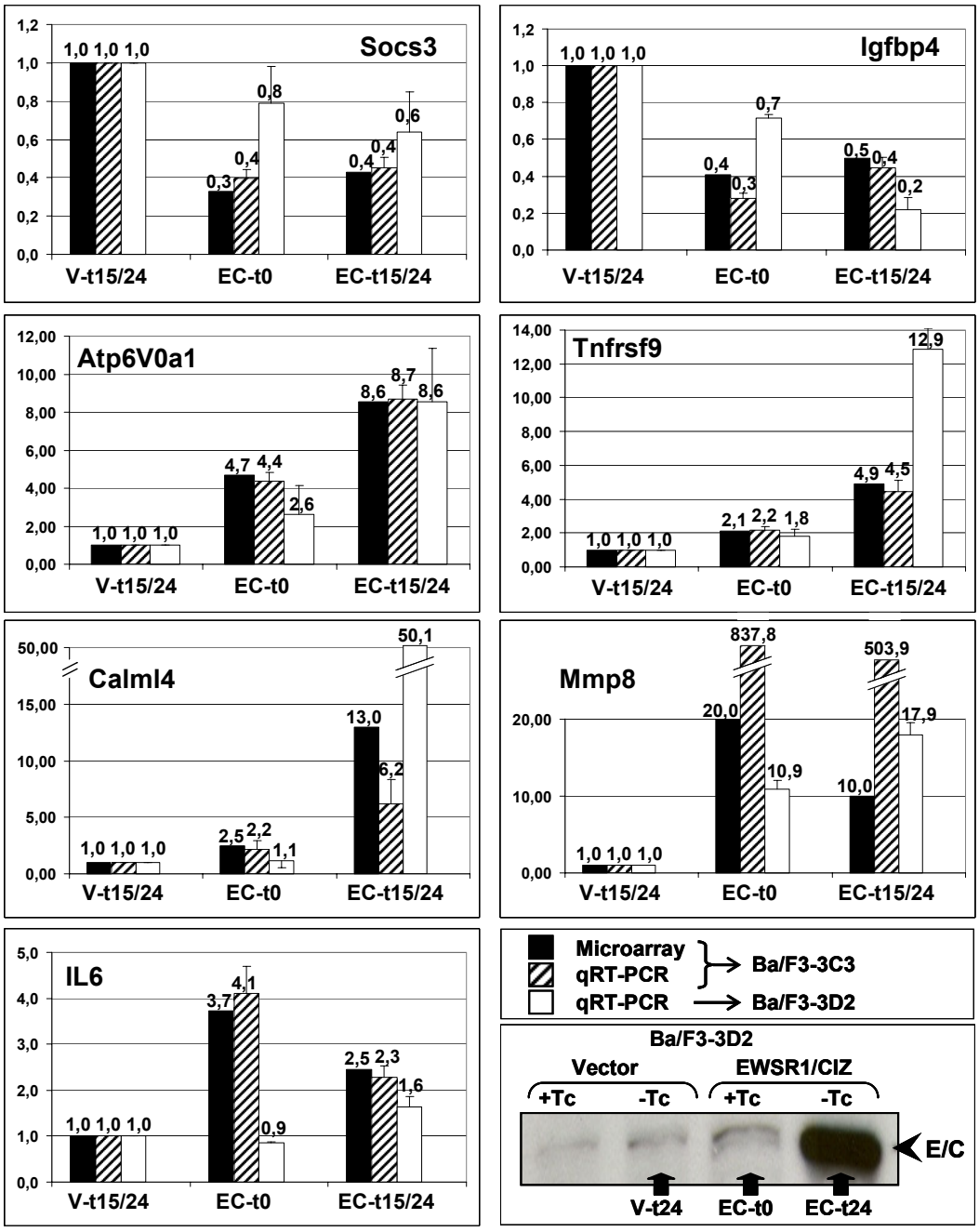

Figure 35 - **qRT-PCR confirmation of the microarray result.** The microarray result was confirmed by qRT-PCR in the original and biologically independent samples. Black bars represent original expression levels obtained by microarray analysis in samples Ba/F3-3C3-V-t15, EC-t0 and EC-t15. Shaded bars indicate qRT-PCR results for the same samples. White bars were obtained by qRT-PCR in the biologically independent samples Ba/F3-3D2-V-t24, EC-t0 and EC-t24. Expression levels were normalized to *Gapdh* and *ßActin*. Each point was performed in triplicate. Error bars represent standard deviations. Bottom right: Western analysis of Ba/F3-3D2 transduced with empty vector or EWSR1/CIZ, in presence of tetracyclin (+Tc) and 24 hours after its removal (-Tc). Leaky EWSR1/CIZ expression is visible in Ba/F3-3D2-EC before Tc removal.

D. Functional profiling of target genes

The microarray analysis results in a list of genes whose transcription is induced or suppressed as a consequence of EWSR1/CIZ expression. Translation of this list into a better understanding of the biological phenomena underlying the effects of EWSR1/CIZ on the cells was performed using OntoExpress software, based on the Gene Ontology nomenclature.

Many databases exist that contain a wealth of information about genes and proteins. Gene Ontology (GO) is a system to organize and query this biological knowledge. It is a universal vocabulary providing terms for all possible roles a gene or protein can exert at three different biological levels: biological process, molecular function and cellular component [Ashburner et al., 2000]. 'Biological process' refers to the biological objective to which the gene or protein contributes, 'molecular function' is defined as its biochemical activity and 'cell compartment' describes the place in the cell were the gene product is active. OntoExpress is an open-access software tool that uses this GO classification to translate lists of differentially expressed genes into functional profiles [Draghici et al., 2003]. For each of the three GO categories, it indicates which functional terms are significantly overrepresented among the group of differentially expressed genes.

The list of 123 genes found to be at least two-fold up- or downregulated 15 hours after EWSR1/CIZ induction as compared to the empty-vector sample, was submitted to OntoExpress using the initial pool of 14,000 genes of the GeneChip® Mouse Genome 430A 2.0 Array as a reference set. We concentrated on those functional categories significant at 5% (p-value < 0.05) and represented by two or more genes. These functional categories are presented in figure 36 for GO classes 'molecular function' and 'biological process'. Several GO terms related to cancer, hematopoiesis, communication between the cell and its environment and splicing appeared to be overrepresented among the differentially expressed genes.

A

Molecular function

p-value	# genes	GO term
0,00004	18	transferase activity
0,00265	13	receptor activity
0,01122	12	ATP binding
0,00732	12	hydrolase activity
0,00170	10	kinase activity
0,00667	7	zinc ion binding
0,01920	6	calcium ion binding
0,00001	5	cytokine activity(3)
0,01201	5	catalytic activity
0,00035	5	glycosyl transferase activity
0,00170	4	sugar binding
0,03681	3	peptidase activity
0,00026	3	hydrogen ion transporter activity
0,03535	3	rhodopsin-like recpetor activity
0,00000	3	sialyltransferase activity
0,00000	2	ATP-gated cation channel activity
0,02466	2	calmodulin binding
0,02250	2	chymotrypsin activity
0,00888	2	cysteine-type peptidase activity
0,00000	2	GTP cyclohydrolase I activity
0,02635	2	trypsin activity
0,00266	2	purinergic nucleotide recpetor activity
0,00003	2	water channel activity
0,00152	2	pre-mRNA splicing factor activity(4)
0,00198	2	neuropeptide Y receptor activity
0,00888	2	metalloendopeptidase activity
0,00113	2	chemokine activity
0,01579	2	cysteine-type endopeptidase activity
0,00001	2	C-C chemokine receptor activity
0,02578	2	metallopeptidase activity
0,00068	2	interleukin receptor activity(3)
0,03572	2	growth factor activity(1)

B

Biological Process

p-value	# genes	GO term
0,00248	7	proteolysis and peptidolysis
0,00014	6	immune response
0,03704	5	signal transduction(3)
0,01364	5	intracellular signaling cascade(3)
0,00873	3	defense response
0,03781	3	cell growth and/or maintenance(1)
0,00572	3	heterophilic cell adhesion(3)
0,02409	3	small GTPase mediated signal transduction
0,00131	3	cell surface receptor linked signal transduction(3)
0,00036	3	regulation of cell growth(1)
0,00023	3	anti-apoptosis(1)
0,00000	3	negative regulation of T-cell proliferation(2)
0,00008	3	protein amino acid glycosylation
0,00032	3	proton transport
0,00252	2	biosynthesis(1)
0,01144	2	inflammatory response
0,02751	2	cytoskeleton organization and biogenesis(3)
0,00677	2	integrin-mediated signaling pathway(3)
0,01180	2	chemotaxis
0,00403	2	cell-matrix adhesion(3)
0,00000	2	immune cell chemotaxis
0,00032	2	mRNA splice site selection(4)
0,00000	2	tetrahydrobiopterin biosynthesis
0,00051	2	lymph gland development
0,03307	2	protein ubiquitination
0,00005	2	water transport
0,00000	2	myeloid blood cell differentiation(2)
0,00000	2	T-cell homeostasis(2)
0,00005	2	collagen catabolism

Figure 36 – Functional categories significantly ($p < 0.05$) altered by induction of EWSR1/CIZ expression in Ba/F3-3C3 TetOff cells. (A) GO class 'molecular function'. (B) GO class 'biological process'. Terms associated with cancer (1), hematopoiesis (2), signaling from the extracellular matrix towards the nucleus (3) and RNA-splicing (4) are indicated.

E. Extension to EWSR1/CIZ-related proteins and other experimental systems

To investigate whether CIZ and other CIZ-fusions have a comparable impact on transcription, wild-type Ba/F3 cells were transduced with CIZ, TAF15/CIZ, EWSR1/CIZ, VP16/CIZ or TAF15/CIZ-ΔZN. Expression of several EWSR1/CIZ targets was then verified by qRT-PCR (Figure 37).

For EWSR1/CIZ, the microarray result was again confirmed in these cells. This allows to exclude influences of the TetOff-system itself on transcription.

CIZ and TAF15/CIZ-ΔZN did not induce any changes in transcription levels (except for the effect of CIZ on *Calml4*). This indicates that in general, both DNA-binding of the zinc finger domain and addition of the extra TET-activation domain are necessary for the effects of EWSR1/CIZ as observed in the microarray analysis. It is also in agreement with previous experiments, where the effect of inducible CIZ

overexpression in Ba/F3-TetOff cells was investigated using microarray technology (Martini, 2004). An effect of CIZ was seen on a number of genes mainly involved in inflammatory processes or the immune response (*Gzmb*, *Tgm2*, *Serpinb1a*, *Prg2* and others), but there was no overlap with the list of genes altered by EWSR1/CIZ.
While VP16/CIZ has the same effect on transcription as EWSR1/CIZ (although for most genes less pronounced), TAF15/CIZ has no or only weak influence. TAF15/CIZ and VP16/CIZ expression was also much weaker than that of EWSR1/CIZ (figure 37).

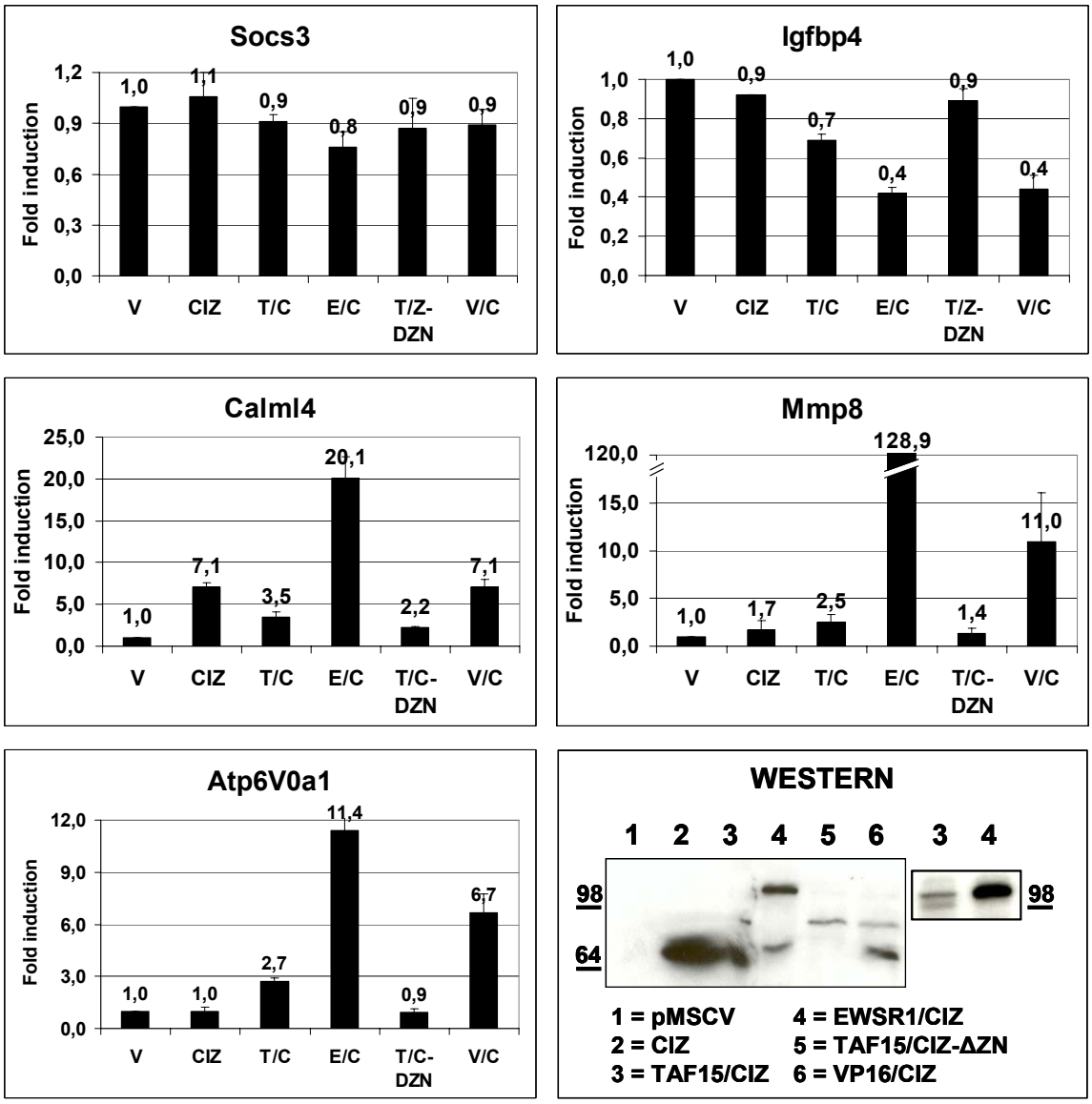

Figure 37 – Effect of CIZ and several CIZ-fusions on transcription of EWSR1/CIZ target genes in Ba/F3 cells. Results of qRT-PCR are shown for *Socs3*, *Igfbp4*, *CalmI4*, *Mmp8* and *Atp6v0a1*. Expression levels were normalized to *Gapdh* and *ßActin*. Each point was performed in triplicate. Error bars represent standard deviations. Bottom right: Western analysis of construct expression.

Expression of the same genes was also verified in bone marrow and spleen of diseased mice after secondary transplantation with TAF15/CIZ- or EWSR1/CIZ-transduced bone marrow. Cells of a similar experiment with a different fusion protein MOZ/TIF2, which also induces AML in mice [Deguchi et al., 2003], were used to compare expression levels. Results are shown in figure 38.

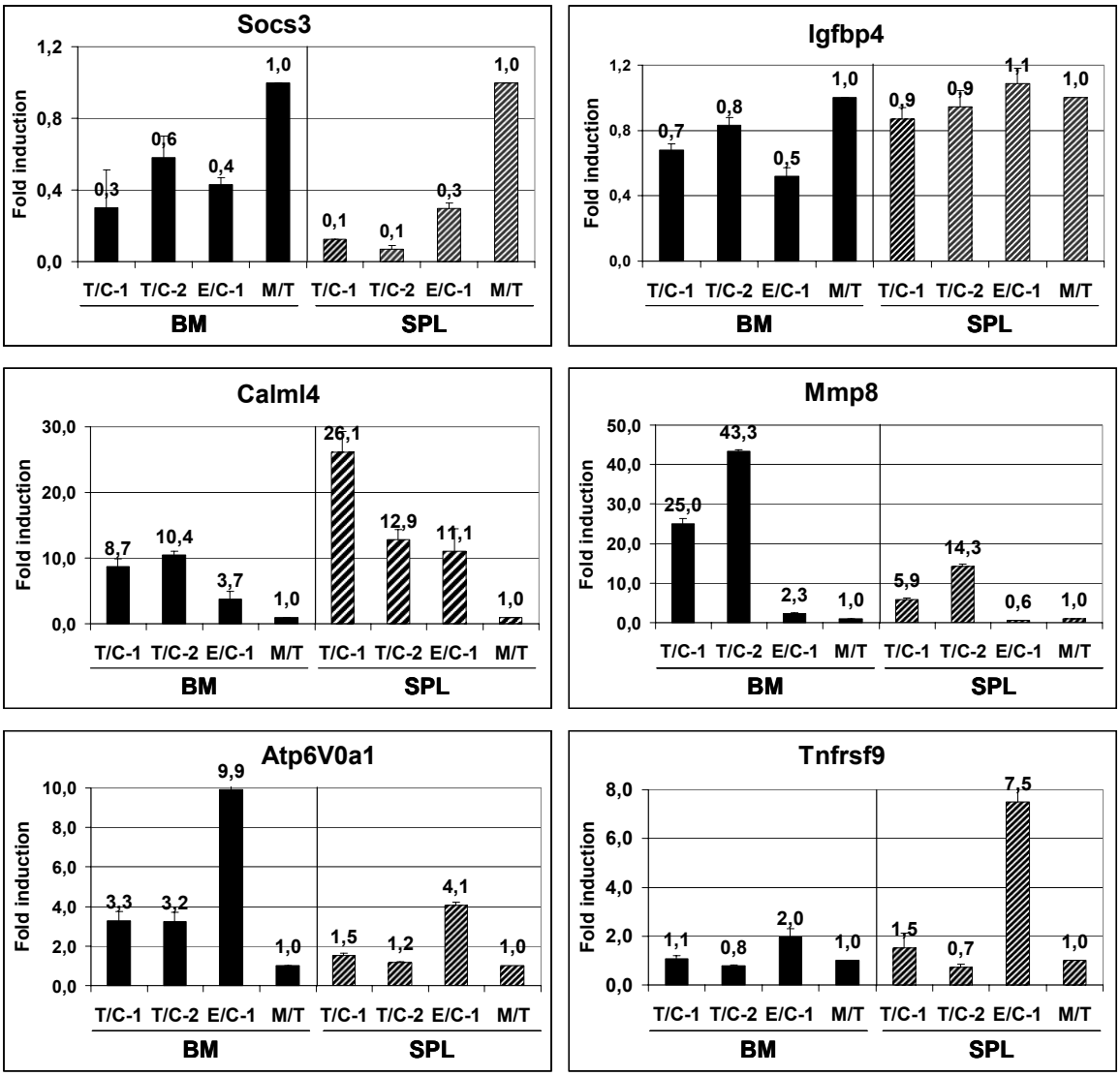

Figure 38 – **Expression of transcriptional targets in secondary bone marrow transplantation mice**. Bone marrow (BM) and spleen (SPL) cells of two TAF15/CIZ (T/C-1, T/C-2) mice and one EWSR1/CIZ (E/C-1) mouse were used. MOZ/TIF2 (M/T) transplanted animals were used as an independent control. Expression levels were normalized to *Gapdh* and *ßActin*. Each point was performed in triplicate. Error bars represent standard deviations.

For all genes tested, expression was higher (*Calml4, Mmp8, Atp6v0a1, Tnfrsf9*) or lower (*Socs3, Igfbp4*) than in the cells of the MOZ/TIF2 mouse, corresponding to an up- or downregulation in the microarray experiment, respectively. The effects on *Igfbp4* in spleen and *Tnfrsf9* in bone marrow were less pronounced. In contrast to the results obtained in the Ba/F3 cells (figure 37), the effect of TAF15/CIZ is for some genes even stronger than the effect of EWSR1/CIZ in the secondary transplant animals. AML appeared also earlier in the TAF15/CIZ than in the EWSR1/CIZ mice.

F. SPI-C: a direct transcriptional target of EWSR1/CIZ

Spi-C is a EWSR1/CIZ target of special interest, not only because of its highest fold induction after both leaky and strong EWSR1/CIZ expression, but also because of its relationship with important hematopoietic regulator proteins. We first confirmed the transcriptional induction of Spi-C by EWSR1/CIZ and analyzed the effect of CIZ and other CIZ-fusion proteins in several experimental systems. Then we investigated whether SPI-C is a direct transcriptional target of the CIZ-fusions in a promoter reporter assay.

First, transcriptional upregulation of Spi-C after induction of EWSR1/CIZ was confirmed by RT-PCR in both the microarray sample (Ba/F3-3C3) and in a biologically independent sample (Ba/F3-3D2). Since expression of Spi-C is turned on rather than only increased upon expression of EWSR1/CIZ, regular RT-PCR was preferred for the validation (figure 39).

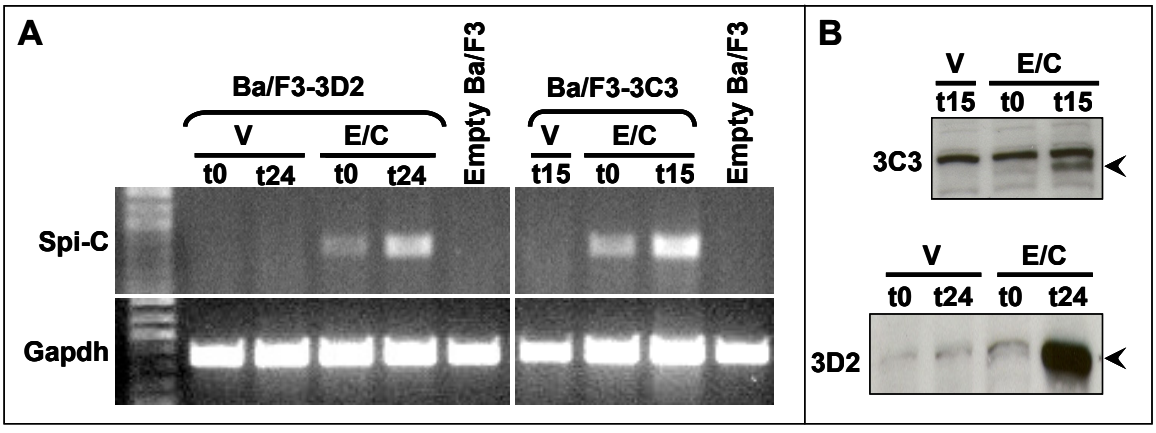

Figure 39 – **RT-PCR confirmation of the microarray result for Spi-C**. (A) RT-PCR for Spi-C and Gapdh in Ba/F3-3C3 and Ba/F3-3D2 cells transduced with EWSR1/CIZ or the empty vector, before (t0) or after (t15, t24) removal of tetracyclin. (B) Western analysis of EWSR1/CIZ expression.

Then, transcriptional upregulation of *Spi-C* was shown upon EWSR1/CIZ or VP16/CIZ expression in Ba/F3, HEK293T (human, embryonic kidney) and LG (mouse, myeloid) cell lines (figure 40). In spite of its low expression level (cfr. figure 37), TAF15/CIZ also induces *Spi-C* expression in Ba/F3 cells. Interestingly, more similarity between the cell line used for the experiment and the t(12;17)-t(12;22) leukemia samples implies a more pronounced effect of the different fusions on induction of Spi-C: in the pro-B Ba/F3 cells, which correspond best to the leukemia samples, all three CIZ-fusions induce expression of Spi-C. In the myeloid LG cell line, an effect of TAF15/CIZ is not visible anymore, while EWSR1/CIZ and VP16/CIZ have less impact than in the Ba/F3 cells. In the embryonical kidney cells (HEK293T), the effects of EWSR1/CIZ and VP16/CIZ are even lower.

In cells of the murine bone marrow colony forming assay, transcription of Spi-C was elevated in the VP16/CIZ-transduced cells (figure 41A). In bone marrow cells of the secondary bone marrow transplantation mice, *Spi-C* showed a higher expression level in the EWSR1/CIZ animal than in the TAF15/CIZ-animals or the BCR/ABL and MOZ/TIF2 mice (figure 41B), although its expression was still lower than in control bone marrow. In spleen cells of the transplanted animals, the *Spi-C* expression level was indistinguishable from the level in the MOZ/TIF2-mouse. In bone marrow cells of patients carrying a translocation t(12;17) or t(12;22), SPI-C expression was lower than in five independent ALL-samples (figure 41C).

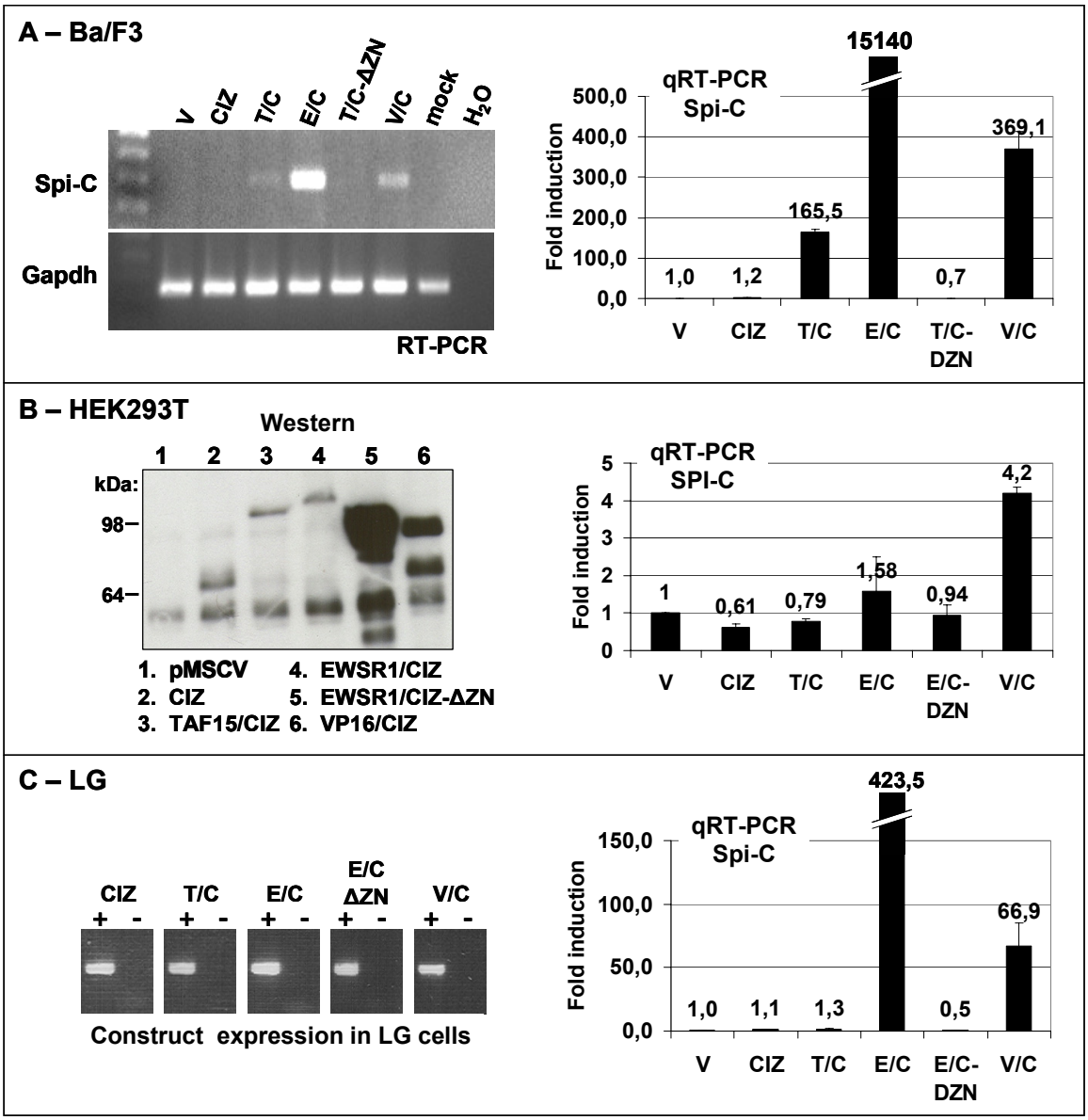

Figure 40 - **Effect of CIZ and CIZ-fusions on *Spi-C* expression.** (A) Stably transduced Ba/F3 cells. Left: RT-PCR for *Spi-C* and *Gapdh*; right: qRT-PCR for *Spi-C*. Expression of constructs in these Ba/F3 cells was shown in figure 37. (B) Transiently transfected HEK293T cells. Left: Western analysis of construct expression; right: qRT-PCR for *SPI-C*. (C) Stably transduced LG cells. Left: RT-PCR to verify construct expression; right: qRT-PCR for *Spi-C*. V: empty vector; T/C: TAF15/CIZ; E/C: EWSR1/CIZ; T/C-ΔZN: TAF15/CIZ-ΔZN; V/C: VP16/CIZ. For qRT-PCRs, expression levels were normalized to *Gapdh* and *ßActin*. Each point was performed in triplicate. Error bars represent standard deviations.

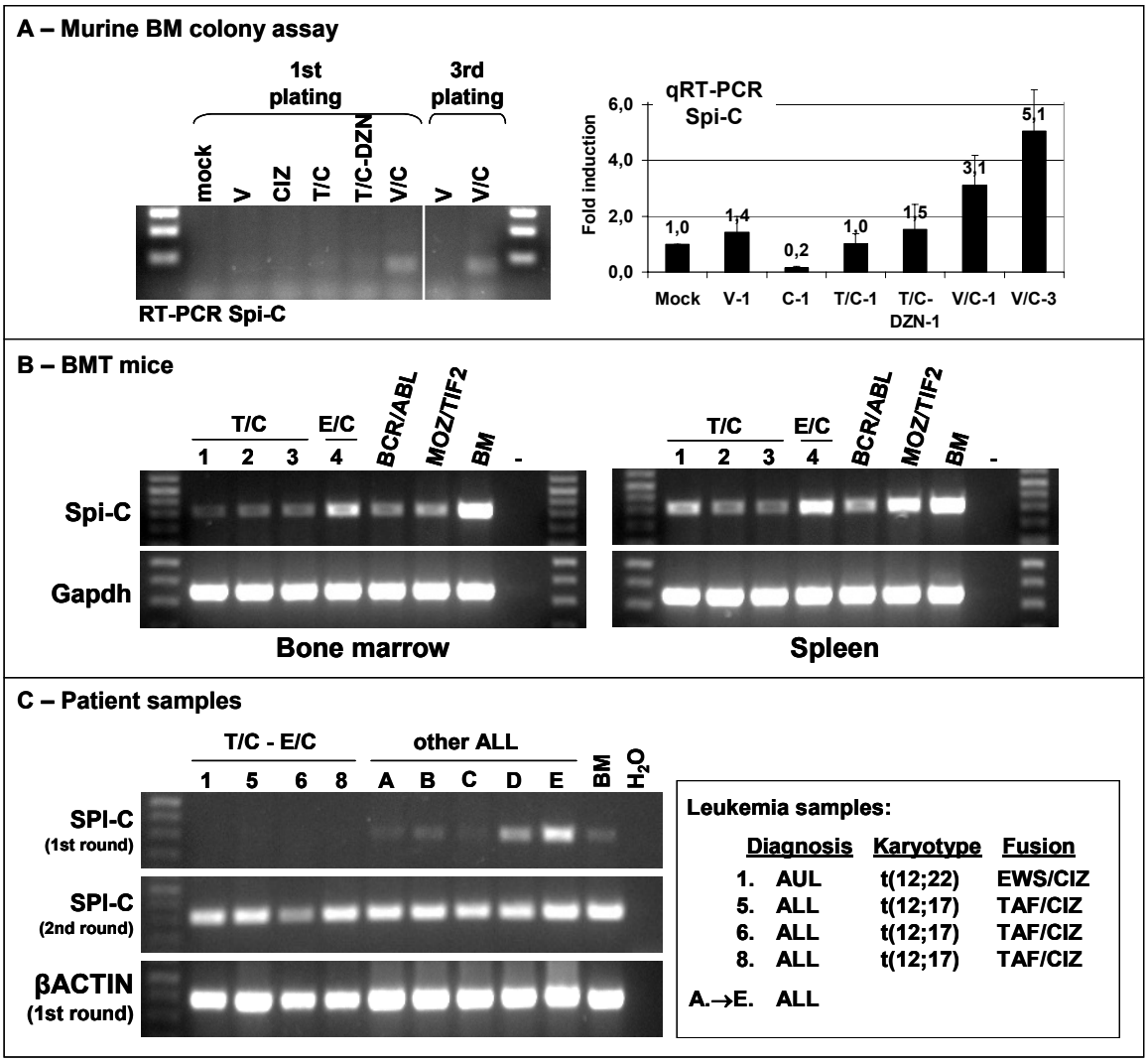

Figure 41 – **RT-PCR analysis of *Spi-C* expression.** (A) In cells of the murine hematopoietic immortalization assay (cfr chapter 4.1). V: empty vector; T/C: TAF15/CIZ; T/C-ΔZN: TAF15/CIZ-ΔZN; V/C: VP16/CIZ. Expression levels were normalized to *Gapdh* (not shown for the regular RT-PCR). For the qRT-PCR, each point was performed in quadruplicate. Error bars represent standard deviations. (B) In bone marrow and spleen cells of secondary bone marrow transplanted mice. Three TAF15/CIZ- (1, 2, 3) and one EWSR1/CIZ- (4) transplanted animal were used. BCR/ABL- and MOZ/TIF2-animals were used as independent AML-mice to compare expression. (C) In bone marrow of four patients (1, 5, 6, 8) carrying TAF15/CIZ or EWSR1/CIZ. Cells of five other, independent ALL-patients were used as an independent control.

To verify whether SPI-C is a direct transcriptional target of EWSR1/CIZ, a luciferase reporter assay was performed to investigate the responsiveness of the human SPI-C promoter to CIZ and several CIZ-fusion proteins. A fragment of 4.5 kb immediately upstream of the *SPI-C* transcription start site was used as a promoter. This fragment

contains 40 CIZ consensus binding sites, which is four times the amount to be expected by coincidence. As shown in figure 42, overexpression of EWSR1/CIZ and VP16/CIZ stimulated luciferase expression up to approximately two-fold, indicating a direct transcription inducing effect of the fusion proteins on SPI-C.

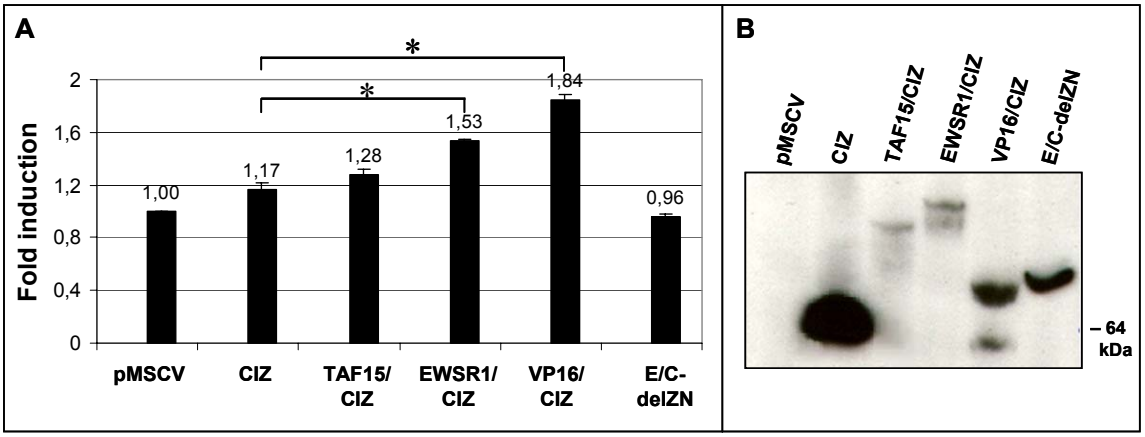

Figure 42 - **Analysis of human *SPI-C* promoter activation by CIZ and different CIZ-fusions**. (A) Luciferase reporter assay. HEK293T cells were cotransfected with a luciferase reporter construct containing 4.5 kb of the human SPI-C proximal promoter, one of the expression plasmids and a β-galactosidase plasmid. Luciferase values were normalized for β-galactosidase activity and the fold induction was calculated as compared to the empty pMSCV sample. Means of three experiments are shown, error bars represent standard deviations. Differences between samples were analyzed with a one-tailed Student's t-test. The cut-off p-value was set at 0.01 and significant differences were indicated with an asterisk (*). (B) Western blot to verify construct expression.

4.3.4 Discussion

Two mechanisms are proposed for cell transformation by TET fusion proteins: they can act as aberrant transcription factors, or they can disturb normal TET-regulated splicing. Several pieces of experimental evidence suggest that TAF15/CIZ and EWSR1/CIZ achieve their oncogenic properties at least in part by acting as aberrant transcription factors. First, the TET/CIZ-fusions still bind to the same DNA consensus as the original transcription factor CIZ, as shown by the EMSA assay. Secondly, experiments with deletion constructs revealed that this DNA-binding capacity is necessary for cell transformation [Corveleyn et al., 2005]. Third, fusion of CIZ to other activation domains (VP16, E2A) resulted in the same cellular phenotype as fusion to 5'-TET, indicating that equipping CIZ with an extra activation domain is important, rather than separating the N- and C-terminal parts of the TET-protein. Moreover, fusion of these different activation domains effectively increased the transcription activation potential of the proteins. Finally, an effect of EWSR1/CIZ on splice site selection was investigated, but not found.

We searched for potential transcriptional target genes of the TET/CIZ-fusions. As shown by RT-PCR and TRAP assays, known transcriptional targets of neither CIZ nor EWSR1/FLI1 were transcriptionally upregulated upon overexpression of TAF15/CIZ or EWSR1/CIZ. To be sure that presence of the myc-epitope had not influenced the outcome of the EMSA assay, the tag was removed and the experiment was repeated, with the same result. Failure to confirm the activation of already described target genes by the TET/CIZ-fusions can be explained in several ways. The cell lines that were used might lack some cofactors that are necessary for transcription, the expression level of the fusion proteins might be too low because of cytotoxicity, target specificity is determined by the transcription factor moiety (CIZ, FLI1) and not the TET-part of the fusion, or the genes tested might simply be not the true TET/CIZ targets.

To broaden our search, we performed a general transcriptional profiling of Ba/F3 cells with stable but inducible EWSR1/CIZ-expression. Given the leaky expression of the construct in the presence of tetracycline, three samples were compared: one without EWSR1/CIZ, one with relatively strong, induced expression and one with an intermediate EWSR1/CIZ-level. This allows subdivision of the genes according to their response to EWSR1/CIZ expression. For some genes an effect on transcription is already visible in the presence of low EWSR1/CIZ levels and is further enhanced upon increase of its expression (e.g. *Spi-C, Atp6v0a1, Calml4, Tnfrsf9*). Some genes

react equally to weak or strong EWSR1/CIZ expression (e.g. *Socs3, Igfbp4*) or require strong EWSR1/CIZ expression before any effect is visible (e.g. *Itgb3*). Finally, for some genes an effect on transcription is present at low EWSR1/CIZ-levels, but is less strong or even reversed when EWSR1/CIZ expression increases (e.g. *IL6, Emp1*). This could be explained by the induction of negative feedback loops upon excess of a EWSR1/CIZ threshold expression level.

A total of 123 genes had an at least two-fold change in transcription after EWSR1/CIZ induction when compared to the empty vector cells. A global functional profiling of this gene set was performed with Onto-Express. It is interesting to note that the results of this analysis include a number of GO-terms known to be associated with cancer and hematopoiesis, like cell growth and/or maintenance, anti-apoptosis and myeloid blood cell differentiation. The analysis also showed that EWSR1/CIZ has an influence on several cell functions involved in the communication between the cell and its environment (e.g. cell-matrix adhesion, interleukin receptor activity) and the subsequent signal transduction toward the nucleus (e.g. cell surface receptor linked signal transduction, integrin-mediated signaling pathway). This fits in the hypothesis that fusion of CIZ to a TET-protein interferes with its function as an ECM signal communicator. Remarkably, EWSR1/CIZ expression also affects some splicing-related functional classes (mRNA splice site selection, pre-mRNA splicing factor activity), referring to the possibility that TET-fusions might interfere with normal splicing.

In the promoters of the same set of 123 genes, the presence of conserved CIZ binding sites was not significantly increased when compared to a random gene set of the same size. However, for 10 of the 13 genes that were selected for qRT-PCR validation of the experiment, changes in expression levels were confirmed. The genes were chosen based on high differences in expression level or interesting functions in the context of CIZ and leukemia.

Spi-C is of particular interest. Together with PU.1 and Spi-B, it constitutes the SPI-subfamily of ETS transcription factors. It is exclusively expressed in specific stages of B-cell development (pre- and mature B-cells) and weakly in macrophages [Bemark et al., 1999], [Carlsson et al., 2002], [Carlsson et al., 2003]. Although its own specific function has not yet been unraveled, it shows strong homology to PU.1, the founding member of the family: Spi-C has the same domain structure and binds to exactly the same DNA-consensus as PU.1.

PU.1 (PU-box binding protein 1) or Spi-1 (SFFV provirus integration site 1) is indispensable for hematopoietic differentiation and is involved in both lineage commitment and cell survival and proliferation. PU.1$^{-/-}$ mice are not viable. They lack normal development of B- and T-cells, neutrophils and macrophages [Scott et al., 1994], [McKercher et al., 1996]. In normal circumstances, low PU.1-levels induce lymphoid differentiation of hematopoietic stem cells (HSC), while high PU.1-levels direct stem cells towards the myeloid lineage [DeKoter and Singh, 2000]. Aberrant PU.1 expression has been shown to cause poor stem cell differentiation and accumulation of an abnormal, pre-leukemic precursor pool, which results in full AML upon further mutations [Rosenbauer et al., 2004]. Important PU.1 interaction partners are GATA-1 and c-JUN. Among its transcriptional targets are a number of growth factors (G-, M-, GM-CSF), cytokines, kinases and proto-oncogenes (c-rel, c-fes).
In the mouse pro-B Ba/F3 cell line, Spi-C expression is switched on upon induction of EWSR1/CIZ expression. A promoter-reporter assay showed that it is likely to be a direct transcriptional target of EWSR1/CIZ. In the mouse bone marrow colony formation assay, only VP16/CIZ transduced cells showed a transformation phenotype. This was accompanied by induction of Spi-C expression. Spi-C expression was higher in normal bone marrow than in bone marrow of the transplanted mice. Likewise, it was higher in a number of randomly chosen ALL bone marrow samples than in bone marrow of the TET-CIZ patients. However, this is matter of PCR controls: it can not be concluded whether this represents upregulation of Spi-C or the expansion of a specific cell type in the 'control samples' that expresses Spi-C.
It is remarkable that Spi-C is an ETS transcription factor. Most oncogenic translocations involving TET-proteins generate TET/ETS chimeric proteins. Translocations t(12;17) and t(12;22) fuse TET-proteins to CIZ and the resulting TET/CIZ-fusions induce transcription of SPI-C. In this way, they indirectly bring about the same effect: increased ETS transcription factor activity.

Transcription of *Socs3* (suppressor of cytokine signaling 3) is downregulated by EWSR1/CIZ. **Socs3** binds to the activation loop of Janus kinases, inhibiting kinase activity and thereby suppressing cytokine signaling [Starr et al., 1997]. Interestingly, it selectively blocks signaling by **IL6** [Croker et al., 2003], which on its turn is upregulated in the Ba/F3-EWSR1/CIZ cells. IL6 or 'B-cell differentiation factor' is a monocyte derived B-cell growth factor involved in the immortalization of EBV-infected B-cells [Tosato et al., 1988]. Whether the changes for *Socs3* and *IL6* are directly caused by EWSR1/CIZ expression or whether *IL6* upregulation is a

consequence of *Socs3* downregulation, they both result in more IL6 signaling and stimulation of B-cells.

Mmp8 or 'neutrophil collagenase' is produced mainly by neutrophils and is involved in inflammatory reactions induced by carcinogens [Balbin et al., 2003]. It is a member of the family of matrix metalloproteinases. Mmp1 or 'fibroblast collagenase', an other MMP family member, is a known transcriptional target of CIZ. Mmp8 and Mmp1 differ in their substrate specificity: while Mmp1 degrades mainly type III collagen, Mmp8 prefers type I collagen. Its 11 kb proximal promoter contains 24 CIZ binding sites.

Calml4 was identified as a breast cancer antigen [Scanlan et al., 2001]. It belongs to the calmodulin family of Ca^{2+}-binding proteins. Binding of Ca^{2+} allows interaction with specific target proteins, which are then (in)activated and are involved in a broad range of cellular processes.

Igfbp4 contributes to the control of IGF-mediated cell growth and metabolism by attenuating IGF activity [Kiefer et al., 1992]. It is downregulated by EWSR1/CIZ.

Atp6v0a1 is a subunit of a vacuolar proton pomp or V-ATPase [Kawasaki-Nishi et al., 2003]. V-ATPases control the pH of intracellular compartments, a parameter that affects many cellular processes. Osteoclasts and some tumor cells are able to target V-ATPases to the plasma membrane were they create an acidic extracellular environment that is necessary for bone resorption or tumor metastasis [Forgac, 1999].

Tnfrsf9 is a member of the TNF receptor family. It is expressed by activated T- and B-lymphocytes and monocytes. It inhibits proliferation and induces programmed cell death [Schwarz et al., 1996].

Emp1 is a tumor marker, involved in cell/cell interactions and cell proliferation. In hematopoietic cells, its expression is restricted to a subpopulation of pro- and pre-B lymphocytes. Its transient expression specifically inhibits proliferation of these cells. The protein is suggested to be involved in the elimination of B cells before productive VDJ rearrangement of Ig loci or in the growth arrest of transformed progenitor B cells [Ruegg et al., 1996].

As a component of integrin-α5β3, **Itgb3** is involved in osteoclast differentiation, cell spreading and organization of cell-matrix adhesions [Nemeth et al., 2003].

Although many of these genes provide interesting links towards leukemogenesis, individual target genes are able to recapitulate only partially the full biology of EWSR1/CIZ cell transformation. It is more likely that the cellular phenotype is dictated by the summed effects of a whole network of modulated genes.

Our experiments indicate that TAF15/CIZ and VP16/CIZ share transcriptional targets with EWSR1/CIZ, while CIZ alone or fusion proteins without the DNA-binding zinc finger domain loose the effect on transcription. Although it is important to take into account differences in expression levels of the constructs in each individual experiment, it can be generally stated that the effects of VP16/CIZ and EWSR1/CIZ on transcription are comparable, and stronger than the effect of TAF15/CIZ. This is in agreement with the *in vitro* and *in vivo* transformation assays: EWSR1/CIZ induced a quicker and more extensive focus formation in NIH/3T3 cells than TAF15/CIZ and an earlier disease onset in mouse bone marrow transplantation experiments.

5 General discussion and perspectives

Translocations t(12;17)(p13;q11) and t(12;22)(p13;q12) generate the fusion proteins TAF15/CIZ and EWSR1/CIZ respectively, in the bone marrow of eight patients with acute leukemia. This extends the involvement of TET-fusion proteins to ALL and suggests a role for CIZ in hematopoiesis. The goal of the present study was to further characterize CIZ and its TET/CIZ fusion proteins.

TET/CIZ transformation properties were demonstrated by an *in vitro* focus formation assay as well as in the more sophisticated *in vivo* mouse bone marrow transplant model. BMT mice develop AML with a relatively long disease latency (3.5 months for the EWSR1/CIZ primary transplant animals). This corresponds with the moderate TET/CIZ transformation potential that was also implied by the negative soft agar and nude mice assays, and suggests the involvement of secondary hits in the initiation and/or maintenance of the disease. This need for collaborative events can be verified using bone marrow cells which already contain an initiating mutation, like p53-deficiency [Hann and Balmain, 2001]. Identification of TET/CIZ complementing mutations after disease manifestation in the BMT mice however, would be no sinecure. One could specifically investigate a number of known, frequent secondary aberrations in other TET-fusion associated diseases, like loss of p16 or mutations in p53 [Kovar et al., 1997], [Deneen and Denny, 2001]. For a more general approach, PCR-based retroviral tagging strategies would be suitable [Mikkers and Berns, 2003]. Finally, to further complete our view of the BMT model, the clonality of the disease and the number and sites of genomic integrations will be investigated by Southern analysis and vectorette PCR. This will probably also shed light on the difference between the disease types in mouse (AML) versus human (ALL).

General concepts emerging from the transformation analysis are (i) that fusion of the 5'-TET domains to CIZ results in chimeric proteins with a weak to moderate oncogenic potential, and (ii) that the cellular microenvironment is likely to play an essential role in TET/CIZ induced leukemia. The analysis also provided a read-out for further structure/function analysis, which indicated that the fusions exert their transforming capacities at least partially as aberrant transcription factors.

The microarray analysis that was subsequently performed to identify EWSR1/CIZ transcriptional target genes in murine pro-B Ba/F3 cells, brought in many interesting candidates with a significant change in expression level after induction of

EWSR1/CIZ. A major hurdle lies in determining the biological significance of individual target genes, since EWSR1/CIZ is unlikely to modulate only target genes that play active roles in cell transformation. So far, we put our major focus on Spi-C, because of its similarity with PU.1 and because altered activity of ETS transcription factors is a common theme in tumorigenesis by TET-fusion proteins. While the crucial role of PU.1 in hematopoiesis and its recurrent involvement in leukemia have been thoroughly investigated (reviewed by [Gangenahalli et al., 2005]), this is not yet the case for SPI-C. It would be interesting to examine its role in a mouse knock-out model, and to specifically investigate whether it mediates EWSR1/CIZ induced hematopoietic cell transformation by knocking down or increasing its expression in the available model systems. As a first step, we showed that it is a direct transcriptional target of EWSR1/CIZ.

A yeast two-hybrid screen was performed to identify CIZ protein interaction partners. While rat Ciz directly interacts with adaptor protein CAS, human CIZ counts one P-residue less in its proline rich region and is not able to interact with CAS. This gap however, is bridged via the interaction of both CIZ and CAS with zyxin. CAS is involved in integrin signaling. After binding of an integrin to an extracellular ligand, CAS assists in assembling a signal transducing protein complex at the cytoplasmic tail of the integrin receptor in focal adhesions. The direct or indirect interaction of CIZ with CAS thus suggests that CIZ is an important transporter and/or effector molecule of ECM signals. This is in agreement with (i) the importance of the cellular microenvironment that appears from the transformation assays, and (ii) the presence of ECM-related GO-categories (cell surface receptor linked signal transduction, integrin-mediated signaling pathway, and others) in the global functional analysis of the Ba/F3 transcription profiling experiment. Interactions via integrins of hematopoietic precursor cells with their microenvironment in the bone marrow determine cell fate to a large extent, and defects in the reading and/or processing of these signals can make the delicate hematopoietic equilibrium of proliferation versus differentiation go awry. After fusion of CIZ to the TET activation domains, both its protein-protein interaction capacities as well as its properties as a transcription factor are altered. As a consequence, ECM-signals might be dealt with differently in cells carrying the translocations.

An other starting point to link altered CIZ properties with leukemia, is the inhibitory effect of CIZ on BMP2/Smad1-5 signaling in osteoblasts [Shen et al., 2002], [Morinobu et al., 2005]. The same pathway is also involved in hematopoiesis, during embryonic development as well as in adult blood formation. Relatively little is known

about the *in vivo* role of BMPs in hematopoiesis, due to the early lethality of most BMP ligand or receptor knock out mice. However, the study of mouse embryos with a targeted disruption of Smad5 allowed Liu and colleagues to define a role for Smad5 in the negative regulation of high-proliferative potential precursors during embryonic hematopoiesis [Liu et al., 2003]. Bhatia and coworkers detected expression of BMP type I receptors and their downstream transducers SMAD1 and 5 in primitive human $CD34^{+}CD38^{-}Lin^{-}$ hematopoietic cells. Treatment with BMP2 of these cells induced dose-dependent changes in proliferation and cell surface phenotype [Bhatia et al., 1999]. A conditional mouse knock out of BMP type I receptor A (BMPRIA, this is part of the BMP2-receptor) revealed an important, yet indirect role for BMP-signaling in the regulation of hematopoietic stem cells by increasing the number of supporting osteoblast cells in the stem cell niche [Zhang et al., 2003]. Finally, overexpression of SMAD7 (which has been shown to block all SMAD-signaling pathways) in human common myeloid/lymphoid precursor cells, caused a shift in cell fate decisions by augmentation of myeloid differentiation at the expense of lymphoid commitment [Chadwick et al., 2005]. It would be worthwhile to investigate whether CIZ also influences BMP2/Smad1-5 signaling in hematopoiesis and whether fusion to TET activation domains might induce a leukemogenic alteration in its properties.

Finally, the oncogenic properties of TET-fusion proteins have also been associated with an effect on splicing, due to the separation of the N- and C- terminal domains of the TET-protein. Specifically in the case of the TET/CIZ-fusions, two extra arguments exist in support of this hypothesis. First, not only TAF15 and EWSR1, but also CIZ itself is involved in the coordination of transcription and posttranscriptional modification via its interaction with PCBP1. After fusion to the TET N-terminus, this interaction is disturbed. Secondly, as an aberrant transcription factor, EWSR1/CIZ also indirectly influences splicing as shown by its effect on several splicing-related GO-classes in the Ba/F3 transcription profiling experiment. Nevertheless, an effect of the TET/CIZ-fusions on splice site selection of CD44 or Ikaros could not be demonstrated. Also the invariable fusion of the TET activation domain to a transcription factor DNA binding domain suggests there must be more than interfering with splicing alone. Perhaps the recurrent generation of chimeric proteins combining components of TET-members with transcription factors provides a powerful mechanism to simultaneously disrupt normal transcription and posttranscriptional regulation. Yet the relative contribution of each remains to be determined.

The ultimate goal of our investigations would be to close the functional gap between cloning of the TET/CIZ fusion oncogenes and the elucidation of the corresponding leukemogenesis. Via several approaches, this study revealed more of the characteristics of both CIZ and the TET/CIZ fusions, and provided a number of concrete lines of thought for further research. Of the different oncogenic mechanisms that were suggested, none excludes the others. The final outcome of hematopoietic cell fate decisions is regulated by a complex crosstalk of many signaling pathways, transcription factors and cell cycle regulators, and deregulation of hematopoiesis is frequently the consequence of alterations in more than one pathway alone.

Summary

Chromosomal translocations are common genetic lesions in leukemia. Their characterization leads to insights into both normal and leukemic hematopoiesis. Transcription factor CIZ is involved in acute leukemia through recurrent translocations t(12;17)(p13;q11) and t(12;22)(p13;q12), which fuse CIZ to TET-family proteins TAF15 and EWSR1, respectively. CIZ is involved in bone metabolism and spermatogenesis. TET family proteins are involved in both transcription regulation and RNA processing. Recurrent fusion of their N-terminal activation domain to transcription factors has been described in – mostly solid – tumors, with the identity of the transcription factor defining the type of the tumor. Therefore, fusion to CIZ extends their oncogenic repertoire towards acute leukemias and suggests a role for CIZ in hematopoiesis too. To better understand leukemogenesis by TET/CIZ-fusions and the role of CIZ in normal hematopoiesis, these proteins were characterized by several approaches.

First, TET/CIZ transformation capacities were investigated. NIH/3T3 fibroblasts stably expressing the chimeric proteins clearly showed a transformed phenotype: both the density and the morphology of the cells were altered, with islands of small round cells appearing among cells with the normal, spindle-like fibroblast shape. In an *in vivo* mouse bone marrow transplant model, TET/CIZ-mice developed a long latency acute myeloid leukemia that was transplantable into secondary recipient mice. Other transformation assays were negative. These data suggest a moderate cell transformation potential for TET/CIZ fusions, and point out the importance of the cellular micro-environment for TET/CIZ-mediated oncogenesis.

The focus forming assay provided a read-out for further structure/function analysis, which indicated that the fusions are at least partially active as aberrant transcription factors. To determine EWSR1/CIZ transcriptional targets, a microarray analysis was performed in Ba/F3 cells. For 10 out of 13 genes that were chosen for qRT-PCR validation of the experiment, the change in expression was confirmed. A global functional profiling of the microarray result pointed out a number of molecular functions related to hematopoiesis/leukemia as well as to cellular communication with the microenvironment. A particularly interesting target was Spi-C, a transcription factor which is exclusively expressed in certain stages of B-cell development. It is a protein family member of PU.1, which is known to be crucially

involved in lymphoid development. As shown by a promoter-reporter analysis, Spi-C is a direct transcriptional target of EWSR1/CIZ.

Finally, CIZ protein interactions partners were identified using yeast two-hybrid technology. In contrast to its rat homolog, human CIZ does not interact with p130CAS. Zyxin, PCBP1 and vimentin however, were identified as CIZ binding partners. Given the interaction between human zyxin and p130CAS, these results suggest that zyxin indirectly enables the interaction of CIZ with p130CAS which is described in rat.

Taken together, this study sheds more light on the characteristics of both CIZ and the TET/CIZ fusion proteins, and provides a lead for further investigations to fully elucidate the oncogenic mechanisms of TET/CIZ induced leukemogenesis.

Samenvatting

Chromosomale translocaties zijn veel voorkomende genetische fouten in leukemie. Hun karakterisering leidt tot inzichten in zowel normale als leukemische hematopoiese. Transcriptiefactor CIZ is betrokken in acute leukemie via translocaties t(12;17)(p13;q11) en t(12;22)(p13;q12), die respectievelijk de fusieproteinen TAF15/CIZ en EWSR1/CIZ genereren. CIZ is belangrijk voor beenvorming en spermatogenese. TAF15 en EWSR1 zijn allebei eiwitten van de TET-familie. Zij spelen een rol in zowel transcriptie als posttranscriptionele modificatie van RNA. TET-eiwitten zijn veelvuldig betrokken in – meestal vaste – tumoren, via translocaties die hun N-terminaal activatie-domein fusioneren aan een transcriptiefactor. Het is deze transcriptiefactor die de aard van de tumor bepaalt. Fusie van TAF15 en EWSR1 aan CIZ breidt dus het repertorium van TET-tumoren uit naar acute leukemie, en suggereert ook een rol voor CIZ in hematopoiese. Om meer inzicht te verwerven in de moleculaire werkingsmechanismen van CIZ en de TET/CIZ-fusies, werden de eigenschappen van deze eiwitten onderzocht vanuit verschillende invalshoeken.

Eerst werden de transformatie-capaciteiten van de TET/CIZ-proteinen onder de loep genomen. NIH/3T3-fibroblasten die de fusies stabiel tot expressie brengen, vertoonden een duidelijk getransformeerd fenotype: zowel de celdensiteit als de vorm van de cellen waren gewijzigd, en eilandjes van kleine, ronde cellen verschenen tussen de normale, spoelvormige fibroblasten. In een *in vivo* muis beenmergtransplantatiemodel werd acute myeloide leukemie waargenomen. Andere transformatietesten waren negatief. Deze resultaten suggereren dat de TET/CIZ-fusies een matig transformerend vermogen bezitten, en wijzen op het belang van de juiste biologische context voor de cellen.

De focusvormingstest leverde een modelsysteem op dat gebruikt werd voor verdere structuur/functie-analyse. Daaruit bleek dat de fusies op zijn minst gedeeltelijk functioneren als aberrante transcriptiefactoren. Om de transcriptionele doelwitgenen van EWSR1/CIZ te identificeren, werd dan een transcriptieprofiel van Ba/F3-cellen opgesteld. Voor 10 van de 13 genen die gekozen werden om het experiment via qRT-PCR te valideren, werd het verschil in transcriptie bevestigd. Uit een algemene functionele analyse van het resultaat kwamen een aantal moleculaire functies naar voren in verband met hematopoiese en leukemie, maar ook in verband met de communicatie tussen de cel en zijn omgeving. Een bijzonder interessant doelwitgen is Spi-C, een transcriptiefactor die uitsluitend tot expressie komt in bepaalde stadia

van B-cel vorming. Het behoort tot dezelfde eiwitfamilie als PU.1, dat op zijn beurt cruciaal is voor de ontwikkeling van lymfocyten. Een promoter-reporter test wees uit dat Spi-C een direct transcriptioneel doelwit is van EWSR1/CIZ.

Daarnaast werden ook CIZ interactiepartners gezocht, gebruik makende van 'two-hybrid' technologie in gist. In tegenstelling tot zijn rat-homoloog, interageert humaan CIZ niet met p130CAS. Zyxin, PCBP1 en vimentin werden wel als CIZ bindingspartners geïdentificeerd. Gezien zyxin wél interageert met CAS, suggereren deze data dat zyxin de communicatie tussen CIZ en CAS in een humane context toch indirect mogelijk maakt.

Deze studie onthult een aantal karakteristieken van CIZ en de TET/CIZ fusie-eiwitten, en levert een aantal concrete aanknopingspunten voor het verder onderzoek naar het precieze moleculaire mechanisme van de TET/CIZ fusies.

List of publications

Articles regarding this work

Janssen H, Marynen P. (2006). Interaction partners for human ZNF384/CIZ/NMP4 - zyxin as a mediator for p130CAS signaling? *Experimental Cell Research*, 312: 1194-1204.

Corveleyn A, Janssen H, Martini A, Somers R, Cools J, Marynen P. (2005). Cellular transformation of NIH3T3 fibroblasts by CIZ/NMP4 fusions. *Journal of Cellular Biochemistry,* 94: 1112-1125.

Martini A, La Starza R, Janssen H, Bilhou-Nabera C, Corveleyn A, Somers R, Aventin A, Foa R, Hagemeijer A, Mecucci C, Marynen P. (2002). Recurrent rearrangement of the Ewing's sarcoma gene, EWSR1, or its homologue, TAF15, with the transcription factor CIZ/NMP4 in acute leukemia. *Cancer Research,* 62: 5408-5412.

Lee BH*, Janssen H*, Williams IR, Gilliland DG, Cools J, Marynen P. Fusion of the Ewing's sarcoma gene, EWSR1, or its homologue TAF15, with the transcription factor CIZ/NMP4 induces acute myeloid leukemia in a murine bone marrow transplant model. In preparation. (*equal contribution)

Other articles

Janssen H, Wlodarska I, Mecucci C, Hagemeijer A, Vandenberghe P, Marynen P, Cools J. (2006). Fusion of ETV6 to GOT1 in a case with myelodysplastic syndrome and t(10;12)(q24;p13). *Haematologica*, 91: 949-51.

Abstracts and presentations

Janssen H, Marynen P. (2003). Interaction partners for human CIZ: does the name still fit the protein? Special FEBS Meeting on Signal Transduction, Brussels, July 3-8. *European Journal of Biochemistry,* 270-s1: 74.

Janssen H, Martini A, Corveleyn A, Marynen P. (2003). Fusion of EWSR1 and TAF15 to a new transcription factor CIZ/NMP4 in acute leukemia. Annual Meeting of the Belgian Association for Cancer Research, Leuven, January 18. *Acta Clinica Belgica* 58: 134.

Martini A, Janssen H, Bilhou-Nabera C, La Starza R, Corveleyn A, Mecucci C, Hagemeijer A, Marynen P. (2002). The TET RNA-binding proteins, EWSR1 and TAF15, are involved in acute lymphoblastic leukemia, through fusion with a new transcription factor, CIZ/NMP4. American Society of Hematology Meeting, Philadelphia, December 5-9. *Blood,* 100: 2066.

Reference list

Abaan OD, Levenson A, Khan O, Furth PA, Uren A, Toretsky JA (2005) PTPL1 is a direct transcriptional target of EWS-FLI1 and modulates Ewing's Sarcoma tumorigenesis. *Oncogene* 24: 2715-2722

Aerts S, Van Loo P, Thijs G, Mayer H, de Martin R, Moreau Y, De Moor B (2005) TOUCAN 2: the all-inclusive open source workbench for regulatory sequence analysis. *Nucleic Acids Res* 33: W393-W396

Alex D, Lee KA (2005) RGG-boxes of the EWS oncoprotein repress a range of transcriptional activation domains. *Nucleic Acids Res* 33: 1323-1331

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403-410

Alvarez M, Shah R, Rhodes SJ, Bidwell JP (2005) Two promoters control the mouse Nmp4/CIZ transcription factor gene. *Gene* 347: 43-54

Alvarez M, Thunyakitpisal P, Morrison P, Onyia J, Hock J, Bidwell JP (1998) PTH-responsive osteoblast nuclear matrix architectural transcription factor binds to the rat type I collagen promoter. *Journal of Cellular Biochemistry* 69: 336-352

Alvarez M, Long H, Onyia J, Hock J, Xu W, Bidwell J (1997) Rat osteoblast and osteosarcoma nuclear matrix proteins bind with sequence specificity to the rat type I collagen promoter. *Endocrinology* 138: 482-489

Aman P, Panagopoulos I, Lassen C, Fioretos T, Mencinger M, Toresson H, Hoglund M, Forster A, Rabbitts TH, Ron D, Mandahl N, Mitelman F (1996) Expression patterns of the human sarcoma-associated genes FUS and EWS and the genomic structure of FUS. *Genomics* 37: 1-8

Araya N, Hiraga H, Kako K, Arao Y, Kato S, Fukamizu A (2005) Transcriptional down-regulation through nuclear exclusion of EWS methylated by PRMT1. *Biochem Biophys Res Commun* 329: 653-660

Armstrong SA, Look AT (2005) Molecular genetics of acute lymphoblastic leukemia. *J Clin Oncol* 23: 6306-6315

Arvand A, Denny CT (2001) Biology of EWS/ETS fusions in Ewing's family tumors. *Oncogene* 20: 5747-5754

Asha H, Nagy I, Kovacs G, Stetson D, Ando I, Dearolf CR (2003) Analysis of Ras-induced overproliferation in Drosophila hemocytes. *Genetics* 163: 203-215

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene Ontology: tool for the unification of biology. *Nature Genetics* 25: 25-29

Astier A, Avraham H, Manie SN, Groopman J, Canty T, Avraham S, Freedman AS (1997) The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after beta 1-integrin stimulation in B cells and binds to p130(cas). *Journal of Biological Chemistry* 272: 228-232

Bailly RA, Bosselut R, Zucman J, Cormier F, Delattre O, Roussel M, Thomas G, Ghysdael J (1994) DNA-binding and transcriptional activation properties of the EWS-FLI-1 fusion protein resulting from the t(11;22) translocation in Ewing sarcoma. *Molecular and Cellular Biology* 14: 3230-3241

Balbin M, Fueyo A, Tester AM, Pendas AM, Pitiot AS, Astudillo A, Overall CM, Shapiro SD, Lopez-Otin C (2003) Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* 35: 252-257

Balmain A, Harris CC (2000) Carcinogenesis in mouse and human cells: parallels and paradoxes. *Carcinogenesis* 21: 371-377

Barna M, Hawe N, Niswander L, Pandolfi PP (2000) Plzf regulates limb and axial skeletal patterning. *Nat Genet* 25: 166-172

Bemark M, Martensson A, Liberg D, Leanderson T (1999) Spi-C, a novel Ets protein that is temporally regulated during B lymphocyte development. *Journal of Biological Chemistry* 274: 10259-10267

Bernardi R, Grisendi S, Pandolfi PP (2002) Modelling haematopoietic malignancies in the mouse and therapeutical implications. *Oncogene* 21: 3445-3458

Bertolotti A, Lutz Y, Heard DJ, Chambon P, Tora L (1996) hTAF(II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins TLS/FUS and EWS is associated with both TFIID and RNA polymerase II. *EMBO Journal* 15: 5022-5031

Bertolotti A, Melot T, Acker J, Vigneron M, Delattre O, Tora L (1998) EWS, but not EWS-FLI-1, is associated with both TFIID and RNA polymerase II: Interactions between two members of the TET family,

EWS and hTAF(II)68, and subunits of TFIID and RNA polymerase II complexes. *Molecular and Cellular Biology* 18: 1489-1497

Bhatia M, Bonnet D, Wu DM, Murdoch B, Wrana J, Gallacher L, Dick JE (1999) Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. *Journal of Experimental Medicine* 189: 1139-1147

Boehm JS, Hahn WC (2005) Cancer genetics: Finding the right mix. *Eur J Hum Genet* 13: 1099-1100

Bosma GC, Custer RP, Bosma MJ (1983) A severe combined immunodeficiency mutation in the mouse. *Nature* 301: 527-530

Bouton AH, Riggins RB, Bruce-Staskal PJ (2001) Functions of the adapter protein Cas: signal convergence and the determination of cellular responses. *Oncogene* 20: 6448-6458

Braun BS, Frieden R, Lessnick SL, May WA, Denny CT (1995) Identification of target genes for the Ewing' s sarcoma EWS/FLI fusion protein by representational difference analysis. *Molecular and Cellular Biology* 15: 4623-4630

Brinkman A, van der Flier S, Kok EM, Dorssers LCJ (2000) BCAR1, a human homologue of the adapter protein p130Cas, and antiestrogen resistance in breast cancer cells. *Journal of the National Cancer Institute* 92: 112-120

Brown D, Kogan S, Lagasse E, Weissman I, Alcalay M, Pelicci PG, Atwater S, Bishop JM (1997) A PML/RARalpha transgene initiates murine acute promyelocytic leukemia. *Proc Natl Acad Sci USA* 94: 2551-2556

Buchholz F, Refaeli Y, Trumpp A, Bishop JM (2000) Inducible chromosomal translocation of AML1 and ETO genes through Cre/loxP-mediated recombination in the mouse. *EMBO Rep* 1: 133-139

Burnham MR, Harte MT, Richardson A, Parsons JT, Bouton AH (1996) The identification of p130cas-binding proteins and their role in cellular transformation. *Oncogene* 12: 2467-2472

Bustelo XR (2000) Regulatory and signaling properties of the Vav family. *Molecular and Cellular Biology* 20: 1461-1477

Bustelo XR, Suen KL, Michael WM, Dreyfuss G, Barbacid M (1995) Association of the Vav protooncogene product with poly(rC)-specific RNA-binding proteins. *Molecular and Cellular Biology* 15: 1324-1332

Caballero OL, de Souza SJ, Brentani RR, Simpson AJG (2001) Alternative spliced transcripts as cancer markers. *Disease Markers* 17: 67-75

Cacciola RR, Stagno F, Impera S, Assisi AR, Cacciola E Jr, Guglielmo P (1997) Beta-1-integrin expression in adult acute lymphoblastic leukemia: possible relationship with the stem cell antigen CD34. *Acta Haematol* 97: 63-66

Calvio C, Neubauer G, Mann M, Lamond AI (1995) Identification of hnRNP P2 as TLS/FUS using electrospray mass spectrometry. *RNA* 1: 724-733

Carlsson R, Hjalmarsson A, Liberg D, Persson C, Leanderson T (2002) Genomic structure of mouse SPI-C and genomic structure and expression pattern of human SPI-C. *Gene* 299: 271-278

Carlsson R, Persson C, Leanderson T (2003) SPI-C, a PU-box binding ETS protein expressed temporarily during B-cell development and in macrophages, contains an acidic transactivation domain located to the N-terminus. *Molecular Immunology* 39: 1035-1043

Castellanos A, Pintado B, Weruaga E, Arevalo R, Lopez A, Orfao A, Sanchez-Garcia I (1997) A BCR-ABL(p190) fusion gene made by homologous recombination causes B-cell acute lymphoblastic leukemias in chimeric mice with independence of the endogenous Bcr product. *Blood* 90: 2168-2174

Chadwick K, Shojaei F, Gallacher L, Bhatia M (2005) Smad7 alters cell fate decisions of human hematopoietic repopulating cells. *Blood* 105: 1905-1915

Chan D, Wilson TJ, Xu D, Cowdery HE, Sanij E, Hertzog PJ, Kola I (2003) Transformation induced by Ewing's sarcoma associated EWS/FLI-I is suppressed by KRAB/FLI-I. *British Journal of Cancer* 88: 137-145

Chansky HA, Barahmand-Pour F, Mei Q, Kahn-Farooqi W, Zielinska-Kwiatkowska A, Blackburn M, Chansky K, Conrad EU, III, Bruckner JD, Greenlee TK, Yang L (2004) Targeting of EWS/FLI-1 by RNA interference attenuates the tumor phenotype of Ewing's sarcoma cells in vitro. *J Orthop Res* 22: 910-917

Chansky HA, Hu M, Hickstein DD, Yang L (2001) Oncogenic TLS/ERG and EWS/Fli-1 fusion proteins inhibit RNA splicing mediated by YB-1 protein. *Cancer Research* 61: 3586-3590

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31: 3497-3500

Chkheidze AN, Liebhaber SA (2003) A novel set of nuclear localization signals determine distributions of the alpha CP RNA-binding proteins. *Molecular and Cellular Biology* 23: 8405-8415

Codrington R, Pannell R, Forster A, Drynan LF, Daser A, Lobato N, Metzler M, Rabbitts TH (2005) The EWS-ERG Fusion protein can initiate neoplasia from lineage-committed haematopoietic Cells. *PLoS Biol* 3: e242

Cohen B (1999) Modelling cancer in the mouse. *Nat Genet* 21: 249-251

Collins EC, Pannell R, Simpson EM, Forster A, Rabbitts TH (2000) Inter-chromosomal recombination of Mll and Af9 genes mediated by cre-loxP in mouse development. *Embo Reports* 1: 127-132

Corral J, Lavenir I, Impey H, Warren AJ, Forster A, Larson TA, Bell S, McKenzie ANJ, King G, Rabbitts TH (1996) An MII-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: A method to create fusion oncogenes. *Cell* 85: 853-861

Corveleyn A, Janssen H, Martini A, Somers R, Cools J, Marynen P (2005) Cellular transformation of NIH3T3 fibroblasts by CIZ/NMP4 fusions. *J Cell Biochem* 94: 1112-1125

Coulombel L, Auffray I, Gaugler MH, Rosemblatt M (1997) Expression and function of integrins on hematopoietic progenitor cells. *Acta Haematol* 97: 13-21

Croker BA, Krebs DL, Zhang JG, Wormald S, Willson TA, Stanley EG, Robb L, Greenhalgh CJ, Forster I, Clausen BE, Nicola NA, Metcalf D, Hilton DJ, Roberts AW, Alexander WS (2003) SOCS3 negatively regulates IL-6 signaling in vivo. *Nat Immunol* 4: 540-545

Crozat A, Aman P, Mandahl N, Ron D (1993) Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. *Nature* 363: 640-644

Daley GQ, Van Etten RA, Baltimore D (1990) Induction of chronic myelogenous leukemia in mice by the p210Bcr/Abl gene of the Philadelphia chromosome. *Science* 247: 824-830

Dauphinot L, De Oliveira C, Melot T, Sevenet N, Thomas V, Weissman BE, Delattre O (2001) Analysis of the expression of cell cycle regulators in Ewing cell lines: EWS-FLI-1 modulates p57(KIP2) and c-Myc expression. *Oncogene* 20: 3258-3265

David G, Terris B, Marchio A, Lavau C, Dejean A (1997) The acute promyelocytic leukemia PML-RAR alpha protein induces hepatic preneoplastic and neoplastic lesions in transgenic mice. *Oncogene* 14: 1547-1554

Deguchi K, Ayton PM, Carapeti M, Kutok JL, Snyder CS, Williams IR, Cross NC, Glass CK, Cleary ML, Gilliland DG (2003) MOZ-TIF2-induced acute myeloid leukemia requires the MOZ nucleosome binding motif and TIF2-mediated recruitment of CBP. *Cancer Cell* 3: 259-271

De Keersmaecker K, Marynen P, Cools J (2005) Genetic insights in the pathogenesis of T-cell acute lymphoblastic leukemia. *Haematologica* 90: 1116-1127

DeKoter RP, Singh H (2000) Regulation of B lymphocyte and macrophage development by graded expression of PU.1. *Science* 288: 1439-1441

Delattre O, Zucman J, Plougastel B, Desmaze C, Melot T, Peter M, Kovar H, Joubert I, Dejong P, Rouleau G, Aurias A, Thomas G (1992) Gene fusion with an ETS DNA-binding domain caused by chromosome-translocation in human tumors. *Nature* 359: 162-165

Deloulme JC, Prichard L, Delattre O, Storm DR (1997) The prooncoprotein EWS binds calmodulin and is phosphorylated by protein kinase C through an IQ domain. *J Biol Chem* 272: 27369-27377

Deneen B, Denny CT (2001) Loss of p16 pathways stabilizes EWS/FLI1 expression and complements EWS/FLI1 mediated transformation. *Oncogene* 20: 6731-6741

Deneen B, Welford SM, Ho T, Hernandez F, Kurland I, Denny CT (2003) PIM3 proto-oncogene kinase is a common transcriptional target of divergent EWS/ETS oncoproteins. *Molecular and Cellular Biology* 23: 3897-3908

Dick JE (1996) Normal and leukemic human stem cells assayed in SCID mice. *Semin Immunol* 8: 197-206

Dietrich C (1998) Cell cycle and cancer. *Med Monatsschr Pharm* 21: 69-75

Dohjima T, Lee NS, Li HT, Ohno T, Rossi JJ (2003) Small interfering RNAs expressed from a pol III promoter suppress the EWS/Fli-1 transcript in an Ewing sarcoma cell line. *Molecular Therapy* 7: 811-816

Draghici S, Khatri P, Martins RP, Ostermeier GC, Krawetz SA (2003) Global functional profiling of gene expression. *Genomics* 81: 98-104

Drees B, Friederich E, Fradelizi J, Louvard D, Beckerle MC, Golsteyn RM (2000) Characterization of the interaction between zyxin and members of the Ena/vasodilator-stimulated phosphoprotein family of proteins. *J Biol Chem* 275: 22503-22511

Eilers M, Picard D, Yamamoto KR, Bishop JM (1989) Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* 340: 66-68

Elefanty AG, Hariharan IK, Cory S (1990) BCR-ABL, the hallmark of chronic myeloid leukaemia in man, induces multiple haemopoietic neoplasms in mice. *EMBO J* 9: 1069-1078

Eliazer S, Spencer J, Ye D, Olson E, Ilaria RL, Jr. (2003) Alteration of mesodermal cell differentiation by EWS/FLI-1, the oncogene implicated in Ewing's sarcoma. *Mol Cell Biol* 23: 482-492

Evans RM (1998) Vimentin: the conundrum of the intermediate filament gene family. *Bioessays* 20: 79-86

Feister HA, Torrungruang K, Thunyakitpisal P, Parker GE, Rhodes SJ, Bidwell JP (2000) NP/NMP4 transcription factors have distinct osteoblast nuclear matrix subdomains. *Journal of Cellular Biochemistry* 79: 506-517

Felsch JS, Lane WS, Peralta EG (1999) Tyrosine kinase Pyk2 mediates G-protein-coupled receptor regulation of the Ewing sarcoma RNA-binding protein EWS. *Curr Biol* 9: 485-488

Feng L, Lee KA (2001) A repetitive element containing a critical tyrosine residue is required for transcriptional activation by the EWS/ATF1 oncogene. *Oncogene* 20: 4161-4168

Ferguson DO, Alt FW (2001) DNA double strand break repair and chromosomal translocation: lessons from animal models. *Oncogene* 20: 5572-5579

Forgac M (1999) Structure and properties of the vacuolar (H+)-ATPases. *J Biol Chem* 274: 12951-12954

Forster A, Pannell R, Drynan LF, Codrington R, Daser A, Metzler M, Lobato MN, Rabbitts TH (2005) The invertor knock-in conditional chromosomal translocation mimic. *Nature Methods* 2: 27-30

Forster A, Pannell R, Drynan LF, McCormack M, Collins EC, Daser A, Rabbitts TH (2003) Engineering de novo reciprocal chromosomal translocations associated with Mll to replicate primary events of human cancer. *Cancer Cell* 3: 449-458

Freedman VH, Shin SI (1974) Cellular tumorigenicity in nude mice: correlation with cell growth in semi-solid medium. *Cell* 3: 355-359

Fuchs B, Inwards C, Scully SP, Janknecht R (2004a) hTERT is highly expressed in Ewing's sarcoma and activated by EWS-ETS oncoproteins. *Clinical Orthopaedics and Related Research* 426: 64-68

Fuchs B, Inwards CY, Janknecht R (2003) Upregulation of the matrix metalloproteinase-1 gene by the Ewing's sarcoma associated EWS-ER81 and EWS-Fli-1 oncoproteins, c-Jun and p300. *FEBS Letters* 553: 104-108

Fuchs B, Inwards CY, Janknecht R (2004b) Vascular endothelial growth factor expression is up-regulated by EWS-ETS oncoproteins and Sp1 and may represent an independent predictor of survival in Ewing's sarcoma. *Clinical Cancer Research* 10: 1344-1353

Fukuma M, Okita H, Hata J, Umezawa A (2003) Upregulation of Id2, an oncogenic helix-loop-helix protein, is mediated by the chimeric EWS/ETS protein in Ewing sarcoma. *Oncogene* 22: 1-9

Furuya K, Nakamoto T, Rittling S, Nifuji A, Tsuji K, Denhardt D, Hirai H, Noda M (2000a) Overexpression of Cas-interacting zinc finger protein (CIZ) suppresses proliferation and enhances expression of type I collagen gene in osteoblast-like MC3T3E1 cells. *Faseb Journal* 14: A1332

Furuya K, Nakamoto T, Shen ZJ, Tsuji K, Nifuji A, Hirai H, Noda M (2000b) Overexpression of Cas-interacting zinc finger protein (CIZ) suppresses proliferation and enhances expression of type I collagen gene in osteoblast-like MC3T3E1 cells. *Experimental Cell Research* 261: 329-335

Futreal PA, Coin L, Marshall M, Down T, Hubbard T, Wooster R, Rahman N, Stratton MR (2004) A census of human cancer genes. *Nature Reviews Cancer* 4: 177-183

Gangenahalli GU, Gupta P, Saluja D, Verma YK, Kishore V, Chandra R, Sharma RK, Ravindranath T (2005) Stem cell fate specification: role of master regulatory switch transcription factor PU.1 in differential hematopoiesis. *Stem Cells Dev* 14: 140-152

Ganss B, Jheon A (2004) Zinc finger transcription factors in skeletal development. *Crit Rev Oral Biol Med* 15: 282-297

Geijtenbeek TB, van Kooyk Y, van Vliet SJ, Renes MH, Raymakers RA, Figdor CG (1999) High frequency of adhesion defects in B-lineage acute lymphoblastic leukemia. *Blood* 94: 754-764

Gerber HP, Seipel K, Georgiev O, Hofferer M, Hug M, Rusconi S, Schaffner W (1994) Transcriptional activation modulated by homopolymeric glutamine and proline stretches. *Science* 263: 808-811

Gershwin ME, Merchant B, Gelfand MC, Vickers J, Steinberg AD, Hansen CT (1975) The natural history and immunopathology of outbred athymic (nude) mice. *Clin Immunol Immunopathol* 4: 324-340

Gietz D, St Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* 20: 1425

Gilliland DG (2001) Hematologic malignancies. *Curr Opin Hematol* 8: 189-191

Greaves MF, Wiemels J (2003) Origins of chromosome translocations in childhood leukaemia. *Nature Reviews Cancer* 3: 639-649

Guinamard R, Fougereau M, Seckinger P (1997) The SH3 domain of Bruton's tyrosine kinase interacts with Vav, Sam68 and EWS. *Scand J Immunol* 45: 587-595

Hahm KB, Cho K, Lee C, Im YH, Chang J, Choi SG, Sorensen PH, Thiele CJ, Kim SJ (1999) Repression of the gene encoding the TGF-beta type II receptor is a major target of the EWS-FLI1 oncoprotein. *Nat Genet* 23: 222-227

Hallier M, Lerga A, Barnache S, Tavitian A, Moreau-Gachelin F (1998) The transcription factor Spi-1/PU.1 interacts with the potential splicing factor TLS. *J Biol Chem* 273: 4838-4842

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100: 57-70

Hann B, Balmain A (2001) Building 'validated' mouse models of human cancer. *Curr Opin Cell Biol* 13: 778-784

He LZ, Bhaumik M, Tribioli C, Rego EM, Ivins S, Zelent A, Pandolfi PP (2000) Two critical hits for promyelocytic leukemia. *Mol Cell* 6: 1131-1141

He LZ, Guidez F, Tribioli C, Peruzzi D, Ruthardt M, Zelent A, Pandolfi PP (1998) Distinct interactions of PML-RARalpha and PLZF-RARalpha with co-repressors determine differential responses to RA in APL. *Nat Genet* 18: 126-135

He LZ, Tribioli C, Rivi R, Peruzzi D, Pelicci PG, Soares V, Cattoretti G, Pandolfi PP (1997) Acute leukemia with promyelocytic features in PML/RARalpha transgenic mice. *Proc Natl Acad Sci USA* 94: 5302-5307

Heisterkamp N, Jenster G, Kioussis D, Pattengale PK, Groffen J (1991) Human BCR-ABL gene has a lethal effect on embryogenesis. *Transgenic Res* 1: 45-53

Heisterkamp N, Jenster G, ten Hoeve J, Zovich D, Pattengale PK, Groffen J (1990) Acute leukaemia in BCR/ABL transgenic mice. *Nature* 344: 251-253

Heisterkamp N, Stam K, Groffen J, de Klein A, Grosveld G (1985) Structural organization of the BCR gene and its role in the Ph' translocation. *Nature* 315: 758-761

Herrmann H, Haner M, Brettel M, Muller SA, Goldie KN, Fedtke B, Lustig A, Franke WW, Aebi U (1996) Structure and assembly properties of the intermediate filament protein vimentin: the role of its head, rod and tail domains. *J Mol Biol* 264: 933-953

Hicks GG, Singh N, Nashabi A, Mai S, Bozek G, Klewes L, Arapovic D, White EK, Koury MJ, Oltz EM, Van Kaer L, Ruley HE (2000) Fus deficiency in mice results in defective B-lymphocyte development and activation, high levels of chromosomal instability and perinatal death. *Nature Genetics* 24: 175-179

Hirota T, Morisaki T, Nishiyama Y, Marumoto T, Tada K, Hara T, Masuko N, Inagaki M, Hatakeyama K, Saya H (2000) Zyxin, a regulator of actin filament assembly, targets the mitotic apparatus by interacting with h-warts/LATS1 tumor suppressor. *J Cell Biol* 149: 1073-1086

Hobert O, Schilling JW, Beckerle MC, Ullrich A, Jallal B (1996) SH3 domain-dependent interaction of the proto-oncogene product Vav with the focal contact protein zyxin. *Oncogene* 12: 1577-1581

Honda H, Oda H, Suzuki T, Takahashi T, Witte ON, Ozawa K, Ishikawa T, Yazaki Y, Hirai H (1998) Development of acute lymphoblastic leukemia and myeloproliferative disorder in transgenic mice expressing p210BCR/ABL: a novel transgenic model for human Ph1-positive leukemias. *Blood* 91: 2067-2075

Hu-Lieskovan S, Zhang J, Wu L, Shimada H, Schofield DE, Triche TJ (2005) EWS-FLI1 fusion protein up-regulates critical genes in neural crest development and is responsible for the observed phenotype of Ewing's family of tumors. *Cancer Res* 65: 4633-4644

Hunger SP, Zhong CH (2002) Cryptic t(12;19) in ALL with lineage switch to AML creates two new E2A fusion proteins. *Blood* 100: 529A

Hynes RO (1987) Integrins: a family of cell surface receptors. *Cell* 48: 549-554

Ichikawa H, Shimizu K, Katsu R, Ohki M (1999) Dual transforming activities of the FUS(TLS)-ERG leukemia fusion protein conferred by two N-terminal domains of FUS(TLS). *Molecular and Cellular Biology* 19: 7639-7650

Ilaria RL, Jr. (2004) Animal models of chronic myelogenous leukemia. *Hematol Oncol Clin North Am* 18: 525-43, vii

Im YH, Kim HT, Lee C, Poulin D, Welford S, Sorensen PH, Denny CT, Kim SJ (2000) EWS-FLI1, EWS-ERG, and EWS-ETV1 oncoproteins of Ewing tumor family all suppress transcription of transforming growth factor beta type II receptor gene. *Cancer Res* 60: 1536-1540

Immanuel D, Zinszner H, Ron D (1995) Association of SARFH (sarcoma-associated RNA-binding fly homolog) with regions of chromatin transcribed by RNA polymerase II. *Mol Cell Biol* 15: 4562-4571

Jaishankar S, Zhang J, Roussel MF, Baker SJ (1999) Transforming activity of EWS/FLI is not strictly dependent upon DNA-binding activity. *Oncogene* 18: 5592-5597

Janssen H, Marynen P (2006) Interaction partners for human ZNF384/CIZ/NMP4 - zyxin as a mediator for p130CAS signaling? *Exp Cell Res* 312: 1194-1204

Jonkers J, Berns A (2002) Conditional mouse models of sporadic cancer. *Nat Rev Cancer* 2: 251-265

Kalev-Zylinska ML, Horsfield JA, Flores MV, Postlethwait JH, Vitas MR, Baas AM, Crosier PS, Crosier KE (2002) Runx1 is required for zebrafish blood and vessel development and expression of a human RUNX1-CBF2T1 transgene advances a model for studies of leukemogenesis. *Development* 129: 2015-2030

Kalnina Z, Zayakin P, Silina K, Line A (2005) Alterations of pre-mRNA splicing in cancer. *Genes Chromosomes Cancer* 42: 342-357

Kawano J, Nakayama T, Takami Y, Kotani T, Sawaguchi A, Nagaike R, Oinuma T, Suganuma T (2001) A monoclonal antibody against insect CALNUC recognizes the prooncoprotein EWS specifically in mammalian cells. Immunohistochemical and biochemical studies of the antigen in rat tissues. *Histochemistry and Cell Biology* 115: 421-428

Kawasaki-Nishi S, Nishi T, Forgac M (2003) Proton translocation driven by ATP hydrolysis in V-ATPases. *FEBS Letters* 545: 76-85

Kiefer MC, Schmid C, Waldvogel M, Schlapfer I, Futo E, Masiarz FR, Green K, Barr PJ, Zapf J (1992) Characterization of recombinant human insulin-like growth factor binding proteins 4, 5, and 6 produced in yeast. *J Biol Chem* 267: 12692-12699

Kiledjian M, Wang XM, Liebhaber SA (1995) Identification of 2 KH domain proteins in the alpha-globin mRNP stability complex. *Embo Journal* 14: 4357-4364

Kirsch KH, Georgescu MM, Hanafusa H (1998) Direct binding of p130(Cas) to the guanine nucleotide exchange factor C3G. *J Biol Chem* 273: 25673-25679

Knoop LL, Baker SJ (2000) The splicing factor U1C represses EWS/FLI-mediated transactivation. *Journal of Biological Chemistry* 275: 24865-24871

Knoop LL, Baker SJ (2001) EWS/FLI alters 5'-splice site selection. *Journal of Biological Chemistry* 276: 22317-22322

Kovar H, Aryee DN, Jug G, Henockl C, Schemper M, Delattre O, Thomas G, Gadner H (1996) EWS/FLI-1 antagonists induce growth inhibition of Ewing tumor cells in vitro. *Cell Growth Differ* 7: 429-437

Kovar H, Jug G, Aryee DNT, Zoubek A, Ambros P, Gruber B, Windhager R, Gadner H (1997) Among genes involved in the RB dependent cell cycle regulatory cascade, the p16 tumor suppressor gene is frequently lost in the Ewing family of tumors. *Oncogene* 15: 2225-2232

Kuroda M, Sok J, Webb L, Baechtold H, Urano F, Yin Y, Chung P, de Rooij DG, Akhmedov A, Ashley T, Ron D (2000) Male sterility and enhanced radiation sensitivity in TLS(-/-) mice. *EMBO Journal* 19: 453-462

La Starza R, Aventin A, Crescenzi B, Gorello P, Specchia G, Cuneo A, Angioni A, Bilhou-Nabera C, Boque C, Foa R, Uyttebroeck A, Talmant P, Cimino G, Martelli MF, Marynen P, Mecucci C, Hagemeijer A (2005) CIZ gene rearrangements in acute leukemia: report of a diagnostic FISH assay and clinical features of nine patients. *Leukemia* 19: 1696-1699

Ladanyi M (2002) EWS-FLI1 and Ewing's sarcoma - Recent molecular data and new insights. *Cancer Biology & Therapy* 1: 330-336

Langenau DM, Traver D, Ferrando AA, Kutok JL, Aster JC, Kanki JP, Lin S, Prochownik E, Trede NS, Zon LI, Look AT (2003) Myc-induced T cell leukemia in transgenic zebrafish. *Science* 299: 887-890

Leder P, Battey J, Lenoir G, Moulding C, Murphy W, Potter H, Stewart T, Taub R (1983) Translocations among antibody genes in human cancer. *Science* 222: 765-771

Lee HJ, Kim S, Pelletier J, Kim J (2004) Stimulation of hTAFII68 (NTD)-mediated transactivation by v-Src. *FEBS Letters* 564: 188-198

Leffers H, Dejgaard K, Celis JE (1995) Characterization of 2 major cellular poly(rC)-binding human proteins, each containing 3 K-homologous (KH) domains. *European Journal of Biochemistry* 230: 447-453

Lerga A, Hallier M, Delva L, Orvain C, Gallais I, Marie J, Moreau-Gachelin F (2001) Identification of an RNA binding specificity for the potential splicing factor TLS. *J Biol Chem* 276: 6807-6816

Lerner C, Harrison DE (1990) 5-Fluorouracil spares hemopoietic stem cells responsible for long-term repopulation. *Exp Hematol* 18: 114-118

Lessnick SL, Braun BS, Denny CT, May WA (1995) Multiple domains mediate transformation by the Ewings-Sarcoma Ews/Fli-1 fusion gene. *Oncogene* 10: 423-431

Lessnick SL, Dacwag CS, Golub TR (2002) The Ewing's sarcoma oncoprotein EWS/FLI induces a p53-dependent growth arrest in primary human fibroblasts. *Cancer Cell* 1: 393-401

Liu B, Sun Y, Jiang F, Zhang S, Wu Y, Lan Y, Yang X, Mao N (2003) Disruption of Smad5 gene leads to enhanced proliferation of high-proliferative potential precursors during embryonic hematopoiesis. *Blood* 101: 124-133

Longhurst CM, Jennings LK (1998) Integrin-mediated signal transduction. *Cellular and Molecular Life Sciences* 54: 514-526

Look AT (1997) Oncogenic transcription factors in the human acute leukemias. *Science* 278: 1059-1064

Loonstra A, Vooijs M, Beverloo HB, Allak BA, Van Drunen E, Kanaar R, Berns A, Jonkers J (2001) Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. *Proc Natl Acad Sci USA* 98: 9209-9214

Macalma T, Otte J, Hensler ME, Bockholt SM, Louis HA, KalffSuske M, Grzeschik KH, vonderAhe D, Beckerle MC (1996) Molecular characterization of human zyxin. *Journal of Biological Chemistry* 271: 31470-31478

Maddison K, Clarke AR (2005) New approaches for modelling cancer mechanisms in the mouse. *J Pathol* 205: 181-193

Makeyev AV, Liebhaber SA (2002) The poly(C)-binding proteins: A multiplicity of functions and a search for mechanisms. *RNA - A publication of the RNA Society* 8: 265-278

Manie SN, Astier A, Haghayeghi N, Canty T, Druker BJ, Hirai H, Freedman AS (1997a) Regulation of integrin-mediated p130(Cas) tyrosine phosphorylation in human B cells. A role for p59(Fyn) and SHP2. *J Biol Chem* 272: 15636-15641

Manie SN, Beck ARP, Astier A, Law SF, Canty T, Hirai H, Druker BJ, Avraham H, Haghayeghi N, Sattler M, Salgia R, Griffin JD, Golemis EA, Freedman AS (1997b) Involvement of p130(Cas) and p105(HEF1), a novel Cas-like docking protein, in a cytoskeleton-dependent signaling pathway initiated by ligation of integrin or antigen receptor on human B cells. *Journal of Biological Chemistry* 272: 4230-4236

Mao X, Miesfeldt S, Yang H, Leiden JM, Thompson CB (1994) The FLI-1 and chimeric EWS-FLI-1 oncoproteins display similar DNA binding specificities. *J Biol Chem* 269: 18216-18222

Martini A, La Starza R, Janssen H, Bilhou-Nabera C, Corveleyn A, Somers R, Aventin A, Foa R, Hagemeijer A, Mecucci C, Marynen P (2002) Recurrent rearrangement of the Ewing's sarcoma gene, EWSR1, or its homologue, TAF15, with the transcription factor CIZ/NMP4 in acute leukemia. *Cancer Research* 62: 5408-5412

Massague J (2000) How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 1: 169-178

Matsui Y, Chansky HA, Barahmand-Pour F, Zielinska-Kwiatkowska A, Tsumaki N, Myoui A, Yoshikawa H, Yang L, Eyre DR (2003) COL11A2 collagen gene transcription is differentially regulated by EWS/ERG sarcoma fusion protein and wild-type ERG. *Journal of Biological Chemistry* 278: 11369-11375

May WA, Arvand A, Thompson AD, Braun BS, Wright M, Denny CT (1997) EWS/FLI1-induced manic fringe renders NIH3T3 cells tumorigenic. *Nature Genetics* 17: 495-497

May WA, Gishizky ML, Lessnick SL, Lunsford LB, Lewis BC, Delattre O, Zucman J, Thomas G, Denny CT (1993) Ewing sarcoma t(11;22) translocation produces a chimeric transcription factor that requires the DNA-binding domain encoded by Fli1 for transformation. *Proc Natl Acad Sci USA* 90: 5752-5756

McKercher SR, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ, Maki RA (1996) Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO Journal* 15: 5647-5658

Merghoub T, Gurrieri C, Piazza F, Pandolfi PP (2001) Modeling acute promyelocytic leukemia in the mouse: new insights in the pathogenesis of human leukemias. *Blood Cells Mol Dis* 27: 231-248

Mikkers H, Berns A (2003) Retroviral insertional mutagenesis: tagging cancer pathways. *Adv Cancer Res* 88: 53-99

Mitelman F, Johansson B, Mertens F (2004) Fusion genes and rearranged genes as a linear function of chromosome aberrations in cancer. *Nat Genet* 36: 331-334

Morinobu M, Nakamoto T, Hino K, Tsuji K, Shen ZJ, Nakashima K, Nifuji A, Yamamoto H, Hirai H, Noda M (2005) The nucleocytoplasmic shuttling protein CIZ reduces adult bone mass by inhibiting bone morphogenetic protein-induced bone formation. *J Exp Med* 201: 961-970

Morinobu M, Nakamoto T, Tsuji K, Shen Z, Nifuji A, Yamamoto H, Hirai H, Noda M (2002) Deficiency of CIZ, a novel negative regulator of BMP, enhances osterix gene expression in bone, osteoblastic differentiation in culture and BMP- as well as ablation-induced bone formation in vivo. *Journal of Bone and Mineral Research* 17: S127

Morohoshi F, Arai K, Takahashi E, Tanigami A, Ohki M (1996) Cloning and mapping of a human RBP56 gene encoding a putative RNA binding protein similar to FUS/TLS and EWS proteins. *Genomics* 38: 51-57

Nabel EG (2002) CDKs and CKIs: molecular targets for tissue remodelling. Nat Rev Drug Discov 1: 587-598

Nakamoto T, Shiratsuchi A, Oda H, Inoue K, Matsumura T, Ichikawa M, Saito T, Seo S, Maki K, Asai T, Suzuki T, Hangaishi A, Yamagata T, Aizawa S, Noda M, Nakanishi Y, Hirai H (2004) Impaired spermatogenesis and male fertility defects in CIZ/Nmp4-disrupted mice. *Genes Cells* 9: 575-589

Nakamoto T, Yamagata T, Sakai R, Ogawa S, Honda H, Ueno H, Hirano N, Yazaki Y, Hirai H (2000) CIZ, a zinc finger protein that interacts with p130(cas) and activates the expression of matrix metalloproteinases. *Molecular and Cellular Biology* 20: 1649-1658

Nasertorabi F, Garcia-Guzman M, Briknarova K, Larsen E, Havert ML, Vuori K, Ely KR (2004) Organization of functional domains in the docking protein p130Cas. *Biochem Biophys Res Commun* 324: 993-998

Nemeth JA, Cher ML, Zhou Z, Mullins C, Bhagat S, Trikha M (2003) Inhibition of alpha v beta 3 integrin reduces angiogenesis, bone turnover, and tumor cell proliferation in experimental prostate cancer bone metastases. *Clinical & Experimental Metastasis* 20: 413-420

Nishimori H, Sasaki Y, Yoshida K, Irifune H, Zembutsu H, Tanaka T, Aoyama T, Hosaka T, Kawaguchi S, Wada T, Hata J, Toguchida J, Nakamura Y, Tokino T (2002) The Id2 gene is a novel target of transcriptional activation by EWS-ETS fusion proteins in Ewing family tumors. *Oncogene* 21: 8302-8309

Nix DA, Beckerle MC (1997) Nuclear-cytoplasmic shuttling of the focal contact protein, zyxin: A potential mechanism for communication between sites of cell adhesion and the nucleus. *Journal of Cell Biology* 138: 1139-1147

Nix DA, Fradelizi J, Bockholt S, Menichi B, Louvard D, Friederich E, Beckerle MC (2001) Targeting of zyxin to sites of actin membrane interaction and to the nucleus. *Journal of Biological Chemistry* 276: 34759-34767

Nozawa S, Ohno T, Banno Y, Dohjima T, Wakahara K, Fan DG, Shimizu K (2005) Inhibition of platelet-derived growth factor-induced cell growth signaling by a short interfering RNA for EWS-Fli1 via down-regulation of phospholipase D2 in Ewing's sarcoma cells. *Journal of Biological Chemistry*

Ohkura N, Yaguchi H, Tsukada T, Yamaguchi K (2002) The EWS/NOR1 fusion gene product gains a novel activity affecting pre-mRNA splicing. *Journal of Biological Chemistry* 277: 535-543

Ohno T, Rao VN, Reddy ESP (1993) Ews/Fli-1 chimeric protein is a transcriptional activator. *Cancer Research* 53: 5859-5863

Onnebo SM, Condron MM, McPhee DO, Lieschke GJ, Ward AC (2005) Hematopoietic perturbation in zebrafish expressing a Tel-Jak2a fusion. *Exp Hematol* 33: 182-188

Ostareck-Lederer A, Ostareck DH (2004) Control of mRNA translation and stability in haematopoietic cells: the function of hnRNPs K and E1/E2. *Biol Cell* 96: 407-411

Ostareck-Lederer A, Ostareck DH, Hentze MW (1998) Cytoplasmic regulatory functions of the KH-domain proteins hnRNPs K and E1/E2. *Trends in Biochemical Sciences* 23: 409-411

Ottensmeier C (2001) The classification of lymphomas and leukemias. *Chem Biol Interact* 135-136: 653-664

Ouchida M, Ohno T, Fujimura Y, Rao VN, Reddy ESP (1995) Loss of tumorigenicity of Ewings-sarcoma cells expressing antisense RNA to EWS-fusion transcripts. *Oncogene* 11: 1049-1054

Palacios R, Steinmetz M (1985) Il-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. *Cell* 41: 727-734

Pear WS, Miller JP, Xu L, Pui JC, Soffer B, Quackenbush RC, Pendergast AM, Bronson R, Aster JC, Scott ML, Baltimore D (1998) Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving p210 Bcr/Abl-transduced bone marrow. *Blood* 92: 3780-3792

Pendergast AM, Muller AJ, Havlik MH, Maru Y, Witte ON (1991) BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. *Cell* 66: 161-171

Pereira DS, Dorrell C, Ito CY, Gan OI, Murdoch B, Rao VN, Zou JP, Reddy ESP, Dick JE (1998) Retroviral transduction of TLS-ERG initiates a leukemogenic program in normal human hematopoietic cells. *Proc Natl Acad Sci USA* 95: 8239-8244

Petermann R, Mossier BM, Aryee DN, Khazak V, Golemis EA, Kovar H (1998) Oncogenic EWS-Fli1 interacts with hsRPB7, a subunit of human RNA polymerase II. *Oncogene* 17: 603-610

Plougastel B, Zucman J, Peter M, Thomas G, Delattre O (1993) Genomic structure of the EWS gene and its relationship to EWSR1, a site of tumor-associated chromosome-translocation. *Genomics* 18: 609-615

Pollock JL, Westervelt P, Kurichety AK, Pelicci PG, Grisolano JL, Ley TJ (1999) A bcr-3 isoform of RARalpha-PML potentiates the development of PML-RARalpha-driven acute promyelocytic leukemia. *Proc Natl Acad Sci USA* 96: 15103-15108

Powers CA, Mathur M, Raaka BM, Ron D, Samuels HH (1998) TLS (translocated-in-liposarcoma) is a high-affinity interactor for steroid, thyroid hormone, and retinoid receptors. *Mol Endocrinol* 12: 4-18

Prieur A, Tirode F, Cohen P, Delattre O (2004) EWS/FLI-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3. *Mol Cell Biol* 24: 7275-7283

Rabbitts TH (1991) Translocations, master genes, and differences between the origins of acute and chronic leukemias. *Cell* 67: 641-644

Rabbitts TH (1994) Chromosomal translocations in human cancer. *Nature* 372: 143-149

Rangarajan A, Weinberg RA (2003) Comparative biology of mouse versus human cells: modelling human cancer in mice. *Nat Rev Cancer* 3: 952-959

Reddy EP, Reynolds RK, Santos E, Barbacid M (1982) A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. *Nature* 300: 149-152

Rego EM, Pandolfi PP (2002) Reciprocal products of chromosomal translocations in human cancer pathogenesis: key players or innocent bystanders? *Trends Mol Med* 8: 396-405

Rego EM, Wang ZG, Peruzzi D, He LZ, Cordon-Cardo C, Pandolfi PP (2001) Role of promyelocytic leukemia (PML) protein in tumor suppression. *J Exp Med* 193: 521-529

Reinhard M, Zumbrunn J, Jaquemar D, Kuhn M, Walter U, Trueb B (1999) An alpha-actinin binding site of zyxin is essential for subcellular zyxin localization and alpha-actinin recruitment. *Journal of Biological Chemistry* 274: 13410-13418

Ren R (2002) The molecular mechanism of chronic myelogenous leukemia and its therapeutic implications: studies in a murine model. *Oncogene* 21: 8629-8642

Ren R (2004) Modeling the dosage effect of oncogenes in leukemogenesis. *Curr Opin Hematol* 11: 25-34

Renan MJ (1993) How many mutations are required for tumorigenesis? Implications from human cancer data. *Mol Carcinog* 7: 139-146

Risser R, Stockert E, Old LJ (1978) Abelson antigen: a viral tumor antigen that is also a differentiation antigen of BALB/c mice. *Proc Natl Acad Sci USA* 75: 3918-3922

Rithidech K, Dunn JJ, Bond VP, Gordon CR, Cronkite EP (1999) Characterization of genetic instability in radiation- and benzene-induced murine acute leukemia. *Mutat Res* 428: 33-39

Rosenbauer F, Wagner K, Kutok JL, Iwasaki H, Le Beau MM, Okuno Y, Akashi K, Fiering S, Tenen DG (2004) Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1. *Nat Genet* 36: 624-630

Rossow KL, Janknecht R (2001) The Ewing's sarcoma gene product functions as a transcriptional activator. *Cancer Research* 61: 2690-2695

Ruegg CL, Wu HY, Fagnoni FF, Engleman EG, Laus R (1996) B4B, a novel growth-arrest gene, is expressed by a subset of progenitor/pre-B lymphocytes negative for cytoplasmic mu-chain. *Journal of Immunology* 157: 72-80

Sadler I, Crawford AW, Michelsen JW, Beckerle MC (1992) Zyxin and cCRP: two interactive LIM domain proteins associated with the cytoskeleton. *J Cell Biol* 119: 1573-1587

Sambrook, J., Fritsch, E. F., and Maniatis, T. Molecular cloning: a laboratory manual. Cold Spring Harbor, ed. 1990. (NY: Cold Spring Harbor Laboratories).

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74: 5463-5467

Sauer B, Henderson N (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci USA* 85: 5166-5170

Scandura JM, Boccuni P, Cammenga J, Nimer SD (2002) Transcription factor fusions in acute leukemia: variations on a theme. *Oncogene* 21: 3422-3444

Scanlan MJ, Gout I, Gordon CM, Williamson B, Stockert E, Gure AO, Jager D, Chen YT, Mackay A, O'Hare MJ, Old LJ (2001) Humoral immunity to human breast cancer: antigen definition and quantitative analysis of mRNA expression. *Cancer Immun* 1: 4

Schmeichel KL, Beckerle MC (1994) The LIM domain is a modular protein-binding interface. *Cell* 79: 211-219

Schwarz H, Blanco FJ, von Kempis J, Valbracht J, Lotz M (1996) ILA, a member of the human nerve growth factor/tumor necrosis factor receptor family, regulates T-lymphocyte proliferation and survival. *Blood* 87: 2839-2845

Scott EW, Simon MC, Anastasi J, Singh H (1994) Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265: 1573-1577

Shah R, Alvarez M, Jones DR, Torrungruang K, Watt AJ, Selvamurugan N, Partridge NC, Quinn CO, Pavalko FM, Rhodes SJ, Bidwell JP (2004) Nmp4/CIZ regulation of matrix metalloproteinase 13 (MMP-13) response to parathyroid hormone in osteoblasts. *Am J Physiol Endocrinol Metab* 287: E289-E296

Shen ZJ, Nakamoto T, Tsuji K, Nifuji A, Miyazono K, Komori T, Hirai H, Noda M (2002) Negative regulation of bone morphogenetic protein/Smad signaling by Cas-interacting zinc finger protein in osteoblasts. *Journal of Biological Chemistry* 277: 29840-29846

Sieber OM, Heinimann K, Tomlinson IPM (2003) Genomic instability - the engine of tumorigenesis? *Nature Reviews Cancer* 3: 701-708

Siligan C, Ban J, Bachmaier R, Spahn L, Kreppel M, Schaefer KL, Poremba C, Aryee DN, Kovar H (2005) EWS-FLI1 target genes recovered from Ewing's sarcoma chromatin. *Oncogene* 24: 2512-2524

Smith AJ, De Sousa MA, Kwabi-Addo B, Heppell-Parton A, Impey H, Rabbitts P (1995) A site-directed chromosomal translocation induced in embryonic stem cells by Cre-loxP recombination. *Nat Genet* 9: 376-385

Spahn L, Petermann R, Siligan C, Schmid JA, Aryee DNT, Kovar H (2002) Interaction of the EWS NH2 terminus with BARD1 links the Ewing's sarcoma gene to a common tumor suppressor pathway. *Cancer Research* 62: 4583-4587

Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA, Hilton DJ (1997) A family of cytokine-inducible inhibitors of signalling. *Nature* 387: 917-921

Svitkina TM, Verkhovsky AB, Borisy GG (1996) Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. *J Cell Biol* 135: 991-1007

Tabin CJ, Bradley SM, Bargmann CI, Weinberg RA, Papageorge AG, Scolnick EM, Dhar R, Lowy DR, Chang EH (1982) Mechanism of activation of a human oncogene. *Nature* 300: 143-149

Takahashi A, Higashino F, Aoyagi M, Yoshida K, Itoh M, Kyo S, Ohno T, Taira T, Ariga H, Nakajima K, Hatta M, Kobayashi M, Sano H, Kohgo T, Shindoh M (2003) EWS/ETS fusions activate telomerase in Ewing's tumors. *Cancer Research* 63: 8338-8344

Taub R, Kirsch I, Morton C, Lenoir G, Swan D, Tronick S, Aaronson S, Leder P (1982) Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. *Proc Natl Acad Sci USA* 79: 7837-7841

Teitell MA, Thompson AD, Sorensen PHB, Shimada H, Triche TJ, Denny CT (1999) EWS/ETS fusion genes induce epithelial and neuroectodermal differentiation in NIH3T3 fibroblasts. *Laboratory Investigation* 79: 1535-1543

Tenen DG (2003) Disruption of differentiation in human cancer: AML shows the way. *Nature Reviews Cancer* 3: 89-101

Thiele BJ, Doller A, Kahne T, Pregla R, Hetzer R, Regitz-Zagrosek V (2004) RNA-binding proteins heterogeneous nuclear ribonucleoprotein A1, E1, and K are involved in post-transcriptional control of collagen I and III synthesis. *Circ Res* 95: 1058-1066

Thomas GR, Latchman DS (2002) The pro-oncoprotein EWS (Ewing's sarcoma protein) interacts with the Brn-3a POU transcription factor and inhibits its ability to activate transcription. *Cancer Biology & Therapy* 1: 428-432

Thompson AD, Braun BS, Arvand A, Stewart SD, May WA, Chen E, Korenberg J, Denny C (1996) EAT-2 is a novel SH2 domain containing protein that is up regulated by Ewing's sarcoma EWS/FLI1 fusion gene. *Oncogene* 13: 2649-2658

Thompson AD, Teitell MA, Arvand A, Denny CT (1999) Divergent Ewing's sarcoma EWS/ETS fusions confer a common tumorigenic phenotype on NIH3T3 cells. *Oncogene* 18: 5506-5513

Thunyakitpisal P, Alvarez M, Tokunaga K, Onyia JE, Hock J, Ohashi N, Feister H, Rhodes SJ, Bidwell JP (2001) Cloning and functional analysis of a family of nuclear matrix transcription factors (NP/NMP4) that regulate type I collagen expression in osteoblasts. *Journal of Bone and Mineral Research* 16: 10-23

Thyagarajan B, Guimaraes MJ, Groth AC, Calos MP (2000) Mammalian genomes contain active recombinase recognition sites. *Gene* 244: 47-54

Tomlins SA, Rhodes DR, Perner S, Dhanasekaran SM, Mehra R, Sun XW, Varambally S, Cao X, Tchinda J, Kuefer R, Lee C, Montie JE, Shah RB, Pienta KJ, Rubin MA, Chinnaiyan AM (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* 310: 644-648

Torrungruang K, Alvarez M, Shah R, Onyia JE, Rhodes SJ, Bidwell JP (2002a) DNA binding and gene activation properties of the Nmp4 nuclear matrix transcription factors. *Journal of Biological Chemistry* 277: 16153-16159

Torrungruang K, Shah R, Alvarez M, Bowen DK, Gerard R, Pavalko FM, Elmendorf JS, Charoonpatrapong K, Hock J, Rhodes SJ, Bidwell JP (2002b) Osteoblast intracellular localization of Nmp4 proteins. *Bone* 30: 931-936

Tosato G, Seamon KB, Goldman ND, Sehgal PB, May LT, Washington GC, Jones KD, Pike SE (1988) Monocyte-derived human B-cell growth factor identified as interferon-beta 2 (BSF-2, IL-6). *Science* 239: 502-504

Van Deursen J, Fornerod M, Van Rees B, Grosveld G (1995) Cre-mediated site-specific translocation between nonhomologous mouse chromosomes. *Proc Natl Acad Sci USA* 92: 7376-7380

Verfaillie CM (1998) Adhesion receptors as regulators of the hematopoietic process. *Blood* 92: 2609-2612

Vodicka MA (2001) Determinants for lentiviral infection of non-dividing cells. *Somat Cell Mol Genet* 26: 35-49

Voncken JW, Kaartinen V, Pattengale PK, Germeraad WT, Groffen J, Heisterkamp N (1995) BCR/ABL p210 and p190 cause distinct leukemia in transgenic mice. *Blood* 86: 4603-4611

Watanabe G, Nishimori H, Irifune H, Sasaki Y, Ishida S, Zembutsu H, Tanaka T, Kawaguchi S, Wada T, Hata J, Kusakabe M, Yoshida K, Nakamura Y, Tokino T (2003) Induction of Tenascin-C by tumor-specific EWS-ETS fusion genes. *Genes Chromosomes & Cancer* 36: 224-232

Weinberg RA (1998) *One renegade cell*. Basic Books: New York

Welford SM, Hebert SP, Deneen B, Arvand A, Denny CT (2001) DNA binding domain-independent pathways are involved in EWS/FLI1-mediated oncogenesis. *Journal of Biological Chemistry* 276: 41977-41984

Wong S, Witte ON (2001) Modeling Philadelphia chromosome positive leukemias. *Oncogene* 20: 5644-5659

Xie X, Brunner N, Jensen G, Albrectsen J, Gotthardsen B, Rygaard J (1992) Comparative studies between nude and scid mice on the growth and metastatic behavior of xenografted human tumors. *Clin Exp Metastasis* 10: 201-210

Yang L, Chansky HA, Hickstein DD (2000a) EWS/Fli-1 fusion protein interacts with hyperphosphorylated RNA polymerase II and interferes with serine-arginine protein-mediated RNA splicing. *Journal of Biological Chemistry* 275: 37612-37618

Yang L, Embree LJ, Hickstein DD (2000b) TLS-ERG leukemia fusion protein inhibits RNA splicing mediated by serine-arginine proteins. *Molecular and Cellular Biology* 20: 3345-3354

Yergeau DA, Hetherington CJ, Wang Q, Zhang P, Sharpe AH, Binder M, Marin-Padilla M, Tenen DG, Speck NA, Zhang DE (1997) Embryonic lethality and impairment of haematopoiesis in mice heterozygous for an AML1-ETO fusion gene. *Nat Genet* 15: 303-306

Yi JS, Kloeker S, Jensen CC, Bockholt S, Honda H, Hirai H, Beckerle MC (2002) Members of the zyxin family of LIM proteins interact with members of the p130(Cas) family of signal transducers. *Journal of Biological Chemistry* 277: 9580-9589

Yilmaz OH, Valdez R, Theisen BK, Guo W, Ferguson DO, Wu H, Morrison SJ (2006) Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature* 441: 475-482

Zhang D, Paley AJ, Childs G (1998) The transcriptional repressor ZFM1 interacts with and modulates the ability of EWS to activate transcription. *Journal of Biological Chemistry* 273: 18086-18091

Zhang J, Niu C, Ye L, Huang H, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li L (2003) Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 425: 836-841

Zhang J, Grindley JC, Yin T, Jayasinghe S, He XC, Ross JT, Haug JS, Rupp D, Porter-Westpfahl KS, Wiedemann LM, Wu H, Li L (2006) PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature* 441: 518-522

Zinszner H, Albalat R, Ron D (1994) A novel effector domain from the RNA-binding protein TLS or EWS is required for oncogenic transformation by CHOP. *Genes Dev* 8: 2513-2526

Zwerner JP, May WA (2001) PDGF-C is an EWS/FLI induced transforming growth factor in Ewing family tumors. *Oncogene* 20: 626-633

Zwerner JP, May WA (2002) Dominant negative PDGF-C inhibits growth of Ewing family tumor cell lines. *Oncogene* 21: 3847-3854