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Functional characterization of the CATS gene with respect to its role in normal hematopoiesis and in leukemia

Thesis Submitted for a Doctoral degree in Human Biology
at the Faculty of Medicine Ludwig-Maximilians-University,
Munich, Germany

Submitted by
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From
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Aus der Medizinischen Klinik und Poliklinik III am Klinikum Großhadern
und GSF, Klinische Kooperations Gruppe ,''Leukämie''
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Charakterisierung des CATS-Gens bezüglich seiner Rolle in der normalen Hämatopoese und bei der Leukämieentstehung

Dissertation zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der Ludwig-Maximilians-
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vorgelegt von
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For my fröhlichen archangels, Lucas and Sophie with love

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I Abbreviations

aa	amino acids
ab	antibody
ALL	acute lymphoblastic leukemia
AML	acute myeloblastic leukemia
Amp	ampicillin
AP	alkaline phosphatase
AP-2	clathrin adaptor protein complex 2
APC	allophycocyanin
approx.	approximately
APS	ammonium persulfate
B-NHL	B-cell non-Hodgkin's lymphoma
bp	base pairs
BSA	bovine serum albumine
cDNA	complementary DNA
cdk	cyclin-dependent kinase
CFP	cyan fluorescent protein
cfu	colony forming unit
Ci	Curie
CIAP	calf intestine alkaline phosphatase
CME	clathrin mediated endocytosis
CoIP	co-immunoprecipitation
d.p.c	days post coitum
DAPI	4',6-DIAMIDINO-2-PHENYINDOLE, DIALACTATE
ddH_2O	double distilled water
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dox	doxycycline
DTT	dithiothreitol
e.g.	example
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tags
FAB	French-American-British classification system for acute leukemia
FACS	fluorescence activated cell sorting
g	gram
GA	glutaraldehyde
GAL4-AD	GAL4 transcriptional activation domain
GAL4-DBD	GAL4 DNA binding domain
GAL4-UAS	GAL4 upstream activating sequence
GAPDH	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GST	glutathione S transferase
HA	hemagglutinin
hnRNP	heterogeneous nuclear ribonucleoprotein
HPRT	hypoxanthine guanine phosphoribosyl transferase
hr(s)	hour(s)
HRP	horse radish peroxidase

INF	interferon
Intr	intron
IPTG	isopropyl β-D thiogalactoside
kb	kilobases
kD	kilodalton
KH	hnRNP K homology domain
l	liter
LacZ	*E. coli* gene encoding beta-galactosidase
LB	Luria Bertani medium
LiAc	lithium acetate
M	Molar
m-	milli (1×10^{-3})
MCS	multi cloning site
min	minute(s)
MOPS	3-N-morpholino-propanesulfonic acid
mRNA	messenger RNA
n	nano (1×10^{-9})
NaAC	sodium acetate
NaOH	sodium hydroxide
NB	northern blot
NHL	Non-Hodgkins Lymphoma
NLS	nuclear localization signal
nt	nucleotide
O/N	overnight
ºC	degree Celsius
OD	optical density
p	pico (1×10^{-12})
PBS	phosphate buffer saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PE	phycoerythrin
PEG	polyethylenglycol
PFA	paraformaldehyde
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
RNA	ribonucleic acid
RNAse A	ribonuclease A
RT	room temperature
SAP	shrimp alkaline phosphatase
SD	synthetic drop out
SDS	sodium dodecylsulfate
sec	second(s)
SSC	saline sodium citrate buffer
TBE	tris-buffered saline
TE	tris-EDTA buffer
Tm	melting temperature
TNF alpha	tumor necrosis factor alpha
TNFR	tumor necrosis factor receptor
TPA	phorbol ester
U	unit
UTR	untranslated region
V	volts

vol.	volume
w/v	weight per volume
WB	western blot
WISH	whole mount *in situ* hybridization
x g	gravity (9.81 m/s^2)
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y2H	yeast two hybrid
YFP	yellow fluorescent protein
YPD	yeast extract, peptone, dextrose
µ-	micro (1×10^{-6})

Single-letter codes for amino acids

A (Ala)	Alanine	M (Met)	Methionine
B	Asparagine or Aspartic acid	N (Asn)	Asparagine
C (Cys)	Cysteine	P (Pro)	Proline
D (Asp)	Aspartic acid	Q (Glu)	Glutamine
E (Glu)	Glutamic acid	R (Arg)	Arginine
F (Phe)	Phenylalanine	S (Ser)	Serine
G (Gly)	Glycine	T (Thr)	Threonine
H (His)	Histidine	V (Val)	Valine
I (Ile)	Isoleucine	W (Trp)	Tryptophan
K (Lys)	Lysine	Y (Tyr)	Tyrosine
L (Leu)	Leucine	Z	Glutamine or Glutamic acid

Single-letter codes for nucleotides

A	Adenine
G	Guanine
U	Uracil
C	Cytosine
T	Thymine

II Index

1 Introduction

1.1 Chromosomal translocations

It has been over 40 years since chromosome aberrations were first associated with particular types of malignancies. Today it is well known that chromosomal abnormalities, such as deletions, inversions, and translocations are crucial events in the malignant process. Balanced chromosomal translocations, in particular, are frequently observed in human leukemias. These lead to the exchange of genetic material between two non-homologous chromosomes (Figure 1.1) resulting in the fusion of genes located at the translocation breakpoints or leading to the deregulated expression of genes close to the translocation breakpoints.

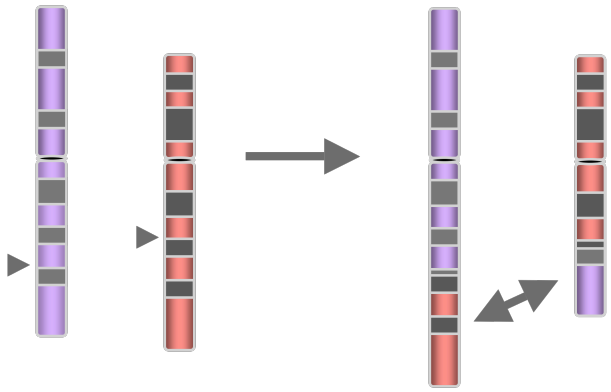
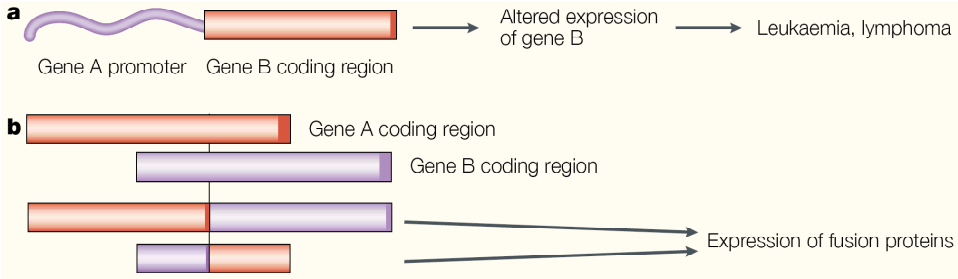


Fig: 1.1: Balanced chromosomal translocation. Arrowheads indicate breakpoint regions on both non-homologous chromosomes. The exchange of chromosome material is depicted by the double-headed arrow.

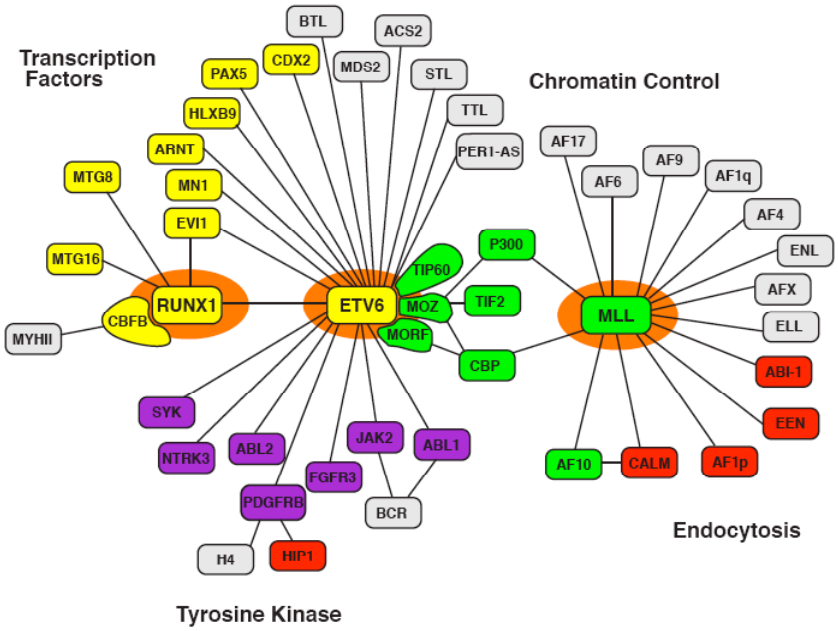
There are basically two molecular mechanisms used by the translocations to alter gene function, (1) the juxtaposition of a coding region from one gene with the promoter of another gene, or (2) fusion of two coding regions to create a new chimeric gene that encodes a fusion protein (Figure 1.2) (Rowley, 1999; Rowley, 2000; Rowley, 2001). Classic examples of these mechanisms are the t(8;14) translocation which juxtaposes the *IGH* promoter region to the *MYC* coding sequences, resulting in an overexpression of the *MYC* oncogene in Burkitt's lymphoma (ar-Rushdi et al., 1983; Taub et al., 1982) and the t(9;22) translocation which fuses the Abelson tyrosine kinase gene (*ABL*) with the breakpoint cluster region gene (*BCR*) resulting in the expression of the BCR-ABL fusion protein encoded by the Philadelphia chromosome in chronic myeloid leukemia (CML) (Shtivelman et al., 1985).



Janet D. Rowley; Nature reviews 2001

Fig. 1.2: Consequences of balanced chromosomal translocation. Chromosomal translocation results (**a**) in the juxtaposition of regulatory elements of a gene A with the intact coding region of a gene B leading to deregulated expression of the last, or (**b**) combination of the coding region of two different genes forming a new chimeric fusion gene leading to the expression of a fusion protein.

In recent years a growing number of chromosomal translocations have been characterized at the molecular level, leading to the identification of many genes involved in inducing malignant transformation. As a consequence of these efforts, a network of fusion genes has emerged where several genes are involved in more than one translocation forming fusion genes with several partner genes. The *RUNX1* (*AML1*), *ETV6* and *MLL* genes occupy central positions in this network, playing pivotal roles in the pathogenesis of various types of leukemias (Figure 1.3) (Bohlander, 2000).



adapted from Bohlander 2000 and 2005

Fig. 1.3: Fusion gene network of RUNX1, ETV6 and MLL. Leukemia-associated gene fusions are indicated by connecting line (not all fusions of PDGFRB and MLL are shown). Genes are grouped into tyrosine kinases: purple; genes with a function in endocytosis: red; chromatin control genes: green; transcription factor genes: yellow.

Translocations involving *the core binding factor complex* (heterodimer of the RUNX1 and CBFβ proteins) are the most frequent AML-associated rearrangements. The *RUNX1/MTG8* (*AML1*/*ETO*) fusion gene resulting from the t(8;21) translocation is found in about half the cases of AML of FAB subtypes M2, whereas the *CBFβ*/*MYH11* fusion resulting from the inversion of chromosome 16 (inv(16)) is strictly found in M4eo. Both rearrangements account for approximately 8-12 % of all cases of AML and are associated with relatively good prognosis (Schnittger, 2002). *RUNX1* is also involved in the t(12;21) translocation fusing with *ETV6*, the resulting *ETV6/RUNX1* fusion being the most common fusion gene found in childhood B-ALL (Bohlander, 2005).

Among the most common chromosomal breakpoint regions in human leukemias is the long arm of chromosome 11 at band q23, where the mixed linage leukemia gene (*MLL*) is located (Ziemin-van der Poel et al., 1991). In translocations involving the 11q23 band the *MLL* gene can become fused to one of more than 39 different partner genes at other chromosomal locations (De Braekeleer et al., 2005). The most frequent fusion products are the MLL/AF4, MLL/AF9, MLL/AF6, MLL/AF10, MLL/ELL and MLL/ENL. These can be found in AML, ALL and therapy associated leukemia with poor prognosis. In these MLL fusion proteins the N-terminal DNA binding domain of MLL is retained whereas the C-terminal SET domain responsible for the H3-K4 methylase activity is lost and replaced by the fusion partner. As a consequence, the chimeric protein binds to the MLL target genes, which are then differently regulated as a result of a gain of function that is associated with the fusion partner proteins. MLL is a major regulator of homeobox (*HOX*) gene expression, directly interacting with *HOX* promoter regions. In leukemias with MLL fusion protein, overexpression of *HOX* genes are found (Daser & Rabbitts, 2004; Daser & Rabbitts, 2005). Some MLL fusion proteins may directly target *HOX* genes for activation by aberrant recruitment of activators (Licht & Sternberg, 2005; Slany, 2005). This is also the case in MLL/AF10 positive leukemias. Okada and coworkers have beautifully shown that leukemogenisis by MLL/AF10 involves upregulation of Hoxa9 mediated through recruitment of histone methyltransferase hDOT1L through interaction with the AF10 octapeptide leucine zipper domain. hDOT1L increases H3-K79 methylation on MLL target genes leading to increased expression of these genes (Okada et al., 2005). There is increasing evidence that nuclear interacting partners of MLL have transcription activity themselves and belong to important chromatin remodeling and transcription modulating complexes, emphasizing the role of epigenetic processes as mediator of malignant transformation (Daser & Rabbitts, 2005; Okada et al., 2005; Slany, 2005; Zeisig et al., 2005).

1.2 The t(10;11)(p13;q14) translocation involving *CALM* and *AF10*

The rare but recurring t(10;11)(p13;q14) translocation was first characterized at the molecular level in the human monocytic cell line U937 (Dreyling et al., 1996). This rearrangement results in the fusion of *CALM* on chromosome 11 band q14 to *AF10* on chromosome 10 band p13 (Figure 1.4).

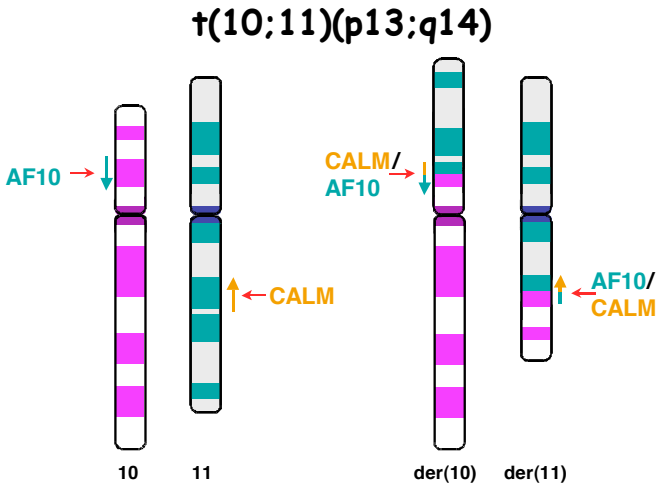


Fig. 1.4: The t(10;11)(p13,q14) translocation. This balanced translocation which results in the CALM/AF10 fusion is found in acute lymphoid and acute myeloid leukemia as well as in malignant lymphoma.

The two reciprocal fusion mRNAs encode fusion proteins of very unequal size. This is due to the location of the breakpoints; in the *CALM* gene the breakpoints are located at the C-terminus of the gene and the breakpoints in *AF10* are located in the N-terminal portion of *AF10* (Dreyling et al., 1996). The *CALM/AF10* fusion encompasses nearly the complete open reading frames of both the *CALM* and *AF10* genes whereas the reciprocal *AF10/CALM* fusion essentially encodes for a truncated AF10 protein of 84 amino acids (Figure 1.5).

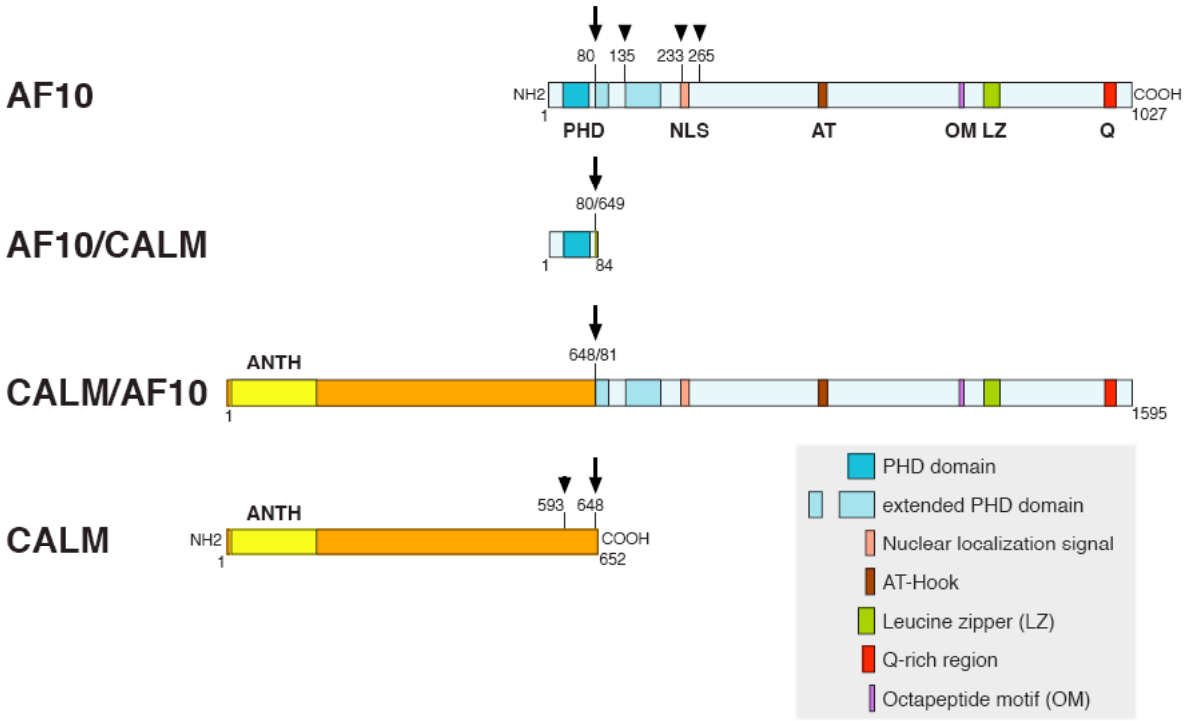


Fig. 1.5: Diagram representing the protein structures of AF10, CALM and the AF10/CALM and CALM/AF10 fusion proteins. Arrows indicate the breakpoints in CALM and AF10 described in U937 cell line. Other breakpoints found in cells from patients are shown as triangles. There is no apparent correlation between breakpoint location and disease phenotype. PHD: plant homeodomain zinc fingers; NLS: nuclear localization signal; AT: AT-hook motif; OM: octapeptide motif; LZ: leucine zipper; Q: glutamic acid rich domain; ANTH: AP180 N-terminal homology domain.

In patients, the occurrence of *CALM/AF10* rearrangement has been observed mainly in undifferentiated acute myeloid leukemia (AML) (FAB M0 or M1) or T-cell acute lymphoblastic leukemia (ALL) and in malignant lymphoma and carries rather poor prognosis (Dreyling et al., 1998; Kumon et al., 1999; Narita et al., 1999). In T-ALL with T-cell receptor (TCR) gamma/delta rearrangement the *CALM/AF10* fusion is found in up to 30 % of cases and constitutes the most frequent rearrangement in this leukemia subtype (Asnafi et al., 2004; Asnafi et al., 2005; Asnafi et al., 2003). Recently, cases of more differentiated AML with a *CALM/AF10* fusion have been reported (FAB M4, M5 and M7) (Abdou et al., 2002; Carlson et al., 2000; Jones et al., 2001; Nakamura et al., 2003; Salmon-Nguyen et al., 2000). The translocation is observed in younger patients (Dreyling et al., 1998; Kobayashi et al., 1997; Kumon et al., 1999; Silliman et al., 1998) and is also associated with mixed-lineage immunophenotype with coexpression of lymphoid (T-cell) and myeloid antigens (Kumon et al., 1999; Narita et al., 1999).

In several patients, the t(10;11)(p13;q14) translocation has been described as a sole chromosomal abnormality (Abdou et al., 2002; Bohlander et al., 2000; Carlson et al., 2000;

Kumon et al., 1999; Narita et al., 1999; Salmon-Nguyen et al., 2000; Silliman et al., 1998) implying that the *CALM/AF10* fusion plays a crucial role in leukemogenesis.

It is very likely that the *CALM/AF10* fusion transcript and not the reciprocal *AF10/CALM* fusion is critical in the leukemogenic process since it is a constant feature observed in all patients with a t(10;11)(p13;q14) whereas the *AF10/CALM* fusion mRNA can only be detected in about half of the patients that carry a *CALM/AF10* fusion (Bohlander et al., 2000; Carlson et al., 2000; Dreyling et al., 1996; Dreyling et al., 1998; Krause, 2004; Narita et al., 1999). Indeed, Deshpande *et al.* could show that the *CALM/AF10* fusion gene causes aggressive biphenotypic leukemia in a murine bone marrow transplantation model (Deshpande, 2005; Deshpande, 2003).

Very recently Delabesse and coworkers and our group have report that *CALM/AF10* positive T-ALL are characterized by overexpression of the *HOXA* and *BMI1* genes. The over-expression of the *HOX* genes is reminiscent of the *HOX* gene expression patterns observed in leukemias involving rearrangements of the *MLL* gene (Dik et al., 2005; Krause, 2004).

The *AF10* gene is one of the few *MLL* partner genes to be independently rearranged with a third gene in leukemia (Ayton & Cleary, 2001; Dreyling et al., 1996). It codes for a 1084-aa nuclear protein which contains an N-terminal LAP/PHD (leukemia associated protein/plant homeodomain) zinc finger motif, an extended LAP/PHD motif, a nuclear localization signal (NLS), an AT-hook motif, an octapeptide motif (OM), a leucine zipper (LZ) motif and a glutamic acid rich region (Q rich region). *AF10* contains sequence motifs common to transcription factors and has been suggested to play a role in heterochromatin-mediated transcriptional silencing (Debernardi et al., 2002; Linder et al., 2001; Linder et al., 2000; Perrin et al., 2003; Perrin & Dura, 2004; Saha et al., 1995).

CALM codes for a 652-aa ubiquitously expressed protein that shows extensive homologies to the neuronal specific endocytic protein AP180 (Morris et al., 1993). It functions as an endocytosis adaptor which takes part in the initial stages of coated pit invagination together with clathrin, AP-2 and PtdIns(4,5)P_2 containing membranes. CALM interacts with the clathrin heavy chain through its C-terminal third and with phophoinositides through its ANTH domain in the N-terminal third, promoting assembly of clathrin triskelia into clathrin cages *in vitro* (Ford et al., 2002; Ford et al., 2001). Both overexpression or down regulation of CALM have been shown to inhibit receptor-mediated endocytosis and impair endosome trafficking in the trans golgi network (Huang et al., 2004; Meyerholz et al., 2005; Tebar et al., 1999). The phenotype of AP180/CALM mutants of *Drosophila* (*lap* mutants) and *Caenorhabditis elegans* (*unc11* mutants) and more recently depletion of CALM protein in mammalian cells

suggested a role of these proteins in regulating the size of endocytic vesicles during the clathrin assembly process (Meyerholz et al., 2005; Nonet et al., 1999; Zhang et al., 1998). Evidences that CALM and its involvement in the clathrin mediated endocytosis processes play an important role for hematopoiesis, iron uptake- metabolism and growth was provided by Klebig and co workers, who have shown that point mutations in the mouse *CALM* homologue *Picalm* are responsible for abnormalities in hematopoiesis and iron metabolism (Klebig et al., 2003).

To further elucidate the function of *CALM* and the leukemogenic CALM/AF10 fusion protein we searched for CALM interacting proteins using a yeast two-hybrid approach. A novel protein, which is strongly expressed in thymus, spleen and colon was identified in this search. The corresponding gene was named *CATS* (CALM interacting protein expressed in thymus and spleen).

2 Material and Methods

2.1 Material

2.1.1 Reagents

1-kb-DNA Ladder	Invitrogen, Karlsruhe, Germany
β-Mercaptoethanol	Sigma, Taufkirchen, Germany
[∝-^{32}P]dCTP 3000 µCi/mmol	Amersham, Freiburg, Germany
10X DIG RNA reaction mix	Roche, Mannheim, Germany
Acetic acid	Merck, Darmstad, Germany
Acrylamid Rotiphorese® Gel 30 (37, 5:1)	Carl Roth, Karlsruhe, Germany
Ag501-X8	BioRad Laboratories, Hercules, CA
Agar	Carl Roth, Karlsruhe, Germany
Agarose	ICN Biomedicals Inc.
Amino acids (yeast two-hybrid)	Sigma, Taufkirchen, Germany
Ammonium acetate (NH_4Ac)	Sigma, Taufkirchen, Germany
Ammonium chloride (NH_4Cl) solution	CellSystems®, Vancouver, Canada
Ammonium persulfate (APS)	Sigma, Taufkirchen, Germany
Ampicillin Na-Salt	Pan Biotech, Aidenbach, Germany
Ampuwa® H_2O	Fresenius, Bad Homburg, Germany
Aprotinin	Sigma, Taufkirchen, Germany
BigDye™ terminator mix	PE Applied Biosystems, Foster City, CA
Blocking reagent	Roche, Mannheim, Germany
BM purple AP substrate	Roche, Mannheim, Germany
Boric acid	Carl Roth, Karlsruhe, Germany
Bovine serum albumin	Sigma, Taufkirchen, Germany
Bromophenol blue	Carl Roth, Karlsruhe, Germany
Calcium chloride ($CaCl_2$)	Sigma, Taufkirchen, Germany
Calf Intestine Alkaline Phosphatase (CIAP)	MBI Fermentas, St. Leon-Rot, Germany
Citric acid	Merck, Darmstadt, Germany
Coomassie® stain solution	BioRad, Hercules, CA
D(+)-Glucose-Monohydrate	Merck, Darmstadt, Germany
Dakocytomation Fluorescent Mounting Medium	DakoCytomation, Hamburg, Germany
DAPI	Sigma, Taufkirchen, Germany
ddH_2O	Millipore, Eschborn, Germany
Deoxyribonuclease I, Amplification Grade	Invitrogen, Karlsruhe, Germany
Dextran sulfate	Carl Roth, Karlsruhe, Germany
Diethyl Pyrocarbonate (DEPC)	Sigma, Taufkirchen, Germany
Dimethyl formamide (DMF)	Carl Roth, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Carl Roth, Karlsruhe, Germany
dNTP Set, PCR Grade	Invitrogen, Karlsruhe, Germany
DPBS	Pan Biotech, Aidenbach, Germany
ECL™ Plus Western Blotting Detection Reagent	Amersham, Freiburg, Germany

ECL™ Western Blot Detection reagents	Amersham, Freiburg, Germany
EDTA	Carl Roth, Karlsruhe, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium bromide	Carl Roth, Karlsruhe, Germany
Fetal bovine serum (FBS)	Gibco Invitrogen cell culture, Karlsruhe, Germany
Ficoll (type 400)	Sigma, Taufkirchen, Germany
Formaldehyde 37 %	Carl Roth, Karlsruhe, Germany
Formamide	Carl Roth, Karlsruhe, Germany
Gelatine	Merck, Darmstadt, Germany
Glutaraldehyde 25 % aqueous solution	Sigma, Taufkirchen, Germany
Glutathione	Sigma, Taufkirchen, Germany
Glutathione-Agarose	Sigma, Taufkirchen, Germany
Glycerin 87 %	neoLab, Heidelberg, Germany
Glycerol	Carl Roth, Karlsruhe, Germany
Glycine	Merck, Darmstadt, Germany
Heparin	Sigma, Taufkirchen, Germany
Herring Testes Carrier DNA denatured	DB Biosciences Clontech, Heidelberg, Germany
Hydrochloridric acid 37 %	Merck, Darmstadt, Germany
Hydrogen peroxide (H_2O_2) solution 35 %	Merck, Darmstadt, Germany
IPTG	Roche, Mannheim, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany
Jung Tissue Freezing Medium™	Leica Instruments, Nussloch, Germany
Kaisers Glyceringelatine	Merck, Darmstadt, Germany
Kanamycin	Pan Biotech, Aidenbach, Germany
Klenow Fragment	MBI Fermentas, St. Leon-Rot, Germany
Leptomycin B (LMB) Streptomyces sp.	Calbiochem, San Diego, USA
Leupeptin	Sigma, Taufkirchen, Germany
Levamisole	Sigma, Taufkirchen, Germany
L-Glutamine	Pan Biotech, Aidenbach, Germany
Lithium acetate	Sigma, Taufkirchen, Germany
Maleic acid	Sigma, Taufkirchen, Germany
Methanol	Merck, Darmstadt, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
MOPS	Carl Roth, Karlsruhe, Germany
NP40	Roche, Mannheim, Germany
Oligo $(dT)_{12-18}$ Primer	Invitrogen, Karlsruhe, Germany
PanScript DNA Polymerase	Pan Biotech, Aidenbach, Germany
Paraformaldehyde	Sigma, Taufkirchen, Germany
Penicillin/streptomycin	Pan Biotech, Aidenbach, Germany
Pepstatin	Sigma, Taufkirchen, Germany
Peptone, meat pancreatic digested	Merck, Darmstadt, Germany
Pfu DNA Polymerase	Promega, Mannheim, Germany
Phenol/chloroform/isoamyl (25:24:1)	Invitrogen, Karlsruhe, Germany
Phenylmethylsulfonylfluorid (PMSF)	Sigma, Taufkirchen, Germany
Platinum® Taq DNA Polymerase	Invitrogen, Karlsruhe, Germany
Polyethylenglycol (PEG) 4000	Merck, Darmstadt, Germany

Polyvinylpyrrolidone (PVP) K 30	Carl Roth, Karlsruhe, Germany
Potassium acetate (KAc)	Calbiochem, San Diego, USA
Potassium chloride (KCl)	Calbiochem, San Diego, USA
Propidium iodide	Sigma, Taufkirchen, Germany
Protein A-Agarose	Roche, Mannheim, Germany
Protein G-Agarose	Roche, Mannheim, Germany
Proteinase inhibitor cocktail	Sigma, Taufkirchen, Germany
Proteinase K	Sigma, Taufkirchen, Germany
Restriction enzymes	MBI Fermentas, Germany
Restriction enzymes	NewEngland Biolabs, Schwalbach, Germany
Ribonuclease A	MBI Fermentas, Sigma or Qiagen, Germany
RNA Ladder, High Range	MBI Fermentas, St. Leon-Rot, Germany
RNA polymerase	Roche, Mannheim, Germany
RNase Away	Carl Roth, Karlsruhe, Germany
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen, Karlsruhe, Germany
Roti®-Fect transfection reagent	Carl Roth, Karlsruhe, Germany
Salmon testes DNA	Sigma, Taufkirchen, Germany
SDS	Carl Roth, Karlsruhe, Germany
SeeBlue® Plus2 pre-stained standard	Invitrogen, Karlsruhe, Germany
Shrimp Alkaline Phosphatase (SAP)	MBI Fermentas, St. Leon-Rot, Germany
Sodium acetate, Anhydrous (NaAC)	Calbiochem, San Diego, USA
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Sodium citrate	Carl Roth, Karlsruhe-, Germany
Sodium deoxycholate	Sigma, Taufkirchen, Germany
Sodium hydroxide (NaOH)	Merck, Darmstadt, Germany
Sodium phosphate dibasic anhydrous (Na_2HPO_4)	Sigma, Taufkirchen, Germany
Sodium phosphate monobasic monohydrate (NaH_2PO_4)	Calbiochem, San Diego, USA
Sucrose	Sigma, Taufkirchen, Germany
SuperScript™ II RNase H^- Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
T4 DNA Ligase	NewEngland Biolabs, Schwalbach, Germany
Tris	Carl Roth, Karlsruhe, Germany
Triton X-100	Carl Roth, Karlsruhe, Germany
tRNA	Roche, Mannheim, Germany
Trypsin-EDTA	Gibco™, Germany
Tween® 20	Sigma, Taufkirchen, Germany
X-α- gal	Clontech, Heidelberg, Germany
Yeast extract	Sigma, Taufkirchen, Germany
Yeast nitrogen base without aa	Difco, Detroit MI, USA

2.1.2 Material and Kits

Blotting paper GB003 and GB002	Schleicher & Schuell, Dassel, Germany
Cell Strainer 40µm Nylon Falcon®	BD Biosciences, Palo Alto, CA
Column Bulk and RediPack GST Purification Modules	Amersham, Freiburg, Germany
Coverslips	Menzel-Gläser®, Braunschweig, Germany
Cryotube™ vials	Nalge Nunc Internacional, Denmark
DNeasy® Tissue Kit (50)	Qiagen, Hilden, Germany
Dual-Luciferase® Reporter Assay System	Promega, Mannheim, Germany
Electroporation cuvettes 2mm	EquiBio, Kent, UK
Endofree® Plasmid Maxi kit (10)	Qiagen, Hilden, Germany
Eppendorf tubes	Eppendorf, Hamburg, Germany
FACS Polystrene round-bottom tubes	Becton Dickinson, Meylan, France
Filter 0.22µm	Millipore, Belford, USA
Glass beads	Sigma, Taufkirchen, Germany
Glassware	Schott, Jena, Germany
Human Multiple Tissue Northern (MTN™) Blot	Clontech, Heidelberg, Germany
Human Multiple Tissue Northern (MTN™) Blot II	Clontech, Heidelberg, Germany
Hybond-N nylon membrane	Amersham, Freiburg, Germany
Hybond™ ECL™ nitrocellulose membrane	Amersham, Freiburg, Germany
Hypercassete™ 18x24 cm	Amersham, Freiburg, Germany
Hyperfilm ECL high performance	Amersham, Freiburg, Germany
JETSorb DNA extraction from Agarose Gels	Genomed, Bad Oeynhausen, Germany
JETSTAR Plasmid Midi Kit	Genomed, Bad Oeynhausen, Germany
Luminometer tube (Disposable Cuvettes 12 mm x 50 mm)	Promega, Mannheim, Germany
Marabu Fixogum Rubber Cement	Marabuwerk GmbH, Tamm, Germany
Megaprime™ DNA labeling system	Amersham, Freiburg, Germany
Microcentrifuge tube (1.5 ml)	Eppendorf, Hamburg, Germany
Microscope slides	Menzel-Gläser®, Braunschweig, Germany
Microspin S-400 HR columns	Amersham, Freiburg, Germany
Mitsubishi thermal papervideo-printer	Mitsubishi, Hatfield, UK
Nescofilm sealing film	Carl Roth, Karlsruhe, Germany
Pipette tips	Carl Roth, Karlsruhe, Germany
Plastic cuvettes for OD	Carl Roth, Karlsruhe, Germany
Plastic ware for cell culture	Greiner Labortechnik, Frickenhausen, Germany
Plastic ware for cell culture	Sarstedt, Nümbrecht, Germany
Plastic ware for cell culture	Corning, USA
Polypropylene conical tubes	Becton Dickinson, Meylan, France
Qiagen RNEasy Mini Kit	Qiagen, Hilden, Germany
QIAquick® Nucleotide Removal Kit (50)	Qiagen, Hilden, Germany
Qiashredder™ columns	Qiagen, Hilden, Germany
Rneasy™ mini kit	Qiagen, Hilden, Germany
Scintillation vials	Sigma, Taufkirchen, Germany
Surgical blade	Feather Safety Razor Co. Med. Div., Japan
Syringe	Braun, Melsungen, Germany

2.1.3 Equipment

Axioplan 2 Imaging microscope	Carl Zeiss, Jena, Germany
AxioCam HR digital camera	Carl Zeiss, Jena, Germany
Axiovert 200M microscope	Carl Zeiss, Jena, Germany
BioPhotometer 6131	Eppendorf, Hamburg, Germany
Centrifuge 5417 C	Eppendorf, Hamburg, Germany
Centrifuge 5417 R	Eppendorf, Hamburg, Germany
Centrifuge RC5B Plus	SORVALL®, Langenselbold, Germany
Centrifuge RC5C	SORVALL®, Langenselbold, Germany
Cryotome CM 1850	Leica Microsystems, Wetzlar, Germany
Electroporator (Easyject Prima)	EquiBio Peqlab, Erlangen, Germany
FACSVantage SE System	BD Biosciences, Palo Alto, CA
Gel Air™ Drying System	BioRad Laboratories, Hercules, CA
GeneAmp® PCR system 2400	Perkin Elmer, Jügesheim, Germany
GeneAmp® PCR system 9700	Perkin Elmer, Jügesheim, Germany
Genetic Analyzer automated sequencer ABI PRISM 310	PE Applied Biosystems, Foster City, CA
GS Gene Linker™ UV Chamber	BioRad Laboratories, Hercules, CA
Icubator	Heraeus Instruments, Langenselbold, Germany
Innova™ 4400 Incubator Shaker	New Brunswick Scientific, Nürtingen, Germany
InsituPro robot	ABIMED, Langenfeld, Germany
MZ APO stereomicroscope	Leica Microsystems, Wetzlar, Germany
JVC KY-F70 digital camera	JVC Professional Europe, London, UK
Savant SpeedVac®	GMI, Minnesota, U.S.A
TD-20/20 luminometer	Turner Designs, Sunnyvale, CA
Thermomixer 5436	Eppendorf, Hamburg, Germany
Tissuemizer	Tekmar-Dohrmann, Manson, OH
Trans-Blot® SD Semi -Dry Transfer Cell	BioRad Laboratories, Hercules, CA
X-Ray Automatic Processor Curix 60	Agfa, Köln Germany

2.1.4 Software

Software INSITU version 4.020 was used in the InsituPro robot (ABIMED) for automated *in situ* hybridization of whole mount mouse embryos. Images of whole mount embryos were recorded using the Leica QWin software (Leica Microsystems Imaging Solutions, Cambridge, UK). AxioVision 3.1 (Carl Zeiss, Jena, Germany) was used for capturing images of sectioned embryos and the Open*lab* 3.08 (Improvision, Coventry, UK) for visualizing and photographic documentation of cells. Pictures were processed with Adobe Photoshop® 7.0 (Adobe Systems, Mountain View, U.S.A) and worked on Macromedia Freehand 10.0 (Freehand Systems, USA). The software used for FACS sorting was the CellQuest Version 3.1(f) (BD Biosciences, Palo Alto, CA). Mac Vector™ 7.2.3 (Oxford Molecular Group) was used for sequence analysis and primers design and AssemblyLIGN™ 1.0.9c (Oxford Molecular Group)

for assembling and generating contig sequences. Microsoft Office 2004 for Mac (Microsoft Corporation, USA) was used for text editing, table calculations and graphic images, Adobe Reader 7.0 (Adobe Systems, Mountain View, U.S.A) was used to covert documents into PDF format, and EndNote 8.0 (Thomson) for managing bibliographies.

2.1.5 Buffers and Solutions

All buffers and solution were prepared with ddH_2O, unless they had to be RNase-free. In this case they were prepared with DEPC-H_2O.

E1 resuspension buffer	50 mM	Tris/HCl pH 8.0
	10 mM	EDTA
	100 µg/mL	RNAse A
		Store at 4°C
E2 lysis buffer	200 mM	NaOH
	1 %	SDS
		Autoclave, store at RT
E3 neutralization buffer	3 M	Potassium acetate pH5.5
		Autoclave, store at RT
10X TE buffer	100 mM	Tris/HCl
	10 mM	EDTA
		pH 8.0 or 7.5 (for Y2H experiments)
5X TBE	445 mM	Tris/HCl pH 8.0
	445 mM	Boric acid
	10 mM	EDTA
Blue Juice loading buffer	65 %	Sucrose
	10 mM	Tris/HCl pH 7.5
	10 mM	EDTA
	0.3 %	Bromophenol blue
		Store at RT
RNA sample buffer (325 µl)	75 µl	Formaldehyde 37 %
	45 µl	10X MOPS RNA running buffer
	180 µl	Formamide
	25 µl	Ethidium bromide (1 µg/µl)
RNA loading buffer (1 ml)	500 µl	Glycerol (autoclaved)
	25 µl	10 % Bromophenol blue
	4 µl	0.25 M EDTA pH 7.4/DEPC-H_2O
	471µl	DEPC-H_2O

10X MOPS RNA running buffer	41.8 g 16.6 ml 20 ml	MOPS in 800 ml DEPC-H_2O, adjust pH 7.0 3 M NaAC/DEPC-H_2O 0.5 M EDTA/DEPC-H_2O Add DEPC-H_2O to complete 1 l vol., filter sterile and store at RT protected from light
Hybridization solution (NB)	5X 5X 10 % 0.5 %	Denhardt's solution SSC Dextran sulfate SDS
Stripping solution for NB	5 mM 2 mM 0.1X	Tris/HCl pH 8.0 EDTA Denhardt's solution
50X Denhardt's solution	1 % 1 % 1 %	BSA Polyvinylpyrrolidone (PVP) Ficoll 400 (type 400) Filter sterile, aliquot and store at –20°C
DEPC-H_2O	0.01 %	Diethyl pyrocarbonate (DEPC) in ddH_2O Mix well, incubate O/N and autoclave
Saline sodium citrate buffer (SSC) 20X	3 M 0.3 M	NaCl Sodium citrate pH 7.0 Autoclave
10 % SDS	10 % (w/v)	Sodium dodecylsulfate
Hybridization solution (RZPD filters)	50 % 4X 50 mM 1 mM 10 % 1 % 50 µg/ml 10X	Formamide SSC Sodium phosphate buffer EDTA Dextran sulfate SDS Denature Salmon testes DNA Denhardt's solution
Sodium phosphate buffer	684 ml 316 ml	1 M Na_2HPO_4 1 M NaH_2PO_4 pH 7.2
Phosphate buffer saline (PBS)	80 g 14.4 g 2.4 g 2 g 800 ml	NaCl Na_2HPO_4 NaH_2PO_4 KCl ddH_2O adjust pH 7.4 with HCl, complete to 1 l vol. and autoclave

Stripping solution for WB	62.5 mM	Tris/HCl pH 6.8
	0.1 M	β-mercaptoethanol
	2 %	SDS
RIPA buffer	1X	PBS
	1 %	Triton X-100
	0.5 %	Sodium deoxycholate
	0.1 %	SDS
1X TBS	10 mM	Tris/HCl pH 8.0
	150 mM	NaCl
1X TBST washing buffer	1X	TBS
	0.1 %	Tween® 20
Transfer buffer(1 l)	5.82 g	Tris
	2.93 g	Glycin
	0.038 g	SDS
	20 %	Methanol
10X Tris-Glycine electrophoresis buffer	250 mM	Tris
	2.5 M	Glycine
	1 %	SDS
2X Laemmli sample buffer	10 %	β-mercaptoethanol
	6 %	SDS
	20 %	Glycerol
	0.2 mg/ml	Bromophenol blue
	0.025X	Laemmli stacking buffer (optional)
		Store up to 2 month at RT
2.5X Laemmli stacking buffer	0.3 M	Tris/HCl pH 6.8
	0.25 %	SDS
		Store up to 1 month at 4°C
FACS buffer	1X	PBS
	2 %	FBS
	1 mg/l	propidium iodide
Ammonium chloride solution	0.8 %	NH_4Cl in sterile water
	0.1 mM	EDTA
PEG/LiAc solution	8 vol.	50 % PEG 4000
	1 vol.	10X TE buffer
	1 vol.	10X LiAc
10X LiAc solution	1 M	Lithium acetate
		Adjust to pH 7.5 with acetic acid and autoclave

1X PEG/LiAc	0.2 ml	10X TE
	0.2 ml	10X LiAc solution
	1.6 ml	ddH_2O
Lysis solution for yeast cells	10 mM	Tris/HCl pH 8.0
	1 mM	EDTA
	100 mM	NaCl
	2 %	Triton X-100
	1 %	SDS
NETN buffer	150 mM	NaCl
(Purification of GST-fusion	20 mM	Tris/HCl pH 8.0
protein)	1 mM	EDTA pH 8.0
	0.5 %	Triton X-100
High salt washing buffer	1 M	NaCl
(Purification of GST-fusion	20 mM	Tris/HCl pH 7.5
protein)	1 %	Triton X-100
	1 mM	DTT
Elution buffer	100 mM	NaCl
(Purification of GST-fusion	50 mM	Tris/HCl pH 8.0
protein)	200 mM	Glutathione
CoIP buffer I	50 mM	Tris/HCl pH 7.5
	150 mM	NaCl
	0.5 %	Triton X-100
	0.25 %	Sodium deoxycholate
	1:100	Proteinase inhibitor cocktail, freshly added
CoIP buffer II	50 mM	Tris/HCl pH 7.5
	0.1 %	Triton X-100
	0.05 %	Sodium deoxycholate
CoIP buffer 3	10 mM	Tris/HCl pH 8.0
	50 mM	NaCl
	0.5 %	Triton X-100
	1:100	Proteinase inhibitor cocktail, freshly added
2 % Paraformaldehyde (PFA)	1 g	PFA
	48 ml	PBS
	47-50 µl	5 M NaOH to dissolve the PFA
	75-90 µl	2 M HCl (37 %; diluted 1:5) to adjust pH to 7.4
		complete with PBS to 50 ml vol.

Tfb 1	30 mM	KAc
	50 mM	$MgCl_2$
	100 mM	KCl
	10 mM	$CaCl_2$
	15 %	Glycerol
		adjust to pH 5.8 (with 0.2 M NaOH), filter sterile, aliquot and store at -20°C
Tfb 2	10 mM	MOPS
	75 mM	$CaCl_2$
	10 mM	KCl
	15 %	Glycerol
		adjust to pH 6.8 (with acetic acid), filter sterile, aliquot and store at -20°C
4 % Paraformaldehyde (PFA) (100 ml)	4 g	PFA
	100 ml	PBS-DEPC
	~50 µl	5 M NaOH and heat up to 55°C to dissolve the PFA
	~90 µl	2 M HCl (37 %; diluted 1:5) to adjust pH to 7.0
4 % PFA/ 0.1 % GA	1 vol.	4 % PFA
	0.1 %	Glutaraldehyde
	1 vol.	PBT
PBS-DEPC	30 ml	5 M NaCl
	15 ml	1 M Sodium phosphate buffer pH 7.3
	955 ml	DEPC-H_2O
PBT	1X	PBS-DEPC
	0,1 %	Tween® 20
Proteinase K buffer	2 mg/ml	Proteinase K (10 mg/ml stock)
	20 mM	Tris/HCl pH 7.0
	1 mM	EDTA
		in DEPC-H_2O
Hybridization solution (WISH)	50 %	Deionized formamide
	5X	SSC-DEPC
	50 µg/ml	Heparin (100 mg/ml stock)
	0.1 %	Tween® 20
		in DEPC-H_2O, adjust to pH 6.0 (with 1 M Citric acid-DEPC)
SSC/FA/ Tween® 20	2X	SSC
	50 %	formamide
	0.1 %	Tween® 20
		in ddH_2O

10X TBST (WISH)	8 g	NaCl
	0.2 g	KCl
	25 ml	1 M Tris/HCl pH 7.5
	10 ml	Tween® 20
		complete with ddH_2O to 100 ml final vol.
MAB	100 mM	Maleic acid
	150 mM	NaCl
		in ddH_2O, adjust to pH 7.5 with solid NaOH
MABT	1X	MAB
	0.1 %	Tween® 20
RNase solution (TNT)	10 mM	Tris/HCl pH 7.5
	0.5 M	NaCl
	0.1 %	Tween® 20
		in ddH_2O
Alkaline phosphatase buffer (NTMT)	100 mM	NaCl
	50 mM	$MgCl_2$
	100 mM	Tris/HCl pH 9.5
	0.1 %	Tween® 20
	2 mM	Levamisole
		in ddH_2O
Blocking stock solution (10 %)	10 %	Blocking reagent Dissolve reagent in MAB with shaking and heating. After autoclaving add 0.1 % Tween® 20, aliquot and store at -20°C
RIPA-DEPC	2.5 ml	10 % SDS-DEPC
	15 ml	5 M NaCl
	5 ml	NP40
	25 ml	10 % Sodium deoxycholate-DEPC
	1 ml	0.5 M EDTA-DEPC
	25 ml	1 M Tris/HCl pH 8.0
		Add to 500 ml DEPC-H_2O
Deionized formamide	10 g	BioRad Ag501-X8
	100 ml	Formamide
		Stir for 1 hr, filtrate and store at -80°C

2.1.6 Culture medium

2.1.6.1 Culture medium for bacteria

Luria Bertani (LB) medium was prepared with ddH_2O and autoclaved

LB medium	1 %	peptone
	0.5 %	yeast extract
	1 %	NaCl
	1.5 %	agar (for LB agar plates only)

In order to select transformed cells ampicillin or kanamycin was added to the medium at final concentration of 50 µg/ml and 25 µg/ml, respectively.
LB-agar medium with antibiotic was poured onto Petri dishes and stored at 4° C.

2.1.6.2 Culture medium for yeast

All yeast media and solutions were prepared and handled on sterile conditions.

YPD medium	20 g/l	peptone
	10 g/l	yeast extract
	20 g/l	agar (for YPD agar plates only)
	950 ml	ddH_2O, autoclave

Adjust the pH to 6.5 (with NaOH), if necessary and autoclave. Allow medium reached approx. 55°C and add 50 ml of 40 % glucose stock solution. At the same temperature YPD-agar medium was poured onto Petri dishes and subsequently stored at 4°C.

A combination of a minimal SD base and a dropout (DO) solution were used to produce a defined minimal yeast medium lacking one or more specific nutrients. The nutrients were added separately for each selective SD/DO medium according to the selection desired. A 10X stock dropout solution was prepared lacking amino acids adenine, histidine, leucine, and tryptophan.

10X DO -Trp, -Leu,		
-His, -Ade	200 mg	Arginine
	300 mg	Isoleucine
	300 mg	Lysine
	200 mg	Methionine
	500 mg	Phenylalanine
	2000 mg	Threonine
	300 mg	Tyrosine
	200 mg	Uracil
	1500 mg	Valine
	1000 ml	ddH_2O, autoclave

SD -Trp, -Leu,		
-His, -Ade	6.7 g	yeast nitrogen base without aa
	20 g	agar (for SD plates only)
	850 ml	ddH_2O
	100 ml	10X dropout solution (-Trp, -Leu, -His, -Ade)
For SD -Trp, -Leu, -His		
add	200 mg	Adenine
For SD -Trp, -Leu add	200 mg	Adenine, Histidine

Adjust the pH to 5.8 if necessary and autoclave. Allow medium reached approx. 55°C and add 50 ml of 40 % glucose stock solution. For SD -Trp, -Leu, -His, -Ade plates supplemented with X-α -gal, 1:1000 volume of X-α -gal (20 mg/ml in DMF) was added to the medium. SD-agar medium was poured onto Petri dishes and subsequently stored at 4°C.

2.1.6.3 Cell culture medium

All cell culture media and solutions were handled on sterile conditions and stored at 4°C. Dulbecco´s Modified Eagle medium (DMEM), Roswell Park Memorial Institute culture medium (RPMI 1640), antibiotics, L-Glutamine and DPBS were purchased from Pan Biotech, Aidenbach, Germany. Fetal bovine serum (FBS) was purchased from Gibco® Invitrogen cell culture.

2.1.7 Bacterial strain (*Escherichia coli*)

Strain	Usage
XL1' Blue	host for plasmid amplification
BL21	host of choice for GST fusion expression. Does not transform well, an alternate strain for cloning and maintenance of the vector is needed

2.1.8 Yeast strain

All yeast reporter strains were purchased from DB Biosciences Clontech, Heidelberg, Germany

Strain	Reporter	Transformation markers
CG 1945	HIS3, lacZ	trp1, leu2
AH 109	HIS3, ADE2, lacZ, MEL1	trp1, leu2

2.1.9 Mouse strain

BALB/c mouse strain was used for whole mount *in situ* hybridization experiments. Animals were obtained from the animal facility of the GSF-Hämatologikum, Grosshadern and Neuherberg.

2.1.10 Mammalian cell lines

All cell lines are available in the Cell Culture Collection from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), unless discriminated.

2.1.10.1 Murine and rat cell lines

Name	Cell type/ Origin
NIH3T3	swiss mouse embryo fibroblast
MEF*	mouse embryonic fibroblast (primary fibroblast from day 15 of the mouse embryo)
BA/F3	mouse pro B-cells, IL-3 dependent line established from peripheral blood
TGR*	rat fibroblast

* courtesy from Dr. Michael Hölzel, GSF, Munich

2.1.10.2 Human cell lines

Normal cell lines

Name	Cell type/ Origin
293T	human embryonal kidney
WI-38*	human normal embryonic lung fibroblast, cells have a finite lifetime of 50 + or – 10 population doublings. Growth of the cells is enhanced by addition of TNF alpha to the medium
JB4**	T-cell line, stimulated bimonthly
TYRF8**	T-cell line, HLA-A2-restricted tyrosinase peptide specific cytotoxic clone. Cells are stimulated bimonthly with IL-2-secreting HLA-A 0201-positive and tyrosinase-positive melanoma cells

* courtesy from Dr. Michael Hölzel, GSF, Munich; ** courtesy from Dr. Elfriede Nössner, GSF, Munich

Tumor cell lines

Name	Cell type/ Origin
HELA	human cervix carcinoma
U2OS*	human osteogenic sarcoma, p53 wild type
SaOS	human osteogenic sarcoma, p53 null
T98G*	human glioblastoma multiforme, cells enter a viable G1 arrest state when deprived of serum or when crowded

* courtesy from Dr. Michael Hölzel, GSF, Munich

B-cell leukemia and B-cell lymphoma cell lines

Name	Cell type/ Origin
NALM6	human B-cell precursor leukemia
Granta 519	human B-cell lymphoma
HBL-2*	human mature B-cell line derived from B-NHL (diffuse large cell), carries t(11;14)(q13;q32) leading to alteration of the BCL1 and IGH genes
JeKo-1	human B-cell lymphoma established from the peripheral blood of a 78-year-old woman with B-cell non-Hodgkin's lymphoma (B-NHL), specifically mantle cell lymphoma
Karpas 422	human B-cell lymphoma established from the pleural effusion of a 73-year-old woman with B-NHL
NCEB-1*	human mature B-cell line derived from B-NHL (diffuse centroblastic-centrocytic), carries t(11;14)(q13;q32) leading to alteration of the BCL1 and IGH

* courtesy from Dr. Martin Dreyling, CCG-Leukemia, Grosshadern, Munich

T-cell leukemia and T-cell lymphoma cell lines

Name	Cell type/ Origin
JURKAT	human T cell leukemia, immature T cell line established from the peripheral blood of a 14-year-old boy with ALL
MOLT-4	human T cell leukemia, immature T cell line established from the peripheral blood of a 19-year-old man with ALL
HUT 78*	human mature T-cell line established from the peripheral blood of a 53-year-old male with cutaneous T-cell lymphoma (Sézary syndrome). Cells carry the t(2;8)(q34;q24) leading to TCL4-MYC fusion gene

* courtesy from Dr. Elfriede Nössner, GSF, Munich

Myeloid leukemia cell lines

Name	Cell type/ Origin
HL-60	human acute myeloid leukemia established from the peripheral blood of a 35-year-old woman with AML (FAB M2). Cells can be used for induction of differentiation studies (responsive to DMSO, TPA and other reagents), carry amplified c-myc gene and lack wild type p53
KASUMI-1	human acute myeloid leukemia. Cell line established from the peripheral blood of a 7-year-old Japanese boy with AML (FAB M2). Cells carry the t(8;21) ETO-AML1 fusion gene
U937	human histiocytic lymphoma. Cells express markers and properties of monocytes and carry the t(10;11) leading to CALM-AF10 fusion gene
K-562	erytroid cell line. Cells carry the t(9;22) leading to BCR-ABL fusion gene

2.1.11 Plasmids

pBluescript II KS (+/-)	Cloning vector, Stratagene, La Jolla, USA
pGEM®-T Easy	Cloning vector with 3'-T overhangs at insertion site for direct cloning of PCR products, Promega, Madison, USA
pGBT9; pGBKT7	Expression of GAL4-DBD (amino acids 1-147) fusion proteins in the yeast system, DB Biosciences Clontech, Heidelberg, Germany
pGADGH; pGADT7	Expression of GAL4-AD (amino acids 768-881) fusion proteins in the yeast system. DB Biosciences Clontech, Heidelberg, Germany
pEGFP-; pECFP-; pEYFP-C1	Expression of green (GFP), cyan (CFP) or yellow (YFP) fluorescent fusion proteins, DB Biosciences Clontech, Heidelberg, Germany
pGEX-4T2	Expression of GST fusion proteins in bacterial cells. Vector contains a tac promoter for protein expression upon IPTG induction, Amersham Pharmacia Biotech
pM1	Expression of GAL4-DBD (1-147) fusion proteins in the mammalian system. The hybrid protein is targeted to the cell's nucleus by the GAL4 NLS, DB Biosciences Clontech, Heidelberg, Germany
pGAL4LUC	Reporter plasmid encoding the luciferase gene under the transcriptional control of the GAL4-binding sites
$pGAL4_5tkLUC$	Reporter plasmid encoding the luciferase gene under the transcriptional control of the Herpes simplex virus thymidine kinase promoter and 5 GAL4-binding sites, (Netzer et al., 2001)
pRL-null	Co-reporter vector encoding the *Renilla* luciferase protein, Promega, Madison, USA
pUC18-HA tag	Provides a COOH-terminal HA tag, (Holzel et al., 2005)
pRTS-1	Episomal all-in-one plasmid with tight doxycycline-dependent gene expression. The bidirectional promoter controls simultaneously the expression of both GFP and the gene of interest, (Bornkamm et al., 2005)

2.1.12 Constructs

pGADGH-CATSv2CT	Joachim Gläsner, Institute für Humangenetik, Göttingen, Germany
pGEX4T-2- CATSv2CT	Joachim Gläsner, Institute für Humangenetik, Göttingen, Germany
YFP-CALM/AF10	Alexandre Krause, CCG Leukemia, GSF, Munich, Germany
pGBKT7-CALM (1-408)	Britta Kaltwasser, CCG Leukemia, GSF, Munich, Germany
GAL4-CALM (1-652)	Sigrun Bartels, Institute für Humangenetik, Göttingen, Germany
pGBT9-CALM (1-335)	Sigrun Bartels, Institute für Humangenetik, Göttingen, Germany
pAS2-CALM (1-294)	Sigrun Bartels, Institute für Humangenetik, Göttingen, Germany
pAS2-CALM (1-221)	Sigrun Bartels, Institute für Humangenetik, Göttingen, Germany
YFP-Nucleostemin	Michael Hölzel, GSF, Munich, Germany
YFP-Nucleophosmin	Angus Lamond, University of Dundee, Dundee, UK
YFP-HAX1	Luciana Fontanari Krause, CCG Leukemia, GSF, Munich, Germany
YFP-SIVA	Luciana Fontanari Krause, CCG Leukemia, GSF, Munich, Germany

2.1.13 Oligonucleotides

All nucleotides were synthesized by Metabion GmbH, Martinsried, Munich, at the concentration of 100 pmol/µl.

CATS specific primers

Oligonucleotide	Sequence	Position at *CATS* sequence*,[1]	Tm °C
CATSE1F1(364)	5'-AAGAGTGCTTTGGGTGCCG-3'	Exon 2 (343-361)	56-60
CATSE4R1(784)	5'-TGAATGAGAGAGACGATGTCACC-3'	Exon 5 (785-763)	56-60
CATSE5R1(828)	5'-CGGTCAGACAGGGCTTGTTTC-3'	Exon 6 (1116-1096)	56-60
FhCATSXhoI	5'-CTACCTCGAGAGATGGCTTCTCGGTGGC-3'	Exon 2 (74-91)	55
RhCATSHindIII	5'-CATTAAGCTTGCAGGCACTTCCTCAGTC-3'	Exon 5 (804-787)	55
hCATS774-793	5'-GGAAGAGAGTGGTGACATCG-3'	Exon 4/5 (753-772)	58
hCATSI4F1	5'-AACCTGCCCTTCCTCCAGAAC-3'	Intr 4 (9997-10017)[1]	56
hCATSf SalI 635(ex3)	5'-TATAGTCGACACTCCTCAACAGAGCCCCTC-3'	Exon 3 (635-654)	55
hCATSr SalI 909(ex6)	5'-TATAGTCGACCCCAGGGGTACTGACGACTCC-3'	Exon 6 (1198-1178)	55
hCATS 5' ORF BglII	5'-GAAGATCTATGGCTTCTCGGTGGC-3'	Exon 2 (76-91)	55
hCATS 3' ORF XhoI	3'-CCGCTCGAGGCAGGCACTTCCTCAGTC-3'	Exon 5 (804-787)	55
hCATSATGf(PUC18)	5'-Pho-GCCACCATGGCTTCTCGGTGGCAG-3'	Exon 2 (76-93)	60
hCATSΔTGAv1r(PUC18)	5'-Pho-GTCATGAATGAGAGAGACGATGTC-3'	Exon 5 (789-766)	60
hCATSΔTGAv2r(PUC18)	5'-Pho-CGGGGAAGCATAGACATCC-3'	Exon 6 (1153-1135)	60

*human CATS cDNA (Figure: 3.2), [1]AC055872.2nt89161-101255 (BAC clone)

Cats specific primers

Oligonucleotide	Sequence	Position at *CATS* sequence*,[1]	Tm °C
mCATSF113	5'-AAACTGGACCAAGCACATGGCG-3'	Exon 2 (270-291)	60
mCATSF364	5'-TCCAGAGCCAGAGCAGCAAG-3'	Exon 2 (501-520)	60
mCATSR630	5'-CAGACTCCCTCCTTAGCCGC-3'	Exon 3 (805-786)	60
mCATS F552	5'-GAAGAACACTCGGCTATGC-3'	Exon 3 (690-708)	55
mCATSE1R(133)	5'-ATGCCCTGCCACTGAGACGC-3'	Exon 2 (308-289)	63
F10-3T319	5'-GTTTCCAGACTCAGAACTATC-3'	Exon 6 (1351-1331)	55
mCATSR886	5'-GGAAGTTCATGGGTTTGGGGTAG-3'	Exon 6 (1065-1045)	62
mCATSR942	5'-GCCAAATCCCTGTTAGCAGTGC-3'	Exon 6 (1121-1099)	60
mCATS 5'ORF EcoRI	5'-CCGGAATTCACATGGCGTCTCAGTGGCAG-3'	Exon 2 (284-303)	55
mCATS 5'ORF XhoI	5'-CTACCTCGAGACATGGCGTCTCAGTGGCAG-3'	Exon 2 (284-303)	55
mCATS 3'ORF Not I	5'-CTAGCGGCCGCATGCTTGCTGTCCTCATTCACG-3'	Exon 5 (994-973)	55
mCATS3'ORFΔTGAXhoI	5'-AATCTCGAGTTCACGGATAAGGGAGACGG-3'	Exon 5 (978-959)	55
mCATS Int.1 L02H-4	5'-TGTGTGGTGTGTCCAAGAGC-3'	Intr 2 (3765-3784)[1]	60
mCATS Ex.4 f	5'-TAGAGACGCACAGTGGAACCTC-3'	Intr 5 (6471-6492)[1]	60
mCATS 3'UTR-R1	5'-TCAGACTCAGACCCACTGTC-3'	3'Cats (7667-7648)[1]	56
mCATS 3'UTR-F1	5'-ACCCCACACTGACACCGTAG-3'	3'Cats (7223-7242)[1]	60
mCATS 5'UTR-R1	5'-CGGAGCAGACTTAGCAATCC-3'	Intr 1 (2532-2513)[1]	60

mCATS 5'UTR-F1	5'-CACAGTAGTTCAGGGAGATTTG-3'	5'Cats (268-289)[1]	56
mCATS 5'UTR-R2	5'-ATCACTGTTACCGACCAGC-3'	Exon 1 (217-199)	56
mCATS 5'UTR-F2	5'-TTGGAGATTTGGTCTGG-3'	5'Cats (694-710)[1]	52
mCATS 5'UTR-R3	5'-GCTCTACGCATCTTTCAATC-3'	5'Cats (1660-1641)[1]	55
mCATS 5'UTR-F3	5'- CCCTGCTACTGTTAGTGAGTG-3'	5'Cats (1022-1042)[1]	55
mCATSInt.3r	5'-CCCTGTGGTTTTCTTACTCC-3'	Intr 5 (6326-6307)[1]	56
mCATSInt.1r	5'-CAGTGTGGTAACTCAGCAGG-3'	Intr 2 (4605-4586)[1]	56
mCATSInt.2f	5'-AGACATCTGGAAGACCGAGG-3'	Intr 3 (5290-5309)[1]	59

*Murine cDNA IMAGp998H174619Q2 clone, [1]L02EcoRI-2 (murine genomic clone)

PCBP-1 specific primers

Oligonucleotide	Sequence	Position at the cDNA sequence*	Tm °C
PCBP1XhoIf	5'-CTACCTCGAGCCATGGATGCCGGTGTGACTG-3'	(176-196)	56
PCBP1HindIIIr	5'- ATTAAGCTTGCAGAAAGGGGTTATTGAGGGAAC-3'	(1282-1259)	56
PCBP1seq0fw	5'-TGTCCGGAGAGAATCATC-3'	(337-354)	54
PCBP1seq1fw	5'-AGTGCGGCTCCCTGATTG-3'	(500-517)	62
PCBP1seq2fw	5'-CTAGATGCCTACTCGATTC-3'	(832-850)	50

*GenBank acc. Nr.: NM_006196

KIS specific primers

Oligonucleotide	Sequence	Position at the cDNA sequence*	Tm °C
FKISXhoI	5'-CTACCTCGAGCTTAACCCACACCGATGGC-3'	(132-150)	56
KISXhoI5'new	5'-CTACCTCGAGCTTTACCCACACCGATGGC-3'	(132-150)	56
RKISHindIII	5'-CATTAAGCTTGGAATAACCCAAGAAATGGAAG-3'	(1460-1439)	56
KISseq1fw	5'-TTCTCCAAATGTGCCATC-3'	(445-462)	55
KISseq2fw	5'-GCAGAGTGATACAGAATGTACC-3'	(784-805)	54
KISseq3fw	5'-TGAATGTGCTGGATGATG -3'	(1122-1139)	54

*GenBank acc. Nr.: NM_175866/AJ536197

Control primers

Oligonucleotide	Sequence	Position at the cDNA sequence*	Tm °C
HPRT fw	5'-GGGGGCTATAAGTTCTTTGC-3'	Exon 3 (295-314)	60
HPRT rev	5'-TCCAACACTTCGAGAGGTCC-3'	Exon 6 (606-587)	60
hGAPDHforward	5'-GCACCACCAACTGCTTAGCACC-3'	Exon 7 (530-551)	60
hGAPDHreverse	5'-GTCTGAGTGTGGCAGGGACTC-3'	Exon 9 (1166-1146)	60
mGAPDHf	5'-CATCACCATCTTCCAGGAGC-3'	(271-290)	60
mGAPDHr	5'-ATGACGTTGCCCACAGCCTT-3'	(714-695)	60
INF 5'	5'-AGAATCTCTCCTTTCTCCTG-3'	(180-199)	56
INF 3'	5'-GATCTCATGATTTCTGCTCTG-3'	(565-545)	56

*GenBank acc. Nr.: NM_013556 (mouse HPRT), NM_002046 (human GAPDH), NM_001001303 (mouse Gapdh), NM_002170 (interferon alpha 8)

Vector primers

Oligonucleotide	Sequence	Position	Tm °C
T7	5'-GTAATACGACTCACTATAGGGC-3'		55
T3	5'-AATTAACCCTCACTAAAGGG-3'		55
SP6	5'-AGGTGACACTATAGAATAC-3'		55
Y2H1	5'-TCATCGGAAGAGAGTAG-3'	(1155-1171 pGBKT7)	55
Y2H3'AD	5'-AGATGGTGCACGATGCACAG-3'	(2102-2083 pGADT7)	55
Y2H2-729-751	5'-CGTTTGGAATCACTACAGGGATG-3'	(1799-1821 pGADT7)	55
M13/pUCRevSeqP	5'-AGCGGATAACAATTTCACACAGG-3'	(2187-2209)	55
pGEX5'	5'-GGGCTGGCAAGCCACGTTTGGTG-3'	(869-891)	55
5'pME18S-FL3	5'-CTTCTGCTCTAAAAGCTGCG-3'	(774-793)	
3'pME18S-FL3	5'-CGACCTGCAGCTCGAGCACA-3'		
pEGFPC-1240-1258	5'-AAAGACCCCAACGAGAAGC-3'	(1240-1258)	55
pUC18-M13fwd	5'-GCGGATAACAATTTCACACAGG-3'	(2188-2209)	55
pUC18-M13rev	5'-GTAAAACGACGGCCAGTGC-3'	(2358-2340)	55
pEBNA_Sfi+55bp (rev)	5'-AATCAAGGGTCCCCAAACTC-3'	(1752-1733)	55

*GenBank acc. Nr.: U55763 (pEGFP-C1), U13854 (pGEX-4T2)

2.1.14 Antibodies

Name	Company	dilution
Anti-GFP rabbit IgG fraction	Molecular Probes, Inc.	1:5000
CALM (S-19, G-17 and C-18)	Santa Cruz Biotechnology, Inc.	1:1000
GAL4 (DBD) (RK5C1)	Santa Cruz Biotechnology, Inc.	1:1000
mouse monoclonal HAX-1	BD Pharmingen, Heidelberg	1:3000
SIVA (C-20)	Santa Cruz Biotechnology, Inc.	1:2000
CD3-12	Dr. Elizabeth Kremmer, GSF, Munich	1:10
CD4-PE labeled	BD Pharmingen, Heidelberg	1:100
CD8-APC labeled	BD Pharmingen, Heidelberg	1:100
Anti-β-Actin clone AC-15	Sigma®	1:2000
Mac1 (CD11b)-APC labeled	BD Pharmingen, Heidelberg	1:250
Gr-1-PE labeled	BD Pharmingen, Heidelberg	1:250
Ter119-PE labeled	BD Pharmingen, Heidelberg	1:250
B220-APC labeled	BD Pharmingen, Heidelberg	1:250
Sca-1-PE labeled	BD Pharmingen, Heidelberg	1:250
c-kit (CD117)-APC labeled	BD Pharmingen, Heidelberg	1:250
Donkey anti-rabbit IgG-HRP conjugated	Santa Cruz Biotechnology, Inc.	1:2000
Donkey anti-goat IgG-HRP conjugated	Santa Cruz Biotechnology, Inc.	1:2000
Sheep anti-mouse IgG-HRP conjugated	Chemicon® International	1:1000-3000
Goat Anti-Rat IgG + IgM -HRP conjugated	Jackson ImmunoResearch Laboratories, Inc.	1:3000
Mouse Anti-Rat IgG-HRP conjugated	Jackson ImmunoResearch Laboratories, Inc.	1:2000
Goat anti-rat IgG + IgM-Cy™3 conjugated	Jackson ImmunoResearch Laboratories, Inc.	1:250
Anti-Digoxigenin-AP conjugate	Roche, Mannheim, Germany	1:5000

2.1.15 Human cDNA IMAG clones

All IMAG cDNA clones were ordered from the Resource Centre of the German Human Genome Project (RZPD, Berlin, Germany).
Human IMAG cDNA clones are IMAGp998G039611 and IMAGp998D169579 (both cloned into pCMV-SPORT6 vector). The murine IMAG cDNA clones are IMAGp998E09899Q2 (cloned into pT7T3D-Pac vector); IMAGp998F103793Q2 and IMAGp998H174619Q2 (both cloned into pME18S-FL3 vector).

2.1.16 Mouse cosmid library

The 129/ ola mouse cosmid library Nr.: 121 as well as the isolated genomic clones were ordered from the Resource Centre of the German Human Genome Project (RZPD, Berlin, Germany). Library was cloned into Lawrist 7 and kept in the host DH5 alpha .

2.1.16.1 Mouse genomic clones

MPMGc121C1938Q2, MPMGc121C0434Q2, MPMGc121L02225Q2, MPMGc121H24352Q2, MPMGc121A14416Q2, MPMGc121I20398Q2, MPMGc121D13437Q2, MPMGc121K17553Q2 and MPMGc121P22586Q2.

2.1.17 cDNAs

cDNA from leukemia patient samples used in 3.3.1, were kindly provided by Alexandre Krause (CCG Leukemia, GSF, Munich).
cDNA from leukemic cells derived from CALM/AF10 murine bone marrow transplant model (used in 3.3.2) were kindly provided by Aniruhddha Deshpande (CCG Leukemia, GSF, Munich). cDNAs were prepared from highly purified $B220^{+}/Mac1^{-}$, $B220^{+}/Mac1^{+}$ and $B220^{-}/Mac1^{+}$ cells obtained after propagation of a single sorted $B220^{+}$/myeloid $marker^{-}$ cell from a bulk leukemic cell population grown in IL3 supplemented medium.

2.1.18 Cellular lysates

Lysates from serum stimulated T98G (3.10.4) as well as from synchronized HeLa and U2OS cells (3.10.5) were kindly provided by Dr. Michael Hölzel (GSF, Munich).

2.2 Methods

All molecular biology standard methods were performed according to the ”Molecular Cloning” (Sambrook, 1989) with minor or no modifications.

2.2.1 Isolation of Nucleic Acids

2.2.1.1 Mini preparation of plasmid DNA (Miniprep)

Miniprep was employed to isolate small amount of DNA from a large number of clones. First, single *E. coli* colonies were inoculated into 3 ml of LB medium plus selective antibiotics and incubated O/N with vigorous shaking at 37°C. From each culture, 1.5 ml was centrifuged for 5 min at 14 000 rpm and the supernatant removed. Pellet was resuspended in 300 µl of E1 resuspension buffer and cells were lysed by adding 300 µl E2 lysis buffer followed by 5 min incubation at RT. 300 µl of E3 neutralization buffer was added to the lysate, gently mixed and reaction was centrifuged for 10 min at 14 000 rpm. The supernatant containing the plasmid DNA was transferred to a fresh 1.5 ml tube. DNA was purified by precipitation with 0.7 vol. of isopropanol followed by 10 min incubation at -20°C and 20 min centrifugation at 4°C (14 000 rpm). The DNA pellet was washed with 300 µl 70 % ethanol (10 min; 14000 rpm, 4°C), air dried and dissolved in 20-30 µl ddH_2O. DNA was stored at -20°C.

Glycerin stocks were prepared for long-term storage of the bacterial clones. For that 700 µl bacterial suspension plus 300 µl glycerin were placed in a fresh 1.5 ml tube, mixed by vortexing and stored at -80°C.

2.2.1.2 Midi preparation of plasmid DNA (Midiprep)

In order to isolate larger amount of plasmid DNA Midi preparation was carried out. JETSTAR Plasmid Midi Kit (Genomed) was used for clean preparation. Single *E. coli* colony or approx. 5 µl of glycerin stock (2.2.1.1) was inoculated into 50 ml LB medium plus selective antibiotics and incubated O/N with vigorous shaking at 37°C. Cells were harvested by centrifugation (4 000 rpm for 15 min). Pellet was resuspended in 4 ml E1 solution. For the cell lysis, 4 ml of solution E2 was added and the suspension incubated for 5 min at RT. 4 ml of solution E3 was added to the reaction and immediately mixed by inversion. The suspension was centrifuged for 10 min and the supernatant containing the plasmid DNA was applied to a previously equilibrated column (10 ml solution E4 had been applied to the column for equilibration). After the suspension has ran through, DNA bound to the column was washed

twice with 10 ml solution E5. The DNA was then eluted from the column with 5 ml solution E6 and isopropanol precipitated as described above (2.2.1.1). DNA was dissolved in 50 µl ddH_2O and stored at -20°C after concentration has been determined (2.2.10).

2.2.1.3 Maxi preparation of plasmid DNA (Maxiprep)

Highly purified endotoxin-free DNA plasmid used in transfection experiments (2.2.21) was isolated with the Endofree® Plasmid Maxi kit (Qiagen). Single *E. coli* colony or approx. 5 µl of glycerin stock was inoculated into 2 ml LB medium plus selective antibiotics and incubated for approx. 6 hrs with vigorous shaking at 37°C. The preculture was diluted 1:500 into a larger vol. (250 ml) of selective medium and expanded to saturation at the same condition as above.

The bacterial cells were harvested by centrifugation at 6 000 x g for 15 min at 4°C. The pellet was resuspended with 10 ml P1 buffer. For cell lysis, 10 ml of P2 buffer was added, mixed by inversion, and the mixture was incubated for 5 min at RT. 10 ml of pre-chilled P3 buffer was added, mixed and the reaction was applied into the QIAfilter Cartridge. After 10 min incubation at RT, cell lysate was filtered and recovered in a 50 ml tube. 2.5 ml buffer ER was added to the lysate, the mixture was mixed and further incubated for 30 min on ice. After that the cell lysate was applied into a previously equilibrated column (column was equilibrated with 10 ml QBT buffer). Plasmid DNA bound to the column was washed twice with 30 ml QC buffer. The DNA eluted with 15 ml QN buffer, precipitated with 0.7 vol. isopropanol, incubated 10 min at -20°C and centrifuged at 15 000 x g for 30 min at 4°C. The DNA pellet was washed with 15 ml 70% ethanol, further centrifuged (20 min, 15 000 x g, at 4°C), air dried and dissolved in 500 µl endotoxin-free buffer (EB). The DNA was stored at -20°C after concentration has been determined (2.2.10).

2.2.2 Cleavage of nucleic acids

2.2.2.1 Digestion of DNA with restriction enzymes

For specific digestion of DNA, type II restriction endonucleases are essential. These enzymes are capable of recognizing, binding and cleaving short defined nucleotide sequences within the target DNA. The restriction of DNA was carried out with defined buffer, when multiple enzymes were used, a Five Buffer Plus System such as Y+/Tango™ (MBI Fermentas) was required. For single digestion the following components were placed in a 1.5 ml tube.

1 µg	DNA
1X	appropriate restriction enzyme buffer
5 U	restriction enzyme
to 20 µl	ddH_2O

Reaction was incubated for 2 hrs to O/N at optimal temperature as recommended for each enzyme. Alternatively, reactions were scaled up to 50 µl vol.

2.2.2.2 Dephosphorylation of digested DNA

Alkaline phosphatase is capable of removing the phosphate group from the 5' free end of the DNA molecule, preventing ligation of DNA, especially re-ligation of previously restricted vectors. The DNA was dephosphorylated as follows:

1 µg	DNA, previously digested (2.2.2.1) and purified (2.2.3 or 2.2.7)
5 µl	10X alkaline phosphatase buffer
1 U	alkaline phosphatase (CIAP or SAP)
to 50 µl	ddH_2O

Reaction was incubated at 37°C for 1 hr and stopped by heating at 85°C (CIAP) or 70°C (SAP) for 15 min. DNA was then purified by ethanol precipitation (2.2.3)

2.2.2.3 Blunting sticky end DNA

Klenow enzyme was used to produce blunt end products, either through its polymerase activity used to fill in 3' recessed ends in the presence of dNTPs or through its 3'-5' exonuclease activity used to cleave protruding 3' overhangs from digested DNA (2.2.2.1). The reaction was carried out as follows:

0.1-4 µg	DNA, previously digested (2.2.2.1) and purified (2.2.3 or 2.2.7)
0.5 µl	2mM dNTP mix
0.5 µl	Klenow Enzyme (10 U/µl)
2 µl	10X Klenow buffer
to 20 µl	ddH_2O

The mixture was incubated for 10 min at 37°C. Reaction was stopped by heat inactivation at 70°C for 10 min. DNA was then purified by ethanol precipitation (2.2.3) and used for ligation (2.2.4).

2.2.3 Precipitation of DNA

Acid nucleic were purified from buffers used in previous reactions (e.g. digestion, dephosphorylation, sequencing, etc.) through ethanol precipitation. Reaction was carried out mixing 1 vol. of DNA sample, with 2.5 vol. of ethanol and 1:10 vol. of 5 M NaAc pH 5.2. Reaction was incubated at -20°C for 10 min and centrifuged at 14 000 rpm for 20 min at 4°C. DNA pellet was washed with 50-200 µl 70 % ethanol, centrifuged at 14 000 rpm for 10 min at 4°C, air dried and diluted in the desired vol. of ddH_2O. Purified DNA was used for subsequent reactions or stored at -20°C.

2.2.4 Ligation

Both vector and restriction fragment (insert) were restricted (2.2.2) in a way to have compatible ends. Vector and insert were used at proportion 3:1 in the ligation reaction as described below:

30 ng	restricted vector DNA (2.2.2) and purified (2.2.3 or 2.2.7)
90 ng	insert
4 µl	5X T4 DNA ligase buffer
1 µl	T4 DNA ligase (5 U/µl)
to 20 µl	ddH_2O

The reaction was incubated for 15 min to 1 hr at RT.

2.2.5 Delivery of plasmid DNA into bacterial cell

2.2.5.1 Preparation of chemically competent cells

An aliquot of frozen cells (*E. coli* strain BL21) was inoculated into 10 ml LB medium free from antibiotics and incubated O/N with vigorous shaking at 37°C. The O/N culture was diluted into a larger volume of medium (300 ml) and further incubated for approx. 2-3 hrs until culture has reach OD_{600} 0.5-0.7. Bacterial suspension was cooled down on ice and cells were harvested by centrifugation. Cell pellet was resuspended with 120 ml Tfb1 (2.1.5) and incubated for 10-15 min on ice. Suspension was centrifuged, supernatant was discarded and cells were ressupended with 12 ml Tfb2 (2.1.5). Bacterial cells were aliquoted (100 µl vol.), frozen in liquid nitrogen and stored at -80°C. All centrifugation steps were carried out at 4000 rpm for 10 min at 4°C.

2.2.5.2 Transformation

Chemically competent cells (100 µl aliquot) (2.2.5.1) were mixed with 1 µl plasmid DNA (50-100 ng) or 20 µl ligation reaction (2.2.4). Reaction was incubated for 45 min on ice, heat shocked for 90 sec at 42°C and placed on ice for 2 more min. 100 µl LB medium was added to the reaction, which was incubated for 30 min shaking at 37°C. Cells were subsequently stricked onto LB agar plate containing appropriate selective antibiotic. Plates were incubated O/N at 37°C.

2.2.5.3 Preparation of electrocompetent cells

O/N culture of *E coli* strain XL1-blue MRF', grown as in 2.2.5.1 was diluted into 400 ml LB medium and incubated for approx. 2-3 hrs until culture has reach OD_{600} 0.5-0.6. Bacterial suspension was cooled on ice and cells were harvested by centrifugation. Pelleted cells were washed twice with 40 ml ice cold ddH_2O, twice with 20 ml ice cold 10 % glycerol (in ddH_2O) and finally resuspended in 800 µl of the same solution (ice cold 10 % glycerol in ddH_2O). Bacterial cells were aliquoted (40 µl vol.) and stored at -80°C. All centrifugation steps were carried out at 4 500 rpm for 5 min at 4°C.

2.2.5.4 Electroporation

Electrocompetent cells (40 µl aliquot) (2.2.5.3) were mixed with 1 µl plasmid DNA (50-100 ng) or 2-7 µl ligation reaction (2.2.4), incubated for 1 min on ice and transferred to a previously cooled electroporation cuvett. Cuvett was placed in an electroporator and cells were electroporated at 2.5 kV. After electroporation, 1 ml pre warmed LB medium (37°C) was immediately added to the cells, the suspension was transferred to a fresh 1.5 ml tube and incubated for 1 hr with vigorous shaking at 37°C. Cells were harvested by brief centrifugation (15 sec, at 14 000 rpm), resuspended with 50 µl LB medium and stricked onto LB agar plates containing appropriate selective antibiotic. Plates were incubated O/N at 37°C.

2.2.6 Agarose-gel electrophoresis for DNA

Non-denaturing gel electrophoresis was employed in the separation of restricted DNA, for both analysis and isolation of the fragments. The concentration of the gels used was determined according to the range of separation of linear DNA in gels containing different amounts of agarose as shown below:

Agarose (%)	Range of separation of linear DNA
0.5	700 bp to 25 kb
0.8	500 bp to 15 kb
1.0	250 bp to 12 kb
1.2	150 bp to 6 kb
1.5	80 bp to 4 kb

Gel was prepared by boiling the desired amount of agarose in 50 ml 0.5X TBE (2.1.5). After gel has cooled to approx. 50°C, 3 µl ethidium bromide was added. The gel was poured onto a horizontal gel bed. DNA samples were loaded on the gel slots with 1 µl Blue Juice loading buffer (2.1.5). Nucleic acids ran under electric current (50-120 V) from the negative to the positive pole.

2.2.7 Isolation of DNA fragments after gel electrophoresis

DNA fragment was cut out of the gel under UV light (312 nm) with a sterile scapel and transfered to a 1.5 ml tube. Gel extraction was performed with JETSorb DNA extraction from Agarose Gels (Genomed, Bad Oeynhausen). For each 100 mg gel slice, 300 µl buffer A1 and 10 µl JETSORB suspension were added. The reaction was incubated at 50°C for 15 min. and centrifuged for 1 min at 10 000 x g. The supernatant was discarded and 300 µl of buffer A1 was added. The pellet was then resuspended and centrifuged once more.

The same procedure was performed twice, with buffer A2. After the second wash step with buffer A2, the pellet was dried at 50°C for 5 min. The DNA was then eluted with 30 µl ddH_2O, incubated at 50°C for 5 min and centrifuged as before. The supernatant containing the purified DNA fragment was transfered to a fresh 1.5 ml tube and was used for subsequent reactions such as ligation (2.2.4).

2.2.8 Extraction of genomic DNA

Genomic DNA from 293T, HeLa and HL-60 cells (2.1.10.2) was isolated using DNeasy® Tissue Kit (Qiagen). For the isolation, 4 x 10^6 cells were harvested and ressuspended in 200 µl PBS. In order to obtain RNA-free genomic DNA, cells were incubated with 4 µl RNase A (100 mg/ml) for 2 min at RT. Cells were lysated by adding 20 µl of proteinase K (>600 mAU/ml) and 200 µl of buffer AL, mixing thoroughly by vortexing and incubating at 70ºC for 10 min. 200 µl of ethanol was added to the lysate, which was applied into the DNeasy spin column (placed in a 2 ml collection tube) and centrifuged for 1 min at 6 000 x g. The flow-through was discarded, the DNeasy spin column was placed in a new collection tube and washed once with 500 µl AW1 buffer (1 min 6 000 x g) and once with 500 µl AW2 buffer (3 min 14 000 rpm). The flow-through was discarded again and the DNeasy spin column was placed in a fresh 1.5 ml tube. In order to obtain a maximal yield of DNA, elution was performed in two steps. In each step 200 µl buffer AE was applied to the DNeasy column, incubated for 1 min at RT and DNA was collected by centrifugation (1 min at 6 000 x g). The isolated genomic DNA was stored at 4°C.

2.2.9 RNA extraction

Total RNA from adult mouse tissue, mouse embryo (2.2.19.1), cultured cell lines (2.1.10) and FACS sorted hematopoietic cell subpopulation (2.2.31) was isolated using the RNeasy® Mini Kit (Qiagen). For extraction from cultured cell lines, approx. 4 x 10^6 cells were lysed by the addition of 350 µl RLT buffer. For extraction from hematopoietic cells, the whole amount of sorted cells (from 807 cells to 2.4 x 10^5 depending on the cell subpopulation) was lysed in 350 µl RTL buffer. For extraction from adult mouse tissue or whole mouse embryos, samples were first weight, 600 µl RLT buffer was added per 30 mg material and subsequently homogenized with a Tissuemizer (Tekmar-Dohrmann). For further homogenization, samples were passed through a QIAshredder spin column placed in a 2 ml collection tube (2 min at 14 000 rpm). 1 vol. of 70 % ethanol was added to the homogenized lysate and mixed thoroughly by pipetting. The lysate was applied to a RNeasy mini column placed in a 2 ml collection tube and centrifuged for 15 sec at 8 000 x g. The flow-through was discarded and the total RNA bound to the column was washed once with 700 µl RW1 buffer and twice with 500 µl RPE buffer. The first and second centrifugation steps were carried out for 15 sec and the third for 2 min at 8 000 x g, the flow-through was discarded after each washing step. Finally, to elute the RNA from the RNeasy column (placed in a fresh 1.5ml tube), 40 µl RNase-free H_2O was

applied to the column and incubated for 1 min at RT. RNA was collected by centrifugation (1 min at 8 000 x g). Yield was determined by measuring RNA concentration and quality was assayed by loading 2 µl of the isolated RNA on 1 % agarose gel (2.2.6) The isolated total RNA was stored at -80°C.

2.2.10 Determination of nucleic acid concentration

Concentration of DNA and RNA was determined in an UV spectrophotometer (BioPhotometer 6131, Eppendorf). 2 µl sample was diluted in 198 µl ddH_2O and measured by absorption at 260 nm and 280 nm. The concentration was calculated according to the ratio A_{260}/A_{280} . $1A_{260}$ unit of double-stranded DNA = 50 µg/ml and of RNA = 40 µg/ml.

2.2.11 Reverse transcriptase reaction (synthesis of cDNA)

Reverse transcriptase reaction followed by PCR (RT-PCR) is a sensitive technique used to determine the presence or absence of RNA templates or to quantify the level of gene expression. It is also an useful tool for amplification of genes of interest or determination of exon-intron boundaries. A cDNA strand, complementary to the RNA template is synthesized with the extension of oligo(dT) primers by the reverse transcriptase. Synthesized cDNA is used as template for subsequent PCR reactions as described in 2.2.12. In order to avoid genomic DNA contamination of the RNA and eventual amplification of these contaminants, RNA (2.2.9) was treated with DNAse I prior to cDNA synthesis as follows: 1 µg of RNA sample was mixed with 1 µl 10X DNase I reaction buffer, 1 µl DNase I (1 U/µl) and DEPC-H_2O to a final vol. of 10 µl. The reaction was incubated for 15 min at RT. The DNAse I was inactivated by adding 1 µl of 25 mM EDTA and heating at 65ºC for 10 min. For the first strand cDNA synthesis, 1 µg DNAse-free RNA (10 µl) was mixed with 1 µl dNTP (10 mM) and 1 µl oligo(dT) primer. The RNA mixture was denatured at 65ºC for 5 min, chilled on ice and the contents of the tube was collected by brief centrifugation. 4 µl of 5X First-Strand Buffer, 2 µl of 0.1 M DTT and 1 µl RNase inhibitor (40 U/µl) was added to the reaction, gently mixed and incubated for 2 min at 42ºC. Then 1 µl of SuperScript II enzyme was added and the reaction was incubated for 50 min at 42°C. Enzyme was inactivated by heating the reaction to 70ºC for 15 min. 1 µl of the synthesized cDNA (20 µl total vol.) was used for subsequent PCR reaction (2.2.12).

2.2.12 PCR

The PCR technique is employed to amplify DNA. The amplification is achieved using synthetic oligonucleotides (primers), which flank the sequence to be amplified. These primers, under appropriate conditions, anneal to the denatured target sequence and, in the presence of DNA polymerase and excess dNTPs, synthesize a complementary strand of the target sequence. As a template 1 µl cDNA (2.2.11), 1 µl genomic DNA (2.2.8) or 0.5 ng plasmid DNA was used. The PCR reaction was performed by mixing template, 2 µl 10X PCR buffer, 0.6 µl $MgCl_2$ (50 mM), 0.4 µl dNTP (10 mM), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 0.16 µl Taq polymerase (5 U/µl) and ddH_2O to a final vol. of 20 µl. Alternatively, reactions were scaled up to 50 µl vol.. The reaction was incubated in a thermocycler with the following program:

95°C 2 min (denaturation)

94°C 45 sec (denaturation), 60°C 1 min (annealing), 72°C 45 sec (extension) (25-35 cycles)

72°C 5 min (final extension)

Depending the primer pair used in the reaction $MgCl_2$ was adjusted to 1.2 mM final concentration instead of 1.5 mM. Annealing temperature varied depending on the primer pair used and extension time varied according to the expected size of the PCR product (1 min per kb).

2.2.13 Sequencing

Sequencing reaction was employed characterize and to confirm the identity of a clone, to check the fidelity of a PCR product or of a ligation junction. The cycle sequencing reaction consists of successive rounds of denaturation, annealing and extension in the presence of polymerase, dNTPs and dye-labeled terminator. For the sequencing reaction 4 µl BigDye terminator was added to the DNA to be sequenced together with 1 µl primer (10 µM) in a 10 µl reaction. 10-100 ng or 1 µg DNA was used to sequence PCR products (0.2-5 kb) and plasmid DNA, respectively. The reaction was incubated in a thermocycler with the following program:

96°C 2 min (denaturation)

96°C 30 sec (denaturation), 50°C 15 sec (annealing), 60°C 4 min (extension) (25 cycles)

After cycle sequencing has been completed, 10 µl ddH_2O was added to complete 20 µl vol. followed by ethanol precipitation (2.2.3). Precipitated DNA was diluted in 20 µl ddH_2O and

10 µl was injected into the capillary of a Genetic Analyzer automated sequencer ABI PRISM 310 (PE Applied Biosystems).

2.2.14 Agarose-gel electrophoresis for RNA

Denaturing agarose-gel was employed in the separation of RNA. For electrophoresis, 1 % agarose-gel was prepared boiling 1 g of agarose in 74 ml DEPC-H_2O plus 10 ml of 10X MOPS RNA running buffer (2.1.5), until completely dissolved. After gel has cooled to approx. 50°C, 16.6 ml Formaldehyde 37 % was added. The gel was poured onto a horizontal gel bed (RNase-free).

To avoid degradation RNA probes were prepared on ice as follows: RNA samples (10 µg) and RNA Ladder were brought to a total vol. of 6 µl with DEPC-H_2O (samples with bigger vol. were previously dried using SpeedVac®) and 12 µl of RNA sample buffer was added. The probes were denatured at 65°C for 13 min and then cooled on ice for 2 min. Finally, 6 µl of RNA loading buffer was added and the probes were loaded into the slots of the gel. The electrophoresis was carried out at RT, at 15 V for the first 15 min and at 60 V for approx. 4 hr.

2.2.15 Northern blot

The technique transfer RNA from the gel to a nitrocellulose membrane, which can be hybridized. The Northern blotting was set up as follows: The membrane was soaked in 20X SSC (2.1.5) and placed onto a pile of blotting paper (20 x G003 and 1 x soaked G002 at the top). The RNA gel was then placed onto the membrane and covered by 4 more layers of G002 (also pre-wet in 20X SSC), the last one having its ends in a 20X SSC-containing reservoir. The RNA was transfered from the gel onto the membrane by the difference of concentration gradient promoted by the salt. Transfer was performed O/N at RT, after that membrane was air dried and RNA was fixed onto the membrane by UV crosslinking with a GS Gene Linker™ UV Chamber (BioRad).

2.2.16 Radioactive labeling of DNA

DNA probes for the hybridization of Northern blots were labeled with Megaprime™ DNA labeling system (Amersham). In a 34 µl vol. reaction, 25 ng DNA probe and 50 pmol oligo$(dN)_9$ random primers were denatured at 95°C for 5 min and subsequently placed on ice. After cooling down, 10 µl labeling buffer, 2 µl Klenow DNA polymerase I and 5 µl [∝-

^{32}P]dCTP were added. Labeling reaction was carried out for 1 hr at 37°C. Labeled probe was precipitated using Microspin S-400 HR column (Amersham), according to the manufacturer's instructions and denatured for 10 min with 150 µl salmon testes DNA (10 mg/ml).

2.2.17 Hybridization

Northern blot membrane placed in a hybridization tube (nucleic acid facing inwards) was rinsed with 2X SSC and then pre-hybridized in 15 ml pre-warmed hybridization solution plus 150 µl salmon testes DNA (10 mg/ml). Pre-hybridization was carried out for 3 hrs in a rotation oven at 65°C. Denatured ^{32}P-labeled probe (2.2.16) was given to the pre-hybridized membrane, which was hybridized O/N under the same conditions. On the next day, ^{32}P-labeled probe/hybridization solution was replaced by 2X SSC. The membrane was then washed for 20 min at RT followed by 2 warm washes, carried out at hybridization temperature. First warm wash was performed with 2X SSC plus 0.1 % SDS for 3-10 min and the second with 0.2X SSC for 1-2 min. Finally, membrane was air dried, wrapped in cling film and placed in a x-ray cassette. For film exposure (2-5 days) cassette was kept at -80°C.
Stripping the Northern blot membrane was performed in order to hybridize the same membrane with a different probe. In this case the membrane was washed in a large vol. of stripping solution for NB (2.1.5) for 2 hrs, rotating at 65°C.

2.2.18 Screening of mouse genomic cosmid library

The mouse genomic cosmid library 129/ola (2.1.16) was screened under stringent conditions according to RZPD's recommendations. Basically, the 11 cosmid filters were pre-hybridized in 100 ml hybridization solution for RZPD filters (2.1.5) plus 500 µl salmon testes DNA (10 mg/ml). Pre-hybridization was carried out for 2 hrs at 65°C in a water bath with gentle shaking. After that, pre-hybridization solution was replaced by 20 ml hybridization solution plus denatured ^{32}P-labeled probe (2.2.16). Hybridization was carried out O/N under the same conditions. Next day filters were washed with 2X SSC for 20 min at RT and 20 min with pre-heated 2X SSC and 0.2X SSC at 65°C. Filters were wrapped in cling film, placed in a x-ray cassette and exposed for 1-2 days at -80°C. Position of the positive signals were calculated according to RZPD's instructions and corresponding clones were ordered on line (http://www.rzpd.de).

Cosmid clones, delivered as stabs were grown on LB agar plates/kanamycin (2.1.6) and Mini preparation of plasmid DNA (2.2.1.1) was used to recover the DNA. Clones were tested for false positive by PCR reaction (2.2.12), using *Cats* specific primers.

2.2.19 Whole mount *in situ* hybridization (WISH)

Whole mount *in situ* hybridization was employed to determine tempo-spatial pattern of *Cats* expression during mouse embryogenesis. Mouse embryos were prepared and hybridized by standard protocols according to Wilkinson, 1992; with some modifications.

2.2.19.1 Preparation of mouse embryo

BALB/c mice (2.1.9) were paired and the females checked the following day for the presence of a vaginal plug, indicating 0.5 days post coitum (d.p.c) embryos. On the 9.5-14.5 d.p.c, the pregnant female mice were dissected, embryos were collected and placed in cold PBS-DEPC. Extra-embryonic membranes were removed and embryos were fixed in 4 % PFA (in PBS-DEPC), rocking O/N at 4°C. Next day embryos were gradually dehydrated (on ice) through 25 %, 50 %, 75 % methanol (in PBS-DEPC) for 10 min each and bleached for 1 hr with freshly prepared 6 % H_2O_2 (in methanol), Treatment with H_2O_2 has been found to decrease considerably the amount of non-specific background. After bleaching, embryos were further dehydrated twice for 5-10 min in large amount of 100 % methanol and stored in this solution at -20°C. Treatment and storage of mouse embryos were carried out in scintillation vials. Embryos dissected for RNA extraction purpose (2.2.9), were stored at -80°C (in 1.5 ml tube) immediately after removal of extra-embryonic membranes and PBS-DEPC wash.

2.2.19.2 DIG-labeling RNA

RNA probes labeled with Digoxigenin-UTP were synthesized by *in vitro* transcription of Cats cDNA templates. The DNA to be transcribed was first cloned into pGEM®-T Easy (2.1.11) which contains promoters for SP6 and T7 RNA polymerases (Figure 2.1). Prior to transcription, enzymes that created a 5'-overhang were used in a digestion reaction (2.2.2.1) to linearise the template avoiding transcription to continue over the insert onto the plasmid itself. Digestion was carried out for 2 hrs with 10 µg DNA template in a 30 µl reaction. Linearised DNA was then purified using a QIAquick® nucleotide removal column (Qiagen) according to manufacturer's instructions and eluted in 32 µl Ampuwa® H_2O.

PI clone (850 bp) was linearised with *Sal*I and transcribed with T7 RNA polymerase to produce anti-sense probe and linearised with *Nco*I and transcribed with SP6 RNA polymerase to produce sense probe. PIII clone (300 bp) was linearised with *Nco*I and transcribed with SP6 RNA polymerase to produce anti-sense probe (Figure 2.1).

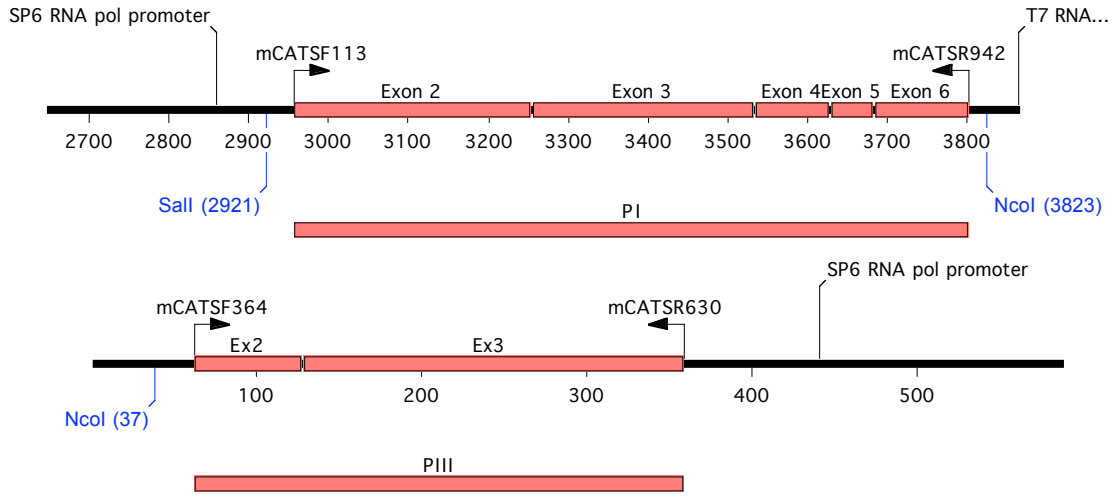


Fig. 2.1: PI and PIII clones used to produce sense and anti-sense WISH probes. PI and PIII clones were generated by RT-PCR from thymus RNA using primer pairs mCATSF113-mCATSR942 and mCATSF364–mCATSR630, respectively and by subsequent cloning of the 850 and 305 bp products into the pGEM®-T Easy vector. PIII was linearised with *Nco*I and transcribed with SP6 RNA polymerase to produce anti-sense probe and PI was linearised with *Nco*I and transcribed with SP6 RNA polymerase to produce sense probe. Arrows indicate binding position of CATS primers.

For the *in vitro* transcription the following components were placed in a 1.5 ml tube.

10 µl	Template DNA (PI or PIII) prepared as described above
3 µl	Ampuwa® H_2O
2 µl	TS-buffer
2 µl	rNTPs DIG-Mix
1 µl	RNase inhibitor
2 µl	RNA polymerase (T7 or SP6)

Reaction was incubated for 2 hrs at 37°C. After that, 2 µl DNase I was added to the reaction, which was incubated for further 15 min. Transcribed RNA was then precipitated by adding 400 µl 100 % ethanol, 1 µl tRNA (20 mg/ml) and 33 µl 7.5 M NH_4Ac, gently mixing and incubating for 1 hr at -80°C. RNA was centrifuged at 15 000 rpm for 30 min at 4°C, pellet was washed with 70 % ethanol (15 000 rpm, 15 min, 4°C) and resuspended in 100 µl Ampuwa® H_2O plus 1 µl RNase inhibitor. Quality and yield were checked, by loading 5 µl of the RNA probe on 1 % agarose-gel (2.2.6). RNA probe was stored at -20°C.

2.2.19.3 Hybridization of mouse embryos

Embryos prepared as described above (2.2.19.1) were re-hydrated through 75 %, 50 % and 25 % methanol (in PBS-DEPC) incubation for 10 min each. Re-hydrated embryos were then washed for 10 min with PBT and transferred to the slots of the InsituPro robot (ABIMED), where an automated hybridization took place. In contrast to manual hybridization, the apparatus allowed rapid changes of solutions and constant temperature throughout all steps. All buffers used were listed in 2.1.5. Briefly, embryos were washed twice with PBT for 10 min. For embryos older than 10.5 d.p.c., 5 min treatment with proteinase K buffer was needed. Proteinase K was used to improve the signal by increasing the accessibility of target RNA. After proteinase K treatment, embryos were washed twice with 2 mg/ml glycine in PBT (10 and 5 min, respectively), then twice with PBT. Prior to pre-hybridization, embryos were treated with RIPA-DEPC and 4 % PFA/ 0.1 % GA (10 min and 20 min, respectively), with two PBT washing steps (10 min) between and after treatments. Pre-hybridization was carried out with hybridization solution (WISH) plus 100 µg/ml tRNA for 3 hrs at 70°C. For hybridization, DIG-labeled RNA probe was previously denatured and then added to the same solution diluted 1:100 dilution. Hybridization was carried out for 16 hrs at 70°C. Post hybridization washes were performed to remove unbound probe. Firstly, embryos were washed for 1 hr (4 x 15 min) with hybridization solution (WISH) free from RNA probe, at 70°C. Then treated with 100 µg/ml RNase A in TNT for 1 hr at 37°C. RNase A treated embryos were washed altogether for 5 hrs (5 min, 3 x 10 min and 9 x 30 min, respectively) in SSC/FA/ Tween® 20 at 70°C, then for 20 min in TBST and MABT (2 x 10 min, each) at RT. Before incubation with antibody, embryos were blocked with 2 % blocking reagent (Roche) in MABT for 3 hrs. Meanwhile a 1:5000 dilution of Anti-Digoxigenin-AP conjugate (Roche) was pre-incubated with 1 % blocking reagent (Roche) in MABT for 1 hr at 4°C with gentle rocking. Immuno-hybridization was carried out for 12 hrs in the AB solution described above. Immuno-hybridized embryos were washed altogether for 14 hrs with TBST (2 x 5 min, 7 x 30 min and 10 x 1 hr, respectively). After washing, embryos were transferred to Cryotube™ vials, washed with NTMT (2 x 5 min) and stained using centrifuged BM purple AP substrate (Roche) O/N at 4°C. The staining reaction was kept in dark and the color development was monitored. When the desired extent was achieved, reaction was stopped and embryos were fixed in 4 % PFA for 1 hr at RT. Stained embryos were stored in the same solution for several month. For documentation, embryos were transferred to PBS and mounted on a thin layer of agarose (in Petri dishes) under a MZ APO stereomicroscope (Leica) in dark field illumination with additional diffuse light from above. All WISHs were recorded on a JVC KY-F70 digital

camera using a Leica QWin software (Leica Microsystems Imaging Solutions, Cambridge, UK) and pictures were adjusted for brightness and contrast in Adobe Photoshop® 7.0 (Adobe Systems, Mountain View, U.S.A).

2.2.19.4 Dissection of hybridized mouse embryos and microscopy

Mouse embryos hybridized and stored as described above (2.2.19.3) were incubated in 30 % sucrose solution (in PBS). After having sank to the bottom of the tube, embryos were incubated in 7.5 % gelatine solution (in 30 % sucrose solution) for 2 h at 42°C in a water bath. In a Petri dish, a thin layer of the 7.5 % gelatine solution was kept at RT until became solid and embryos were then embedded with the same solution onto this layer. After O/N incubation at 4°C, embryos were recovered into small gelatine blocks. Gelatine blocks were placed in alu-folio boxes (1 x 1 cm), which were filled with Jung Tissue Freezing Medium™, frozen in dry ice and stored at -80°C. Embedded embryos were sectioned in 35 μm slices with a Cryotome CM 1850 (Leica) at -30°C and placed onto pre-cooled slides. Cover slips were mounted on the slides with Kaisers Glyceringelatin and stored at RT. Sections were observed under an automated Axioplan 2 Imaging microscope (Carl Zeiss, Jena, Germany) equipped with an AxioCam HR digital camera. Images were recorded using the AxioVision 3.1 software (Carl Zeiss, Jena, Germany) and pictures were processed as in 2.2.19.3.

2.2.20 Culture of mammalian cells

Cells (2.1.10) were grown either in DMEM or RPMI medium (2.1.6.3) supplemented with 10-20 % heat inactivated FBS and 1 % penicillin/streptomycin, in some cases 1 % L-Glutamine was also added to the medium. Cells were grown at 37°C in a humidified incubator supplemented with 5 % carbon dioxide. Confluent cultures were split 1:3 - 1:5 every 2-3 days. Suspension cells were subcultured by simply aliquoting part of the growing culture in a new flask containing fresh growth medium. Cultures of adherent cells were washed with DPBS, incubated with trypsin/EDTA for approx. 5 min at 37°C, detached cells were resuspended in growth medium and replated in a new flask. For long-term storage, harvested cells were washed in DPBS, resuspended in freezing medium (70 % growth medium supplemented with 20 % FCS and 10 % DMSO), aliquoted in Cryotube™ vials and stored in liquid nitrogen.

2.2.21 Transient transfection of adherent cells

Transient transfection of adherent cells was performed using Roti®-Fect transfection reagent. The day before the transfection, 3.2 x 10^5 cells were seeded per reaction, they were placed in 6 well plates with 2 ml of the appropriate growth medium and incubated for 16 hrs at 37°C in a CO_2 incubator until they were 60 % confluent. Cells, which were transfected with the purpose of colocalization and microscopy were grown on 18 x 18 mm coverslips placed in the wells. For the transfection the following reactions were mixed in parallel: 1-2 µg of plasmid DNA (2.2.1.3) plus 60 µl medium free of serum and antibiotic (reaction A) and 10 µl Roti®-Fect transfection reagent plus 60 µl medium free of serum and antibiotics (reaction B). Reactions A and B were mixed and incubated at RT for 40 min in order to allow DNA-lipid complex formation. Meanwhile cells were washed once with DPBS and refilled with 0.8 ml antibiotica-free medium with serum. The DNA-lipid complexes were added to the cells and after 3 hrs incubation 1.2 ml of complete growth medium was added to the cells, which were incubated for another 20-48 hrs. After that, cells were either fixed for microscopy (2.2.21.2) or harvested for protein extraction (2.2.24.1) and reporter gene assay (2.2.23.1). Alternatively, when greater vol. of protein extracts from transfected cells was required, transfections were scaled up to be performed in a 100 mm or 150 mm Petri dish.

2.2.21.1 Nuclear export inhibition of overexpressed protein

Nuclear export inhibition was employed to determine cytoplasmic-nuclear shuttling of proteins. For that NIH-3T3 cells were transfected with YFP-CATS (both isoforms) (3.4.1), YFP-CALM (3.7) or YFP-CALM/AF10 (2.1.12) constructs as described in 2.2.21. 16 hrs after transfection, cellular medium was replaced by 100 nM Leptomycin B in fresh growth medium. Nuclear export inhibition was carried out for 4 hrs in cell culture conditions. Cells were subsequently fixed and mounted for microscopy as described below (2.2.21.2).

2.2.21.2 Fixation of transfected cells and microscopy

At different time points after transfection (2.2.21), the cells were washed with PBS (3 x 3 min) and fixed for 10 min with freshly prepared 3.7 % formaldehyde (in PBS). After fixation, cells were washed with PBS and stained with 2 ml DAPI solution (5 mg/ml stock diluted 1:20000 in PBS) for 5 min. DAPI solution was replaced by PBS and coverslips were mounted on slides with Dakocytomation Fluorescent Mounting Medium and sealed with Fixogum Rubber Cement (Marabu). Cells were observed under an automated Axiovert 200M

microscope (Carl Zeiss, Jena, Germany) equipped with single-band pass filter sets for visualization of DAPI, cyan (CFP) and yellow (YFP) fluorescence. Images were recorded using the Open*lab* 3.08 software (Improvision, Coventry, UK) and pictures were processed with Adobe Photoshop® 7.0 (Adobe Systems, Mountain View, CA)

2.2.22 Yeast two-hybrid screen

Y2H is a GAL4-based system that provides a transcriptional assay for detecting protein interactions in living yeast cells. This system can be employed to identify novel protein interactions, confirm suspected interactions, and to define interacting domains. The Y2H screen is based on the coexpression of the bait protein (CATS fused in frame to the GAL4-DBD) and a prey protein (a library of random cDNA expressing protein X fused in frame to the GAL4-AD). If there is interaction between the bait and prey proteins the GAL4-DBD and -AD are brought into proximity thus a transcriptional activation complex is formed which activates the expression of reporter genes in the yeast (Figure 2.2). The yeast strain AH109 (2.1.8), used for the screen, contains reporter constructs in which *HIS3*, *ADE2* and *MEL1*/*lacZ* genes are under the control of distinct GAL4-UAS and TATA boxes. Expression of *HIS3* and *ADE2* provides nutritional selection which enable the yeast cells to grow on plates lacking certain nutrients (2.1.6.2) and MEL1 provides a catalytic color reaction on the same plates supplemented with X-∝-GAL (X-∝-GAL is converted into a blue color by MEL1 gene product).

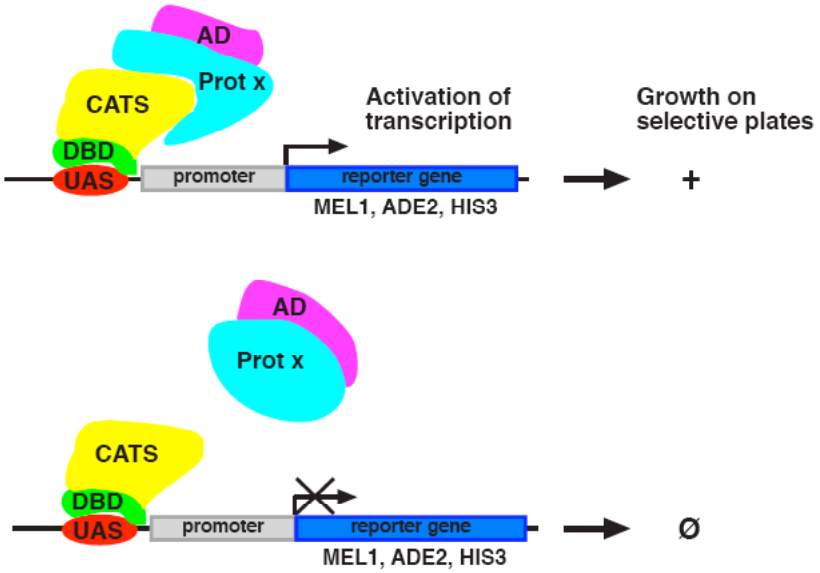


Fig. 2.2: The two-hybrid principle. The DBD is amino acids 1-147 of the yeast GAL4 protein, which binds to the GAL4-UAS upstream of the reporter genes. The AD is the amino acids 768-881 of the GAL4 protein and functions as a transcriptional activator.

Potential interaction between proteins identified with the screen should be confirmed by independent methods. Interaction was confirmed cotransforming the prey and bait plasmids into yeast strain AH109 (2.2.22.2) and assaying for growth on selective plates as well as by colocalization (2.2.21) and co-immunoprecipitation experiments (2.2.28).

2.2.22.1 Test of bait plasmid for autonomous activation

Some bait proteins may have intrinsic DNA-binding and/or transcriptional activating properties when fused to the GAL4-DBD activate the yeast reporter genes. Hence deletion of certain portions of the bait protein may be required to eliminate unwanted activity before the protein can be used in a two-hybrid screen. In order to test the bait protein, pGBT9-CATS construct was transformed in the yeast strain CG 1945 (2.1.8) as described in 2.2.22.2. As a control, CG 1945 cells were transformed with the empty pGBT9 plasmid (2.1.11). Since the bait expression plasmid pGBT9 harbor *TRP1* gene, transformants were selected by growth on SD -Trp plates (2.1.6.2). The test of self-activation transformants were stricked onto SD -Trp, -His plates (2.1.6.2).

2.2.22.2 Transformation in yeast cell

A polyethylene/lithium acetate mediated transformation was performed to transform yeast strains AH109 or CG 1945 with plasmids expressing either/both GAL4-DBD and -AD fusion proteins (or cDNA library). Yeast cells were made competent as follow: one or more yeast colonies (≤ 4 weeks old) were resuspended in 1 ml YPD medium, mixed by vortexing and transferred to 20 ml culture medium (2.1.6). O/N culture grown at 30°C for 16-18 hrs shaking at 250 rpm (stationary phase OD_{600} >1.5) was transferred to 300 ml YPD and incubated for approx. 3 hrs to OD_{600} 0.4-0.6. Cells were collected by centrifugation at 1 000 x g for 5 min at RT and resuspended in 30 ml ddH_2O. After a second centrifugation step, the supernatant was discarded, and the cells were resuspended in 1.5 ml freshly prepared 1X TE/LiAc (2.1.5). For sequential transformation, 100 µl of the freshly prepared yeast competent cells were mixed with 10 µl carrier DNA (10 mg/ml), 100 ng bait or prey plasmid (or 1 µg library DNA) and 600 µl PEG/LiAc. For simultaneous cotransformation both bait and prey plasmids were added together in the reaction above at a molar ratio of 2:1 (200 ng DNA-DBD: 100 ng DNA-AD). The reaction was incubated for 30 min shaking at 30°C (200 rpm). 70 µl DMSO was added and gently mixed by inversion. Cells were heat shocked for 15 min in a 42°C water bath, chilled on ice for 2 min, collected by centrifugation at 14 000 rpm at RT for 5 sec and

resuspended in 1 ml YPD. In order to allow cells to recover from the stress, they were incubated for 1 hr at 30°C, shaking at 230 rpm. After that, cells were washed twice in 1 ml 1X TE buffer (14 000 rpm, 5 sec at RT) and finally stricked onto SD plates lacking tryptophane and/or leucine (selection for the presence of plasmid expressing the GAL4-DBD and -AD, respectively). Colonies positive for both bait and prey plasmids were then assayed for protein interaction by re-plating transformants from the SD -Trp, -Leu plate onto SD -Trp, -Leu, -His, -Ade plate supplemented with X-α-GAL. Plates were incubated at 30°C and growth monitored for several days.

Glycerin stocks were prepared for long-term storage of the yeast clones. Single colony was inoculated into 3 ml YPD and incubated O/N shaking at 30°C. 1 ml of the yeast culture plus 300 µl glycerin were placed in a fresh 1.5 ml tube, mixed by vortexing and stored at -80°C.

2.2.22.3 Large-scale cDNA library transformation

For screening the HeLa S3-cDNA library thus obtaining a high number of cells expressing an individual prey protein, large-scale transformation of the yeast AH109 was performed. In a sequential transformation, the pGBKT7-CATS bait plasmid was firstly introduced through a small-scale transformation as described in 2.2.22.2. Colonies positive for pGBKT7-CATS were assayed by growth on SD -Trp plates. Selected transformants (pGBKT7-CATS/AH109 cells) were then grown up and made competent as described in 2.2.22.2. In order to obtain a maximal amount of transformation efficiency ($4x10^4$ cfu /µg cDNA library), the amount of the library DNA was increased to 4X and reaction was scaled up to be performed in a 11X vol. of the small-scale reaction. Thus 1.1 ml of the freshly prepared bait-containing competent cells was mixed with 44 µg cDNA library, 1.1 mg carrier DNA and 6.6 ml PEG/LiAc. Transformation reaction, divided in 11 reaction tubes (1.5 ml) proceeded as described before. Selection of cotransformants was performed using medium-stringency conditions. For that, cells transformed with the library cDNA were plated on SD -Trp, -Leu, -His plates. 200 µl vol. was used to strick each of the 150 mm selective plates (42 plates in total). Plates were grown at 30°C for 7 days. Subsequently, His positive colonies were replicated onto SD -Trp, -Leu, -His, -Ade plate supplemented with X-α-GAL to screen for *ADE2* and *MEL1* expression. After 7 days incubation at 30°C, the positive colonies were identified by the blue color.

In order to assay the number of transformants screened in this experiment, 0.1 and 0.2 % of the transformation reaction (1 and 2 µl, respectively) was plated onto SD -Trp, -Leu plates and the number of cfu/ reaction vol. was determined.

2.2.22.4 Isolation of plasmid DNA from yeast cells

Prey plasmids were recovered from positive colonies identified in 2.2.22.3 by a rapid isolation of plasmid-DNA. For that, single colonies were inoculated into 2 ml YPD medium and grown at 30°C, O/N at 200 rpm. 1.5 ml of the O/N culture was transferred to a 1.5 ml tube and spin down for 5 sec at high speed. Supernatant was removed and the pellet was briefly vortexed. Cells were lysed in 200 µl Lysis solution for yeast cells (2.1.5) plus 0.3 g glass beads and 200 µl of phenol/chloroform/isoamyl (25:24:1), vortexed for 2 min and centrifuged for 5 min at 14 000 rpm. Approx. 250 µl of supernatant was transferred to a fresh 1.5 ml tube and ethanol precipitated as described in 2.2.3. DNA was resuspended in 20 µl ddH_2O and 5 µl was used for electroporation (2.2.5.4).

2.2.23 GAL4-based transcription assay

The GAL4-based transcription assay is based on the expression of GAL4-DBD fusion protein in the mammalian system. The hybrid protein is targeted to the cell's nucleus by the GAL4 NLS from the pM1 plasmid (2.1.11) where the GAL4-DBD of the fusion protein binds to the GAL4-UAS in the promoter region of the reporter gene plasmid. If the protein posses any function as a transcriptional regulator, a transcription activation or repression complex will induce or repress the expression of the luciferase reporter gene (Figure 2.3).

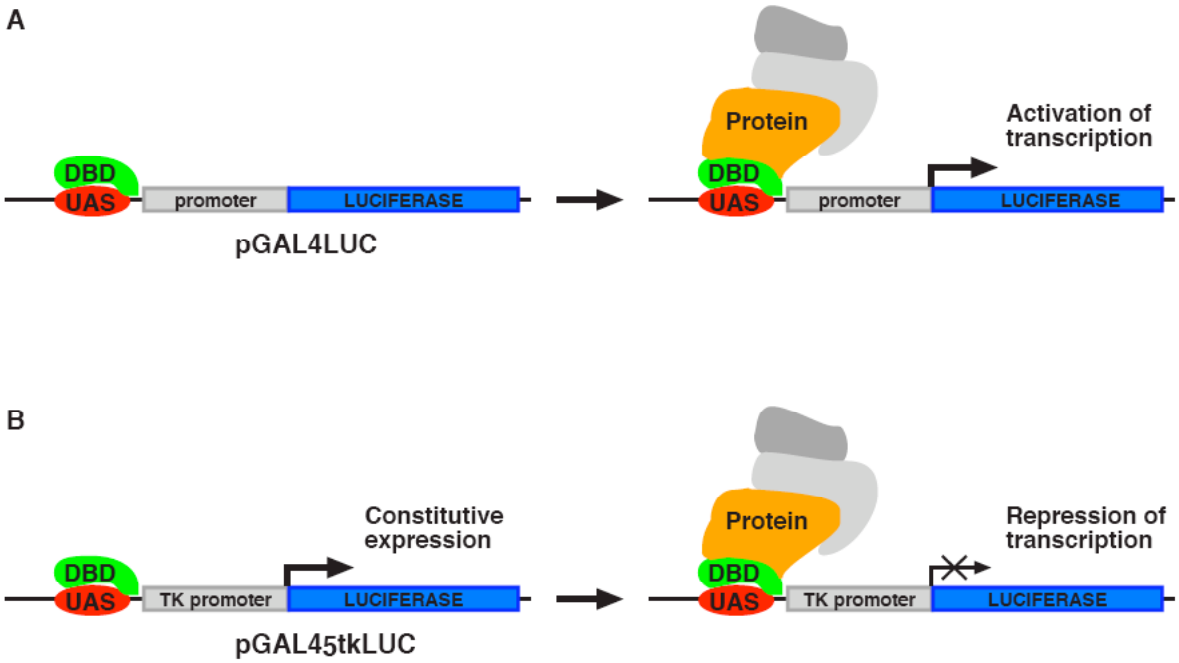


Fig. 2.3: Principle of GAL-4-based transcription assay. Vectors expressing both the GAL4-DBD fusion protein and reporter gene are coexpressed in mammalian cells. The GAL4-DBD of the fusion protein binds to the GAL4-UAS in the promoter region of the reporter gene. (**A**) If the expressed protein has a function as

activator of transcription, it will induce the transcription of the luciferase reporter gene from the pGAL4LUC plasmid. (**B**) If the protein functions as repressor of transcription it will repress the constitutive expression of the luciferase reporter gene (under the transcriptional control of the Herpes simplex virus thymidine kinase promoter) from the pGAL4$_5$tkLUC plasmid.

2.2.23.1 Luciferase reporter gene assay

Human embryonal kidney 293T cells were transfected as described in 2.2.21. 48 hrs after transfection, cells were washed with 1 ml PBS and harvested in 200 µl to 1 ml of lysis buffer (PLB). Cell lysates were transferred to a 1.5 ml tube and alternately incubated for 5 min in ice and at 37ºC. Cell lysates were assayed for firefly and *Renilla* luciferase activities using a Dual-Luciferase® Reporter Assay System (Promega) and a Turner Designs TD-20/20 luminometer. The luminometer was programmed to perform a 2-sec pre measurement delay, followed by a 10-sec measurement period for each reporter assay. Measurements were performed by transferring 8-20 µl of cell lysate into a luminometer tube containing 100 µl LAR II, placing the tube in the luminometer and initiating the reading for the firefly luciferase activity. The sample was removed from the luminometer and 100 µl of Stop & Glo® reagent was added. After briefly vortexing, the tube was placed in the luminometer again and the *Renilla* luciferase activity was read. Measurements of *Renilla* luciferase activity were used for normalization. Experiments were performed at least in triplicate.

2.2.24 Protein extraction

2.2.24.1 Protein extraction from mammalian cells

Protein extraction from both transfected and nontransfected cells were performed as follows. Cells were harvested and washed twice in ice-cold PBS (1 100 rpm for 5 min). Cell pellets were lysed in an appropriate vol. of RIPA buffer (2.1.5) with freshly added protease inhibitors (protease inhibitor cocktail diluted 1:100). After 30 min. of incubation on ice, whole cell extracts were cleared by centrifugation at 10 000 x g for 10 min at 4º C. The supernatant was the total cell lysate.

2.2.24.2 Protein extraction from human tissue

Proteins were extracted from human tissue similarly to what described above (2.2.24.1). Maintaining the temperature at 4°C throughout all procedures, 60 mg of human thymus was homogenized in 200 µl RIPA buffer with freshly added protease inhibitor (in general approx. 3 ml RIPA buffer per gram of tissue) with a Tissuemizer (Tekmar-Dohrmann). 200 µl of

PMSF (100 mM) was added to the homogenized tissue, which was incubated on ice for 30 min. Protein extract was cleared by centrifuging at 10 000 x g for 10 min at 4°C, twice. The supernatant fluid was the total protein extract. Protein extracts were mixed with an equal volume of 2X Laemmli sample buffer (2.1.5) and denatured at 95º C for 5 min. Lysates were stored at 20º C for several months.

2.2.24.3 Protein extraction from yeast cells

One or more yeast colonies (≤ 4 weeks old) were inoculated in 5 ml YPD medium, mixed by vortexing and incubated O/N at 30°C shaking at 230 rpm. The O/N culture was diluted in 5 ml YPD medium (OD_{600} 0.2) and further incubated for approx. 4-6 hrs to OD_{600} 0.45-0.7. 1.5 ml of the culture suspension was transferred to a fresh tube and centrifuged at 13 000 x g for 3 min at RT. Cells were resuspended in 50 µl 2X Laemmli sample buffer, vortexed and frozen on dry ice (or liquid nitrogen). Samples were subsequently boiled for 5 min and centrifuged for 5 sec at 14 000 rpm. The supernatant was the total cell lysate. 25 µl of cell lysate was used per lane of SDS-PAGE gel (2.2.26).

2.2.25 Determination of protein concentration using the Bradford method

Standard BSA curve was calibrated by diluting the stock solution (1 µg/µl; 30 mg BSA in 30 ml ddH_2O) in the appropriate vol. of ddH_2O as shown in the table below.

BSA (µl)	0	1	5	10	15	20	25
ddH_2O (µl)	800	799	795	790	785	780	775

In parallel, 1 µl of the protein extract was diluted in 799 µl of ddH_2O and 200 µl of Bradford reagent was added to each reaction tube, both to the BSA and sample dilutions. Measurement of the protein against the BSA calibration curve was performed in an Eppendorf BioPhotometer 6131.

2.2.26 PAGE gel

SDS-Polyacrylamide (SDS-PAGE) gel electrophoresis was used for separation of proteins under denaturing conditions. The concentration of the gels used was determined according to the effective range of separation of SDS-PAGE shown below:

Acrylamide concentration (%)	Linear range of separation (kDa)
15	12-43
10	16-68
7.5	36-94
5	57-212

SDS-PAGE gel was prepared according to the range of protein separation desired. For the resolving gel the following components were mixed together:

Components (5 ml)	8 %	10 %	12 %
ddH_2O	2.3 ml	1.9 ml	1.6 ml
30 % Acrylamide mix	1.3 ml	1.7 ml	2.0 ml
1.5 M Tris/HCl (pH 8.8)	1.3 ml	1.3 ml	1.3 ml
10 % SDS	50 µl	50 µl	50 µl
10 % APS	50 µl	50 µl	50 µl
TEMED	3 µl	2 µl	2 µl

Resolving gel solution was poured into the gap between glass plates placed at vertical position at RT. After polymerization of the resolving gel was completed (approx. 30 min at RT), stacking gel was prepared by mixing the following components:

Components (2 ml)	Stacking gel 5 %
ddH_2O	1.4 ml
30 % Acrylamide mix	330 µl
1 M Tris/HCl (pH 6.8)	250 µl
10 % SDS	20 µl
10 % APS	20 µl
TEMED	2 µl

Stacking gel solution was poured directly onto the surface of the polymerized resolving gel.

2.2.26.1 Coomassie staining of PAGE gel

Coomassie staining of PAGE gel was used for detection of proteins on the gel after electrophoresis. Gel was immersed in Coomassie® solution (BioRad) and incubated for approx. 1 hr at RT with shaking. Coomassie® solution bound unspecifically was removed by washing the gel with 10 % methanol plus 10 % acetic acid in ddH_2O. Washing was performed until protein bands became visible. Gel was then washed in 90 % methanol/10 % acetic acid for approx. 10 min and fixed for 15 min with 20 % ethanol plus 2 % glycerol in ddH_2O. Finally, gel was dried for approx. 1 hr between 2 sheets of cellophane using a Gel Air™ Drying System (BioRad).

2.2.27 Western blot

Immunocomplexes and cellular lysates were electrophoresed on 8-12 % SDS-PAGE gels and transferred to a nitrocellulose membrane using a semi-dry system, consisting of 3 sheets of blotting paper (GB003), a nitrocellulose membrane, the gel and 3 layers of blotting paper (GB003), one on the top of the other one laid on the surface of the Trans-Blot® SD Semi -Dry Transfer Cell (BioRad). Except for the gel, papers and membrane were pre-wet in transfer buffer (2.1.5). Transfer of protein from the gel to the membrane occurred under electric current from the negative to the positive pole at 0.15 A for 1 hour. The membranes were blocked for nonspecific binding sites with 5 % nonfat dried milk for 1 hr at RT (alternatively, O/N at 4°C). Immunoblotting was carried out with specific antibodies diluted in 5 % nonfat dried milk at optimal concentration. Incubation with primary antibody was carried out in most cases for 1 hr at RT (alternatively, O/N at 4°C) followed by extensive washing steps with TBST (rinse twice, wash 1 x 15 min and 3 x 5 min, respectively). Secondary antibodies conjugated with horseradish peroxidase (HPR) were used for detection of the primary antibody. Incubation was carried out for 45 min at RT followed by extensive washing steps as described above. Finally, proteins were detected by incubating the membrane with enhanced chemiluminescent reagent for 5 min at RT (ECL, Amersham Pharmacia biotech). The membrane was then wrapped in cling film and placed in an x-ray film cassette. Autoradiography films were exposed between 5 sec to 2 min.

Stripping the immunoblotings was performed in order to probe the same membrane with a different antibody. Immunoblotings were stripped by immersing the membranes in stripping solution for WB (2.1.5) under shaking at 56°C for 30 min. Membranes were washed twice with TBST for 10 min at RT followed by blocking and probing as described above.

2.2.28 Immunoprecipitation

Cell lysates (2.2.24.1) were precleared in order to remove proteins, which bind in an unspecific manner. For this, 2 µg of normal control IgG and 30 µl Protein A- or G-Agarose beads were incubated with 400 µg total cell lysate in 1 ml CoIP buffer (reaction A). Protein A was used to bind rabbit or mouse antibodies and G to bind rat antibody. In parallel antibody-beads conjugates were formed by incubating 2 µg of specific antibody (100 µl when α-CATS 2C4 was used for precipitation) or normal IgG control with 30 µl Protein A- or G-Agarose beads in 500 µl vol. of the same buffer (reaction B). After 1 $^{1}/_{2}$ hr incubation, reactions A and B were centrifuged at 2 200 rpm for 5 min at 4°C. Antibody-bead conjugates (reaction B) were washed in 1 ml CoIP buffer and pelleted by centrifugation at 2 200 rpm for 5 min at 4°C. Precipitation of immunocomplexes was performed by incubating the precleared cell lysates (reaction A) with the antibody-beads conjugates (reaction B) for 5 hrs at 4°C with 10 rpm rotation. Precipitates were washed 3 times with 1 ml CoIP buffer, resuspended in 15 µl 2x Laemmli sample buffer and denatured for 5 min at 95°C before loading on SDS-PAGE gel for analysis. The CoIP buffer used for the immunopreciptation was determine according to the appropriate stringency necessary to detect the coimmunoprecipitates. When the high stringent CoIP buffer I was used for the precipitation reaction, the last two washing steps were performed with the CoIP buffer II without proteinase inhibitors. When the low stringent CoIP buffer 3 was used, all the washing steps were performed with the same buffer.

2.2.29 Immunofluorescence

2.5 x 10^5 U2OS cells were seeded and grown on 18 x 18 mm coverslips placed in 6 well plates. After 24 hrs incubation at 37°C in a CO_2 incubator, cells were washed in PBS and fixed for 10 min in 2 % PFA (2.1.5). Cells were then washed three times with PBS (3 min each), incubated for 15 min with PBS plus 0.1 % Tween® 20 and blocked with 10 % FCS in PBS for 1 hr at RT with gentle rocking. For the immunofuorescence reaction, CATS antibody was diluted 1:10 in PBS plus 1.5 % FCS and incubation was carried out O/N at 4°C in a humidified chamber. Next day, cells were washed with PBS plus 0.1 % Tween® 20 (3 x 5 min) at RT with gentle rocking. Secondary antibody Cy™ 3-conjugated diluted 1:250 was used for detection of the primary antibody for 30 min at RT in dark followed by washing as described above (also in dark). Finally, cells were stained for 2 min in DAPI solution (5 mg/ml stock, diluted 1: 20 000 in PBS) and washed with PBS. Coverslips were mounted on slides with Dakocytomation Fluorescent Mounting Medium and sealed with nail varnish.

Cells were observed under an automated Axiovert 200M microscope (Carl Zeiss, Jena, Germany) equipped with single-band pass filter sets for visualization of DAPI and Texas red fluorescence. Image documentation was performed as described in 2.2.21.2.

2.2.30 Expression and purification of GST-CATS fusion protein

For expression of the GST-CATS fusion protein, the bacteria strain BL21 (2.1.7) was transformed with the pGEX4T-2-CATSv1 and pGEX4T-2-CATSv2CT constructs as described in 2.2.5.2. Transformed colony was inoculated into 10 ml LB medium plus ampicillin and incubated O/N at 37°C with vigorous shaking. The O/N culture was diluted into 100 ml LB medium plus ampicillin and further grown to OD_{600} 0.6-0.8 at the same conditions. Induction of GST-fusion protein expression was performed incubating the cell culture for 2 hrs in the presence of 1 mM IPTG. As a control, a parallel culture grown at the same conditions was grown deprived from IPTG treatment. After induction, cells were harvested by centrifugation at 4 500 rpm for 15 min at 4°C and resuspended in 5 ml cold NETN buffer plus protease inhibitors (1 mM PMSF and 10 µg/ml pepstatin, leupeptin and aprotinin). Suspension was then sonicated for 1 min on ice. Cell lysate was transferred to 1.5 ml tubes, centrifuged at 14 000 rpm for 5 min at 4°C and supernatant was transferred to a fresh tube. Protein extract was stored at -80°C. In order to check whether induction of protein expression has occurred, a PAGE gel electrophoresis was performed with 20 µl protein extract from both induced and non-induced cultures. Overexpressed protein was visualized by staining the gel with coomassie solution (2.2.26.1). GST fusion protein was purified from the bacterial lysate by affinity chromatography using glutathione immobilized to a matrix (such as agarose) and gravity flow. For that GST-CATS protein extract was incubated with 250 µl Glutathione-agarose beads (which had been firstly washed 3 times in NETN buffer) per ml of lysate. Incubation was carried out for 4 hrs at 4°C with 10 rpm rotation, protein-Glutathione beads were applied to the GST purification column followed by extensive wash with high salt buffer (2.1.5) (3 x 2.5 ml), PBS plus 0.1 % Triton X-100 (2 x 2 ml) and PBS plus 1 mM DTT and protease inhibitors (1 x 2 ml), respectively. Protein was finally recovered from the ligand through 3 elution steps with 500 µl elution buffer each.

The integrity of the purified protein was examined in 12 % SDS-PAGE and Coomassie blue staining. GST-CATS fusion protein was stored at -80°C.

2.2.31 FACS sorting of hematopoietic cell sub populations

Hematopoietic cells were obtained from the thymus and bone marrow of 4 weeks old mice. Bone marrow cells were flushed from both femurs and tibias with DMEM medium supplemented with 2 % FBS. Cell from thymus were obtained through smashing the tissue onto a cell strainer and recovering them with DMEM medium. Cellular suspensions (from thymus and bone marrow) were centrifuged for 3 min at 2 000 rpm and cell pellets were resuspended in 5 ml DPBS. For the cellular suspension originated from bone marrow, treatment with ammonium chloride was needed in order to lysate the red blood cells. For that, the same vol. of ammonium chloride solution was added to that suspension followed by 20 min incubation on ice. Ammonium chloride treated cells were harvested by centrifugation and resuspended in 1 ml DPBS. Cellular suspensions were divided into 250 µl or 500 µl aliquots and each of them was incubated with the appropriated PE or/and APC conjugated antibody at optimal concentration (2.1.14). Antibody incubation was carried out for 30 min on ice protected from light. The cells were then washed with DPBS and pellets were resuspended in 0.5 ml FACS buffer (2.1.5). Cells were sorted using FACSVantage SE System (BD Biosciences) and cell subpopulations were collected in DMEM medium supplemented with 50 % FBS. After sorting, cells were rinsed in PBS and immediately lysate in RLT buffer for RNA extraction as described in 2.2.9.

3 Results

3.1 Isolation and characterizing the *CATS* genes

3.1.1 The human *CATS* gene

In order to obtain the complete mRNA sequence of the human *CATS* gene, the human EST database was searched with the sequence (834 bp) from the pGADGH-CATSv2CT prey clone (2.1.12) isolated from a previous Y2H screen (Gläsner, 1999). Three overlapping ESTs were identified (GenBank AA604379, BE888231, BE799806) which were assembled into a 1532 bp long contig. Two IMAGE cDNA clones (IMAGp998G039611, IMAGp998D169579) corresponding to the EST contig were obtained from RZPD, Berlin, Germany (2.1.15) and completely sequenced (2.2.13). Genbank searches revealed that the complete *CATS* genomic locus is contained in the sequence of a BAC (EMBL accession number AC055872). Alignment of CATS cDNA sequence to the whole human genomic sequence was used to characterize the genomic locus as well as to identify exon-intron boundaries and pseudogenes of CATS.

CATS is located on chromosome 17 band p13, 6.29 megabase pairs from the telomere of the short arm (chromosome 17 genomic contig NT_010718). 4 processed pseudogenes of *CATS* were identified at other chromosomal locations. Two of them are located on chromosome 4 (4p15; 4q24), one on chromosome 6 (6q15) and the fourth on chromosome 2 (2q33). The genomic locus of *CATS* spans 7 kb and contains 6 exons with a non-coding first exon. The splice donor and acceptor sites of the *CATS* are canonical except for the splice donor in the third intron (GC-AG (splice donor - splice acceptor)) (Table 3.1). At least three alternatively spliced variants of *CATS* were identified which code for two protein isoforms of 238 and 248 amino acids in length (isoform 1 and 2, respectively) (Figure 3.1). The calculated molecular weight for isoform 1 is 26.24 kDa (pI 10.23) and for isoform 2 is 27.47 kDa (pI 10.56). Both isoforms share the same sequence from residues 1-228, encoded by exons 2-4. The two proteins differ in their C-terminus. The 10 last amino acids of isoform 1 are encoded by exon 5, which is not present in the transcript coding for isoform 2. The last 20 amino acids of isoform 2 are encoded by exon 6 (Figure 3.2).

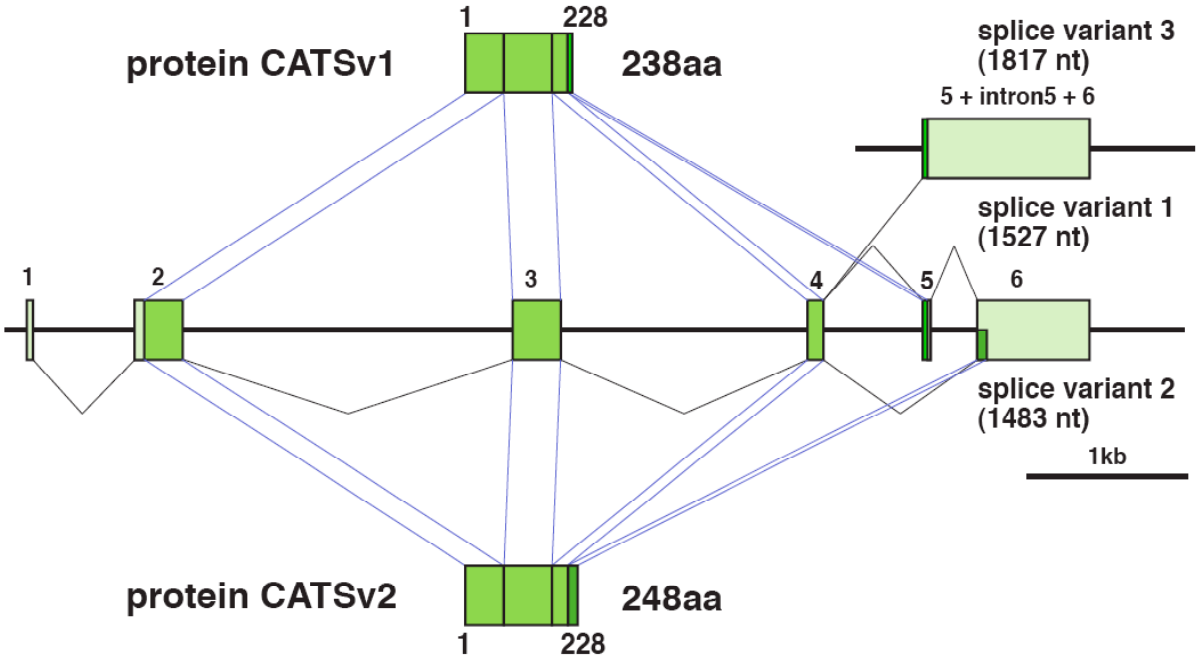


Fig. 3.1: Genomic organization, alternative splicing, and protein isoforms of CATS. Boxes represent exons, with filled parts symbolizing coding region. 5' and 3' UTRs are indicated by lighter coloring of the boxes. There are 3 splice variants (sizes are indicated in brackets). In splice variant 2, exon 5 is spliced out resulting in a longer protein (CATSv2) encoded by exons 2, 3, 4 and 6. The shorter isoform (CATSv1) is encoded by exons 2, 3, 4 and 5 from splice variants 1 and 3. Both CATS isoforms are identical from amino acids 1-228, they differ in their last 10 and 20 C-terminal residues (CATSv1 and CATSv2, respectively).

Intron	splice acceptor	Exon	splice donor
		Exon 1 (39 bp)	GTGGTGTCGGgtgagtgcgg
Intron 1 (588 bp)	tcctccccagGGTCTAGTGG	Exon 2 (328 bp)	CGTGTCCCAGgtaatactga
Intron 2 (2059 bp)	cctgttccagAGAATCCAGG	Exon 3 (295 bp)	GCTCTCCCAG**gc**aagtggga
Intron 3 (1554 bp)	tttcatgcagCGAGTCTGAC	Exon 4 (95 bp)	CGGAAGAGAGgtgagttggc
Intron 4 (621 bp)	ttctctctagTGGTGACATC	Exon 5 (44 bp)	GTGCCTGCAGgtaatgccca
Intron 5 (290 bp)	tgtcttgaagGAAACAAGCC	Exon 6 (702 bp)	

Table 3.1: Exon-intron boundaries of human *CATS*. Sequences of the splice donor and acceptor sites of *CATS* as determined by comparison of the cDNA and genomic sequences are underline. Exons are in upper-case and introns are in lower-case. The non-canonical splice donor in intron 3 is in bold. Exon and intron sizes are indicated in brackets.

Exon 1 **Exon 2**

```
          10          20          30          40          50          60
GCA GCT GTG CTG CGG CTG TGC TCG GCC TTA GTG GTG TCG GGG TCT AGT GGA CAG AGA AGA

          70          80          90         100         110         120
CTC TTG GCC AGG CAG ATG GCT TCT CGG TGG CAG AAC ATG GGG ACC TCC GTG CGC CGG AGA
                    M   A   S   R   W   Q   N   M   G   T   S   V   R   R   R
                    1                                                      15

         130         140         150         160         170         180
TCT CTC CAG CAC CAG GAG CAG CTG GAG GAC AGC AAG GAG CTG CAG CCT GTG GTC AGC CAT
 S   L   Q   H   Q   E   Q   L   E   D   S   K   E   L   Q   P   V   V   S   H
 16                                                                     35

         190         200         210         220         230         240
CAG GAG ACC TCT GTA GGG GCC CTG GGG TCC CTG TGC AGA CAG TTC CAA AGG AGG CTG CCC
 Q   E   T   S   V   G   A   L   G   S   L   C   R   Q   F   Q   R   R   L   P
 36                                                                     55

         250         260         270         280         290         300
CTG AGA GCC GTC AAC CTC AAC CTC CGC GCA GGG CCC TCC TGG AAA CGC CTG GAA ACC CCA
 L   R   A   V   N   L   N   L   R   A   G   P   S   W   K   R   L   E   T   P
 56                                                                     75

         310         320         330         340         350         360
GAG CCA GGT CAG CAG GGC CTC CAG GCT GCA GCT CGC TCA GCT AAG AGT GCT TTG GGT GCC
 E   P   G   Q   Q   G   L   Q   A   A   A   R   S   A   K   S   A   L   G   A
 76                                                                     95

           Exon 3
         370         380         390         400         410         420
GTG TCC CAG AGA ATC CAG GAG TCC TGC CAA AGT GGC ACC AAG TGG CTG GTG GAG ACC CAG
 V   S   Q   R   I   Q   E   S   C   Q   S   G   T   K   W   L   V   E   T   Q
 96                                                                    115

         430         440         450         460         470         480
GTG AAG GCC AGG AGG CGG AAG AGA GGA GCA CAG AAG GGC AGT GGA TCC CCA ACT CAC AGC
 V   K   A   R   R   R   K   R   G   A   Q   K   G   S   G   S   P   T   H   S
116                                                                    135

         490         500         510         520         530         540
CTG AGC CAG AAG AGC ACC CGG CTG TCT GGA GCC GCC CCT GCC CAC TCA GCC GCA GAC CCC
 L   S   Q   K   S   T   R   L   S   G   A   A   P   A   H   S   A   A   D   P
136                                                                    155

         550         560         570         580         590         600
TGG GAG AAG GAG CAT CAC CGC CTC TCT GTC CGG ATG GGC TCA CAT GCC CAC CCA TTA CGG
 W   E   K   E   H   H   R   L   S   V   R   M   G   S   H   A   H   P   L   R
156                                                                    175

         610         620         630         640         650         660
CGA TCA AGG CGG GAG GCT GCC TTC CGG AGC CCC TAC TCC TCA ACA GAG CCC CTC TGC TCT
 R   S   R   R   E   A   A   F   R   S   P   Y   S   S   T   E   P   L   C   S
176                                                                    195

      Exon 4
         670         680         690         700         710         720
CCC AGC GAG TCT GAC AGT GAC CTA GAG CCT GTG GGG GCG GGA ATT CAG CAT CTC CAG AAG
 P   S   E   S   D   S   D   L   E   P   V   G   A   G   I   Q   H   L   Q   K
196                                                                    215

                                                    Exon 5
         730         740         750         760         770         780
CTG TCC CAA GAG CTA GAT GAA GCC ATT ATG GCG GAA GAG AGT GGT GAC ATC GTC TCT CTC
 L   S   Q   E   L   D   E   A   I   M   A   E   E   S   G   D   I   V   S   L
216                                                 228                    235
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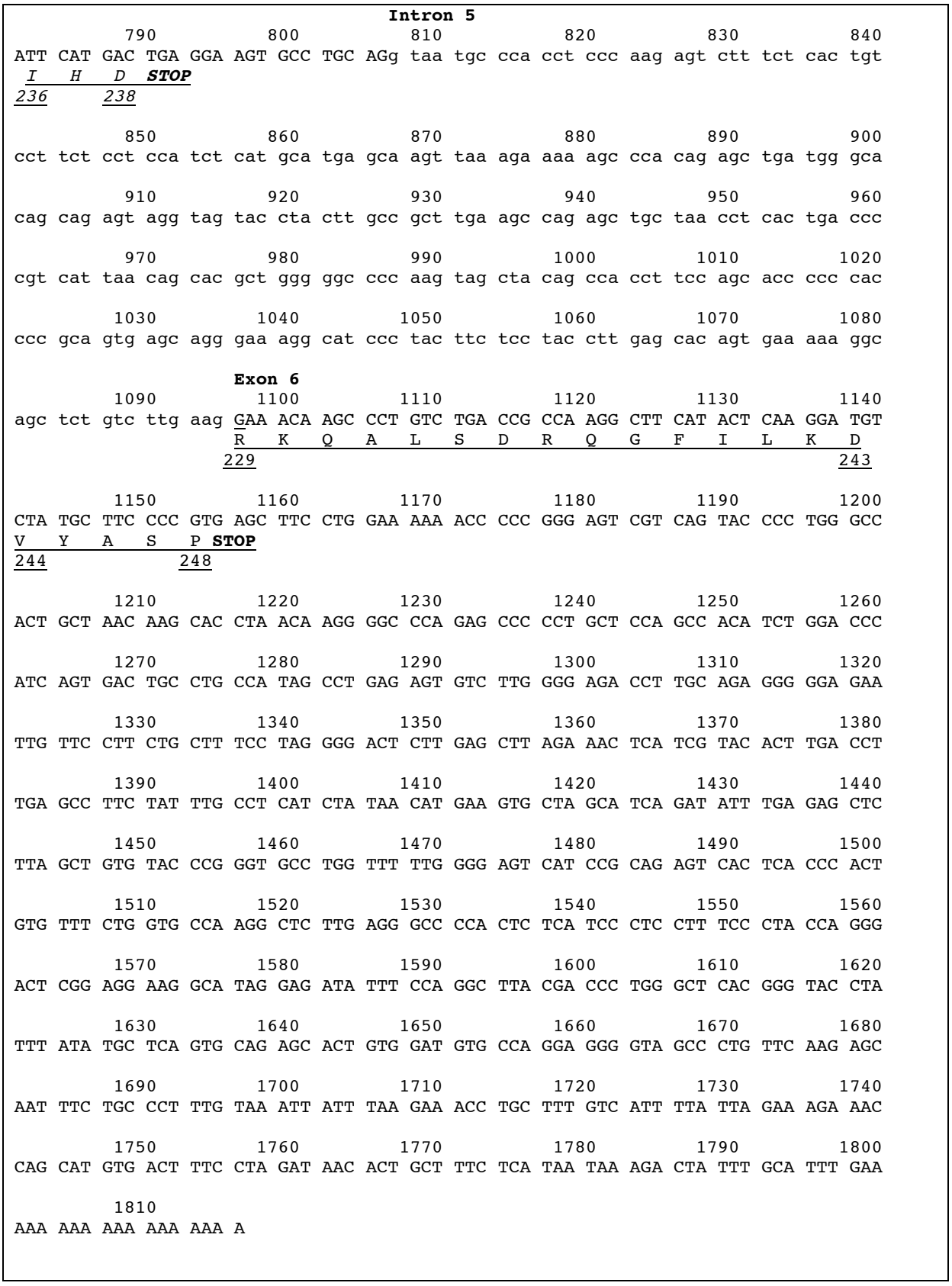


Fig. 3.2: Human CATS nucleotide and amino acid sequences: Splice variant 1, 2 and 3. The first nucleotide of each exon is underlined. The last 10 C-terminal amino acids of CATS isoform 1 coded by the alternatively spliced exon 5 (splice variant 1 and 3) are italicized and underlined. The last 20 C-terminal amino acids of CATS isoform 2 coded by exon 6 (splice variant 2) are underlined. Stop codons for the both isoforms are marked. Note

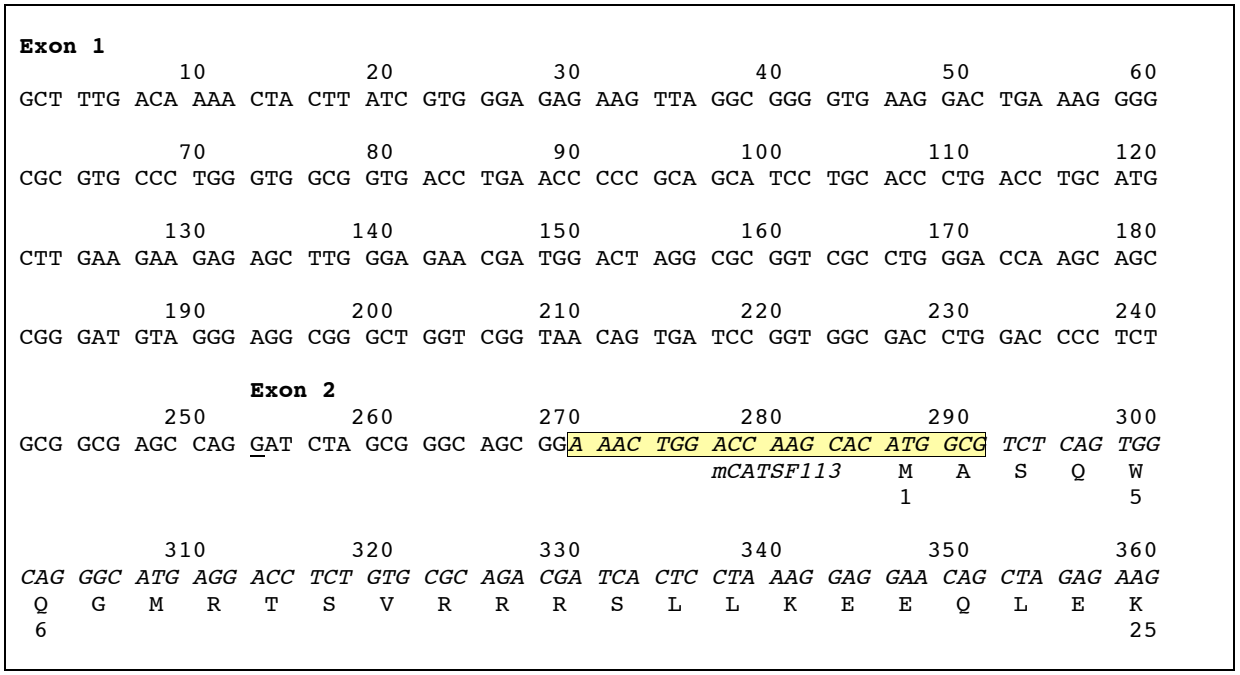
the presence of not removed intron 5 sequences (only present in splice variant 3), indicated in lower-case. Splice variant 1 (1527 nt) corresponds to cDNA clone IMAGp998D169579. Splice variant 3 (1817 nt) corresponds to the cDNA clone IMAGp998G039611.

3.1.2 The murine *Cats* gene

In order to obtain the full sequence of the murine *Cats* gene, the murine EST database was searched with the human cDNA sequence (3.1.1). Four overlapping ESTs were identified (GenBank W65832, AA103173, BF167691, W80053) and a contig of the murine *Cats* gene was assembled. Three IMAGE cDNA clones (IMAGp998E09899Q2, IMAGp998F103793Q2, IMAGp998H174619Q2) corresponding to the contig EST sequences were obtained from the RZPD, Berlin, Germany (2.1.15) and were completely sequenced (2.2.13) (Figure 3.3).

3.1.2.1 Cloning of *Cats* cDNA (mCats cDNA F113-R942 fragment)

The murine *Cats* contig sequence was used to design primers flanking the Cats ORF. A 850 bp murine *Cats* cDNA fragment was amplified by RT-PCR from thymus RNA (2.2.11 and 2.2.12) using the mCATSF113 and mCATSR942 primer pair (2.1.13). The RT-PCR product was cloned into the pGEM®-T Easy vector and fully sequenced (Figure 3.3). The cDNA fragment was radioactive labeled (2.2.16) and used as a probe for hybridization of the mouse cosmid library (3.1.2.2) and Northern blot (3.2.1). Moreover the cloned RT-PCR fragment was used to produce the anti-sense probe I (PI) for whole mount *in situ* hybridization (2.2.19.2).



```
          370          380          390          400          410          420
AAG GAG GTG ACC CGG TCT GCT GGG GGT CAT CCG GAG ACC GGT CCC TTG GGC TCC CTG TGC
 K   E   V   T   R   S   A   G   G   H   P   E   T   G   P   L   G   S   L   C
 26                                                                          45

          430          440          450          460          470          480
AGA CAA TTC CAA AGG AGG CTG CCT CTG AGA GCG GTC AGC CTG AAC CTC GGT AAC GGC CCC
 R   Q   F   Q   R   R   L   P   L   R   A   V   S   L   N   L   G   N   G   P
 46                                                                          65

          490          500          510          520          530          540
TCC TGG AAA CGC CTG GAA AGT CCA GAG CCA GAG CAG CAA GGT CTT CAG GCT GCA GCT CGC
 S   W   K   R   L   E   S   P   E   P   E   Q   Q   G   L   Q   A   A   A   R
 66                                                                          85

                                                  Exon 3
          550          560          570          580          590          600
TCA GCC AAA AGC GCC TTG GGT GCC ATG TCC CAG AGG ATC CAG GAG TCC TGT CAG AGT GGC
 S   A   K   S   A   L   G   A   M   S   Q   R   I   Q   E   S   C   Q   S   G
 86                                                                         105

          610          620          630          640          650          660
ACC AAG TGG CTG ATG GAG ACA CAG GTG AAA GTG AGA AGG AAG CGA GGA GCC CAG AAA GAC
 T   K   W   L   M   E   T   Q   V   K   V   R   R   K   R   G   A   Q   K   D
106                                                                         125

          670          680          690          700          710          720
AGA GGG TCA CCA CCA CCC AGT CTG AGC CAG AAG AAC ACT CGG CTA TGC AGG GCC AAC CGG
 R   G   S   P   P   P   S   L   S   Q   K   N   T   R   L   C   R   A   N   R
126                                                                         145

          730          740          750          760          770          780
GAT GCT AGA GTC GGG GGA CAT CTT CGC CTC TCT GGC CAG ATG GGT CCG CAT GCC CAT CGC
 D   A   R   V   G   G   H   L   R   L   S   G   Q   M   G   P   H   A   H   R
146                                                                         165

          790          800          810          820          830          840
CGT CAG CGG CTA AGG AGG GAG TCT GCC CTC CGG AGC CCC TGT TCC TCC ACA GAA CCT CTC
 R   Q   R   L   R   R   E   S   A   L   R   S   P   C   S   S   T   E   P   L
166                                                                         185

              Exon 4
          850          860          870          880          890          900
TGC TCT CCC AGT GAG TCT GAC AGT GAC CTA GAG CCT GTG GGA GCC GGA ATC CAG CAT CTT
 C   S   P   S   E   S   D   S   D   L   E   P   V   G   A   G   I   Q   H   L
186                                                                         205

                                                              Exon 5
          910          920          930          940          950          960
CAG AAG CTG TCT CAA AGG CTG GAC AGA GCC ATC AAG GCT GAA GAG AGT GGT GAC ATG ACC
 Q   K   L   S   Q   R   L   D   R   A   I   K   A   E   E   S   G   D   M   T
206                                                                         225

                                                          Exon 6
          970          980          990         1000         1010         1020
GTC TCC CTT ATC CGT GAA TGA GGA CAG CAA GCA TCC TAC AGG AAG CCG GCT CTG TCC AAC
 V   S   L   I   R   E  STOP
226                 231

         1030         1040         1050         1060         1070         1080
CAC CAA GCC ACC AGG ATG TCC ATG CTG CCC AAC CCA TGA ACT TCC TGG AGA CAG CAT TGG

         1090         1100         1110         1120         1130         1140
GTT TAT CAG CAT CCC CAG GCC ACT GCT AAC AAG GAT TTG ACT AAC ATA GGG TCC AGA ACC
                                mCATSR942

         1150         1160         1170         1180         1190         1200
CTT CTA CTT CAG CCA TAT CTG GGC CCA CTG GTA ACC ACT GGC CAC AGC CTG AGA GTT ATT
```

```
         1210         1220         1230         1240         1250         1260
TAG GAA GAC CTT GAA GAG GTG ATG GGA ACT GCC CAT GTT CCC CCA GGG GAC TCC TGG ACT

         1270         1280         1290         1300         1310         1320
CGG GCA CAC CCA TCT GGC TCT CCA GCT TCA TAG TTG TCC ATC CAT ATT AGG AAT GTA CCA

         1330         1340         1350         1360         1370         1380
GAA GAT GCT AGA TAG TTC TGA GTC TGG AAA CTA GGA CCA AGG ACC TGC AGG AGT CAT CCC

         1390         1400         1410         1420         1430         1440
ACA AAC CCT CCC CCT GTA TTC CCA GGG CAG AGA TTC CGG AGG ACC CAC TTT CAC TGC TGC

         1450         1460         1470         1480         1490         1500
TTC CCA CCC CCA GGA CCC CAC AGG AAG AGA GGA GGT ATT TGC AGA CTG CAA GCC TCA GCT

         1510         1520         1530         1540         1550         1560
CAC GGA GCC TAC TTA TCT TCT CCA TGT AGG GGC ACT AGC GTG CCT GGA GGG GAA TGC TGT

         1570         1580         1590         1600         1610
TCG AAA GCA GTT TCT TCT CTT TGT AAA TTA TTT AAG ACA TCT GCT ATG TCA TT
```

Fig. 3.3: Murine *Cats* nucleotide and amino acid sequences. The cDNA sequence (1613 nt) corresponds to the cDNA clone IMAGp998H174619Q2. The first nucleotide of each exon is underlined. The stop codon is marked. Yellow filled boxes indicate the primers used to amplify *Cats* ORF by RT-PCR from thymus RNA. The underlined and bold sequence is absent both in the RT-PCR product (italicized) and in the genomic clone L02EcoRI-2 (3.1.2.2).

3.1.2.2 Cloning of the murine *Cats* genomic locus

The arrayed mouse genomic cosmid library 129/ola was screened as described in 2.2.18 using the *Cats* cDNA F113-R942 fragment (3.1.2.1) as a probe. Nine clones were detected with the *Cats* probe and ordered from RZPD (2.1.16.1). PCR analysis using these clones as template and *Cats* specific primers mCATSF364 and mCATSF630 showed *Cats* amplification in six out of the nine clones (MPMGc121C1938Q2, MPMGc121C0434Q2, MPMGc121L02225Q2, MPMGc121H24352Q2, MPMGc121D13437Q2 and MPMGc121K17553Q2). Restriction mapping and further PCR analysis revealed the presence of the whole *Cats* genomic locus in an *Eco*RI fragment of approx. 7.9 kb from cosmid MPMGc121L02225Q5. This *Eco*RI fragment was subcloned into the *Eco*RI site of the pBluescript II KS (+/-) vector. The corresponding clone, named L02EcoRI-2 was fully sequenced and characterized in further detail. The murine *Cats* is located on chromosome 11 (genomic contig NT_096135) in a region which is syntenic to human chromosome 17. The murine *Cats* genomic locus spans approx. 6 kb and similarly to the human counterpart comprises 6 exons with a non-coding first exon. Coding exons are of similar size in both human and mouse genes. Except for intron 1, introns are shorter in the murine locus (Figure 3.4). The non-canonical splice site of *CATS* is also found in the third intron of the murine *Cats* gene (Table 3.2).

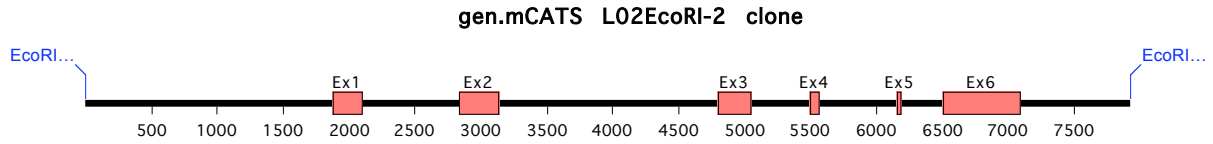


Fig. 3.4: Murine genomic *Cats* locus in clone L02EcoRI-2. The EcoRI fragment (7.9 kb) was isolated from cosmid clone MPMGc121L02225Q5 and subcloned into the pBluescript II KS (+/-) vector. Ruler represents the nucleotide. Exons (Ex) are represented as filled boxes. Restriction enzymes used for cloning are depicted in blue.

Intron	splice acceptor	Exon	splice donor
		Exon 1 (253 bp)	GGCGAGCCAGgtgggggtc
Intron 1 (714 bp)	acctccccagGATCTAGCGG	Exon 2 (317 bp)	CATGTCCCAGgtaacaatga
Intron 2 (1642 bp)	aacccatcagAGGATCCAGG	Exon 3 (279 bp)	GCTCTCCCAG**gc**aagtagtc
Intron 3 (422 bp)	ttgtgttcagTGAGTCTGAC	Exon 4 (97 bp)	CTGAAGAGAGgtgagctgca
Intron 4 (565 bp)	ctgtccttagTGGTGACATG	Exon 5 (55 bp)	CATCCTACAGgtaatgaccc
Intron 5 (293 bp)	tgtcttacagGAAGCCGGCT	Exon 6 (669 bp)	

Table 3.2: Exon-intron boundaries of *Cats*. Sequences of the splice donor and acceptor sites in murine *Cats* as determined by comparison of genomic sequence and RT-PCR product (3.1.2.1). Exons are in upper-case and introns are in lower-case. The non-canonical splice donor is in bold. Exon and intron sizes are indicated in brackets.

3.1.3 CATS homology among different species

Protein databases were searched with the CATS amino acid sequence in order to identify evolutionarily conserved domains in CATS. The Cats protein was identified in four other species, *Bos Taurus (*genomic contig BtUn_WGA322593_1), *Rattus norvegicus* (genomic contig Rn10_WGA1855_3), *Fugu rubripes* and *Gallus gallus* (genomic contig Gga19_WGA443_1) (Figure 3.5). Comparison of the amino acid sequences of the CATS proteins revealed that the human isoform 1 rather than isoform 2 has a higher overall homology to the Cats proteins of other species. Closest to the human CATS protein is the bovine (*Bos taurus*) protein, with an overall homology of 91 % and 85 % to the human isoform 1 and 2, respectively. Human CATS isoform 1 revealed to be 76 % homologous to both murine and rat Cats (*Rattus norvegicus*) proteins, 47 % homologous to the chicken (*Gallus gallus*) protein and 34 % homologous to the puffer fish (*Fugu rubripes*) protein (Table 3.3). Four regions within the proteins were conserved from *Fugu* to man suggesting that these regions are important for function protein (Figure 3.5).

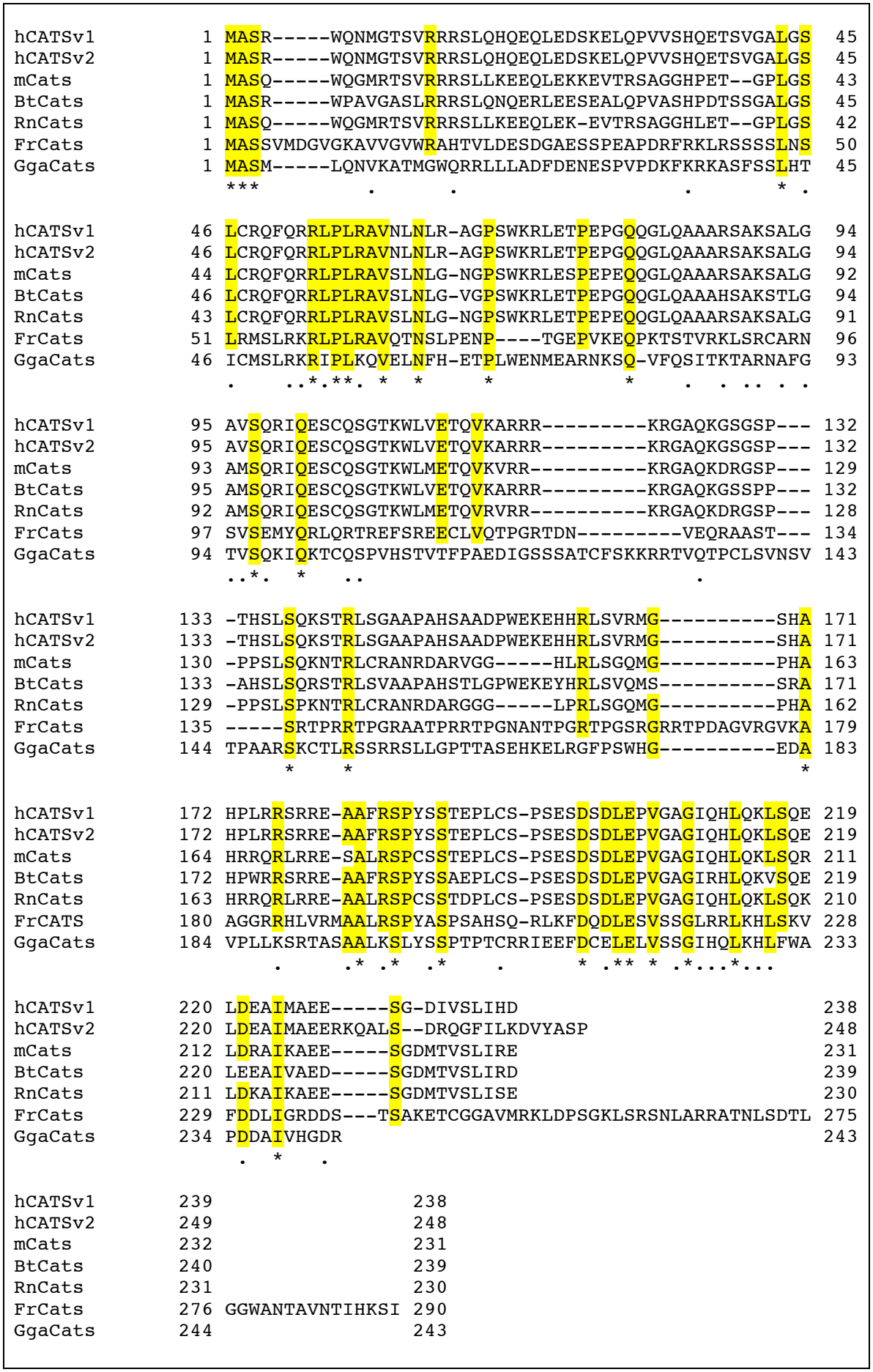

hCATSv1 1 MASR-----WQNMGTSVRRRSLQHQEQLEDSKELQPVVSHQETSVGALGS 45
hCATSv2 1 MASR-----WQNMGTSVRRRSLQHQEQLEDSKELQPVVSHQETSVGALGS 45
mCats 1 MASQ-----WQGMRTSVRRRSLLKEEQLEKKEVTRSAGGHPET--GPLGS 43
BtCats 1 MASR-----WPAVGASLRRRSLQNQERLEESEALQPVASHPDTSSGALGS 45
RnCats 1 MASQ-----WQGMRTSVRRRSLLKEEQLEK-EVTRSAGGHLET--GPLGS 42
FrCats 1 MASSVMDGVGKAVVGVWRAHTVLDESDGAESSPEAPDRFRKLRSSSSLNS 50
GgaCats 1 MASM-----LQNVKATMGWQRRLLLADFDENESPVPDKFKRKASFSSLHT 45
hCATSv1 46 LCRQFQRRLPLRAVNLNLR-AGPSWKRLETPEPGQQGLQAAARSAKSALG 94
hCATSv2 46 LCRQFQRRLPLRAVNLNLR-AGPSWKRLETPEPGQQGLQAAARSAKSALG 94
mCats 44 LCRQFQRRLPLRAVSLNLG-NGPSWKRLESPEPEQQGLQAAARSAKSALG 92
BtCats 46 LCRQFQRRLPLRAVSLNLG-VGPSWKRLETPEPGQQGLQAAAHSAKSTLG 94
RnCats 43 LCRQFQRRLPLRAVSLNLG-NGPSWKRLETPEPEQQGLQAAARSAKSALG 91
FrCats 51 LRMSLRKRLPLRAVQTNSLPENP----TGEPVKEQPKTSTVRKLSRCARN 96
GgaCats 46 ICMSLRKRIPLKQVELNFH-ETPLWENMEARNKSQ-VFQSITKTARNAFG 93
hCATSv1 95 AVSQRIQESCQSGTKWLVETQVKARRR---------KRGAQKGSGSP--- 132
hCATSv2 95 AVSQRIQESCQSGTKWLVETQVKARRR---------KRGAQKGSGSP--- 132
mCats 93 AMSQRIQESCQSGTKWLMETQVKVRR----------KRGAQKDRGSP--- 129
BtCats 95 AMSQRIQESCQSGTKWLVETQVKARRR---------KRGAQKGSSPP--- 132
RnCats 92 AMSQRIQESCQSGTKWLMETQVRVRR----------KRGAQKDRGSP--- 128
FrCats 97 SVSEMYQRLQRTREFSREECLVQTPGRTDN---------VEQRAAST--- 134
GgaCats 94 TVSQKIQKTCQSPVHSTVTFPAEDIGSSSATCFSKKRRTVQTPCLSVNSV 143
hCATSv1 133 -THSLSQKSTRLSGAAPAHSAADPWEKEHHRLSVRMG----------SHA 171
hCATSv2 133 -THSLSQKSTRLSGAAPAHSAADPWEKEHHRLSVRMG----------SHA 171
mCats 130 -PPSLSQKNTRLCRANRDARVGG-----HLRLSGQMG----------PHA 163
BtCats 133 -AHSLSQRSTRLSVAAPAHSTLGPWEKEYHRLSVQMS----------SRA 171
RnCats 129 -PPSLSPKNTRLCRANRDARGGG-----LPRLSGQMG----------PHA 162
FrCats 135 -----SRTPRRTPGRAATPRRTPGNANTPGRTPGSRGRRTPDAGVRGVKA 179
GgaCats 144 TPAARSKCTLRSSRRSLLGPTTASEHKELRGFPSWHG----------EDA 183
hCATSv1 172 HPLRRSRRE-AAFRSPYSSTEPLCS-PSESDSDLEPVGAGIQHLQKLSQE 219
hCATSv2 172 HPLRRSRRE-AAFRSPYSSTEPLCS-PSESDSDLEPVGAGIQHLQKLSQE 219
mCats 164 HRRQRLRRE-SALRSPCSSTEPLCS-PSESDSDLEPVGAGIQHLQKLSQR 211
BtCats 172 HPWRRSRRE-AAFRSPYSSAEPLCS-PSESDSDLEPVGAGIRHLQKVSQE 219
RnCats 163 HRRQRLRRE-AALRSPCSSTDPLCS-PSESDSDLEPVGAGIQHLQKLSQK 210
FrCATS 180 AGGRRHLVRMAALRSPYASPSAHSQ-RLKFDQDLESVSSGLRRLKHLSKV 228
GgaCats 184 VPLLKSRTASAALKSLYSSPTPTCRRIEEFDCELELVSSGIHQLKHLFWA 233
hCATSv1 220 LDEAIMAEE-----SG-DIVSLIHD 238
hCATSv2 220 LDEAIMAEERKQALS--DRQGFILKDVYASP 248
mCats 212 LDRAIKAEE-----SGDMTVSLIRE 231
BtCats 220 LEEAIVAED-----SGDMTVSLIRD 239
RnCats 211 LDKAIKAEE-----SGDMTVSLISE 230
FrCats 229 FDDLIGRDDS---TSAKETCGGAVMRKLDPSGKLSRSNLARRATNLSDTL 275
GgaCats 234 PDDAIVHGDR 243
hCATSv1 239 238
hCATSv2 249 248
mCats 232 231
BtCats 240 239
RnCats 231 230
FrCats 276 GGWANTAVNTIHKSI 290
GgaCats 244 243

Fig. 3.5: Alignment of Cats proteins. Comparison between Cats proteins from human, mouse, cow, rat, fugo and chicken. Asterisks and points under the alignment represent identical and similar amino acids, respectively. Regions conserved from *Fugu* to man are marked in yellow. hCATSv1 and hCATSv2 (human protein isoform 1 and 2, respectively), mCats (*M. musculus*), BtCats (*Bos taurus*), RnCats (*Rattus norvegicus*), FrCats (*Fugu rubripes*) and GgaCats (*Gallus gallus*).

	hCATSv1	hCATSv2	mCats
mCats	76 %	71 %	
BtCats	91 %	85 %	77 %
RnCats	76 %	70 %	96 %
FrCats	34 %	35 %	30 %
GgaCats	47 %	47 %	18 %

Table 3.3: Homology between CATS proteins from different vertebrate species.

3.2 Expression analysis of CATS at the RNA level in normal tissue

3.2.1 *CATS* is predominantly expressed in lymphoid organs

The complete CATS open reading frame (ORF) (exons 2-5) was amplified by PCR using the CATS cDNA IMAGp998G039611 clone (2.1.15) as template and the primer pair FhCATSXhoI and RhCATSHindIII (2.1.13). The 730 bp product was radioactively labeled and used to probe human multiple tissue Northern blots. The hybridization revealed a *CATS* transcript of approximately 1.6 kb expressed predominantly in lymphoid organs. High levels of expression can be seen in thymus and spleen and to a lesser extent in the small and large intestines. Low levels of transcript are also present in ovary and brain. No transcripts were detected in placenta, lung, liver, kidney, pancreas, prostate, testis or peripheral blood. In skeletal muscle and heart, transcripts of higher molecular weight (of approximately 4 kb and 6.5 kb) are detected. These are most likely due to transcripts containing a longer 3' untranslated region (Figure 3.6).

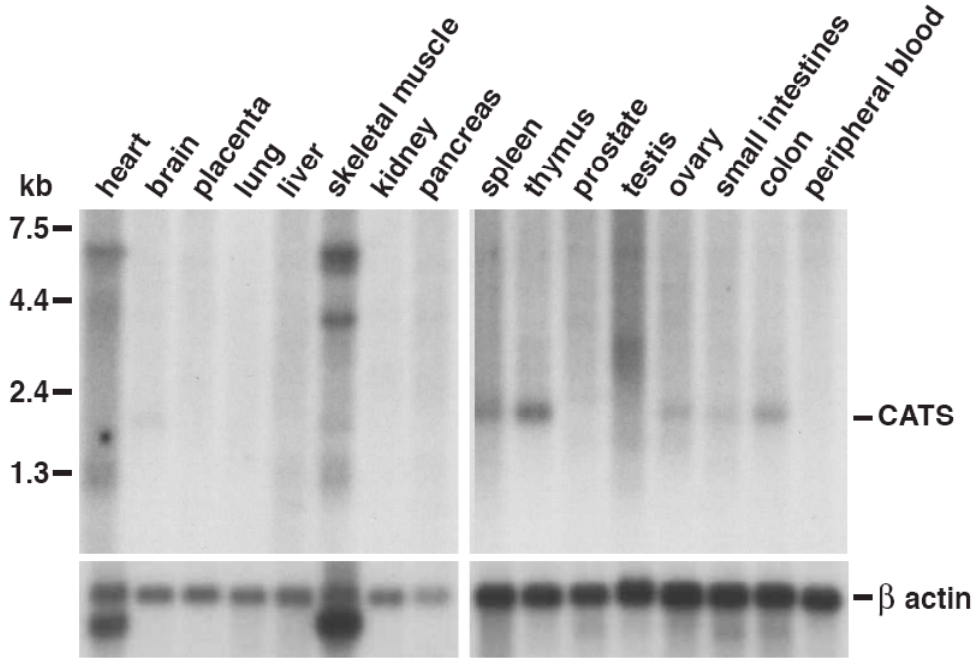


Fig. 3.6: Predominant expression of *CATS* in lymphoid organs. Multiple tissue Northern blots (Clontech) containing 2µg poly(A)$^+$ RNA per lane from 16 different human adult tissues. Membranes were hybridized with a cDNA probe for *CATS* and human β-actin, which serves as a control for the loading and the integrity of the RNA preparation.

3.2.2 Expression of the murine *Cats* in adult multiple tissue

The complete Cats ORF was used as a probe for murine Northern blot analysis. The approximately 1.5 kb long murine transcript showed high levels of expression in thymus and ovary and low level of expression in heart. *Cats* transcripts were absent in brain, kidney, liver, bone marrow and surprisingly, in spleen of adult mice (Figure 3.7).

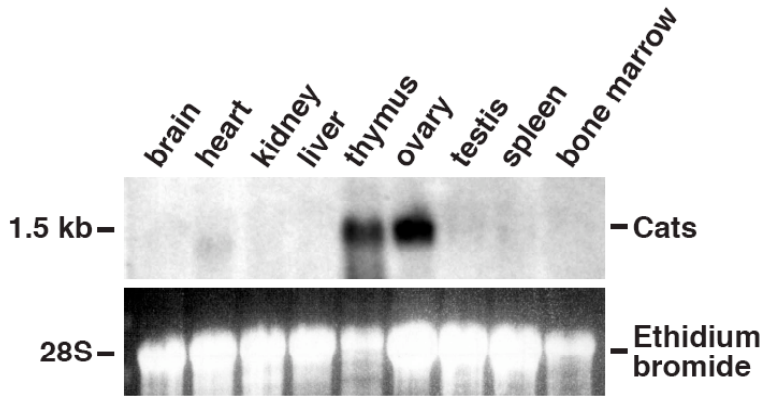


Fig. 3.7: Expression of *Cats* in adult multiple tissue Northern blot. Northern blot containing 10 µg total RNA per lane from nine different tissues of adult mice was hybridized with Cats cDNA F113-R942 fragment (3.1.2.1). The 1.5 kb murine transcript is strongly expressed in thymus and ovary and to a much lesser extent in heart. Bottom panel shows Ethidium bromide staining as a control for loading and integrity of the RNA preparation.

Since the RT-PCR technique is far more sensitive than Northern blot analysis, it was used to analyze *Cats* expression in tissues with either low levels of gene expression or with small amount of material available (e.g. lymph nodes). In a semi quantitative RT-PCR analysis, equal amounts of cDNA were used from each tissue to be amplified with *Cats* specific primers mCATSF364 and mCATSR630 (2.1.13). Amplification of a 305 bp product under stringent conditions (1.2 mM MgCl final concentration) revealed stronger *Cats* expression in thymus and ovary, confirming the results obtained with Northern blot analysis. However, after 35 PCR cycles *Cats* expression is also clearly seen in bone marrow, lymph node and testis although not as strong as in thymus and ovary. Except for liver, low levels of *Cats* transcripts were also detected in brain, heart, kidney and spleen (Figure 3.8).

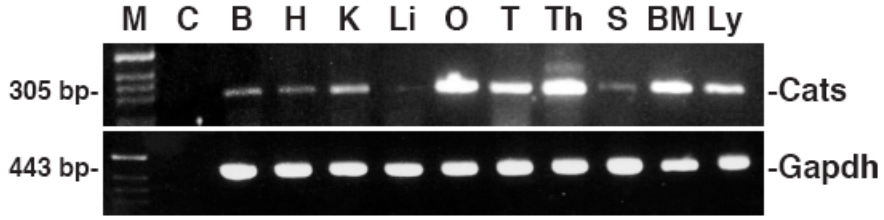


Fig. 3.8: RT-PCR with total RNA from 10 different tissues of adult mice. Strong *Cats* expression is found in thymus and ovary. Less expression is detected in bone marrow, lymph node and testis. After 35 PCR cycles *Cats* transcripts could also be amplified from brain, heart, kidney and spleen but not from liver. Control RT-PCR amplification of a 443 bp mGapdh fragment from the same RNA samples reveals that comparable amounts of cDNA were used as template in the reactions. M: DNA size marker. C: negative control PCR reaction with no template shows no amplification. Tissues are: brain (B), heart (H), kidney (K), liver (Li), ovary (O), testis (T), thymus (Th), spleen (S), bone marrow (BM) and lymph node (Ly).

3.2.3 *Cats* is strongly expressed throughout mouse embryogenesis

Northern blot analysis of *Cats* with total RNA prepared from mouse embryos revealed a strong expression of *Cats* transcript in every stage of development tested. However, *Cats* expression decreased gradually while embryos developed to later stages. Stages 9.5-14.5 days post coitum (d.p.c.) used for this analysis correspond to the time of organogenesis, suggesting an important role for *Cats* during organ formation (Figure 3.9).

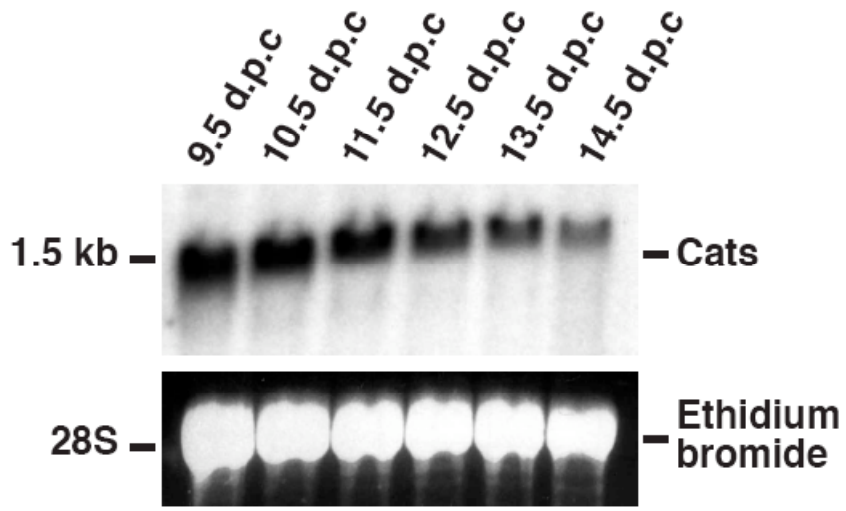


Fig. 3.9: *Cats* expression throughout mouse embryogenesis. Northern blot containing 10 µg total RNA per lane from whole mouse embryos of day 9.5 to 14.5 d.p.c.. The blot was hybridized with *Cats* cDNA F113-R942 fragment (3.1.2.1). The 1.5 kb murine transcript is strongly expressed in all stages examined. Note reduction of *Cats* expression during the course of embryo development. Bottom panel shows Ethidium bromide staining as a control for loading and integrity of the RNA preparation.

Whole mount *in situ* hybridizations (2.2.19) were performed to analyze the temporo-spatial expression pattern of *Cats* during mouse development. Hybridization with either the PI sense or the PIII anti-sense probe (2.2.19.2) revealed widespread expression of *Cats* at stage 9.5 d.p.c with prominent expression in the neural tube, somites, posterior region of the midbrain, olfactory placode and the branchial arches but no expression in the primitive heart. At 10.5 d.p.c there is a reduction of the widespread expression and stronger *Cats* expression is seen in the neural tube, branchial arches, developing limbs, telencephalon, nasal process, lense vesicle, anterior and posterior regions of the midbrain and hindbrain. From 11.5 d.p.c on strong staining is also seen in the genital tubercle and hair and vibrissae follicles. A reduction of *Cats* expression is seen from stage 12.5 d.p.c onwards with completely lack of *Cats* expression in the cephalic region and the neural tube in 14.5 d.p.c embryos (Figure 3.10).

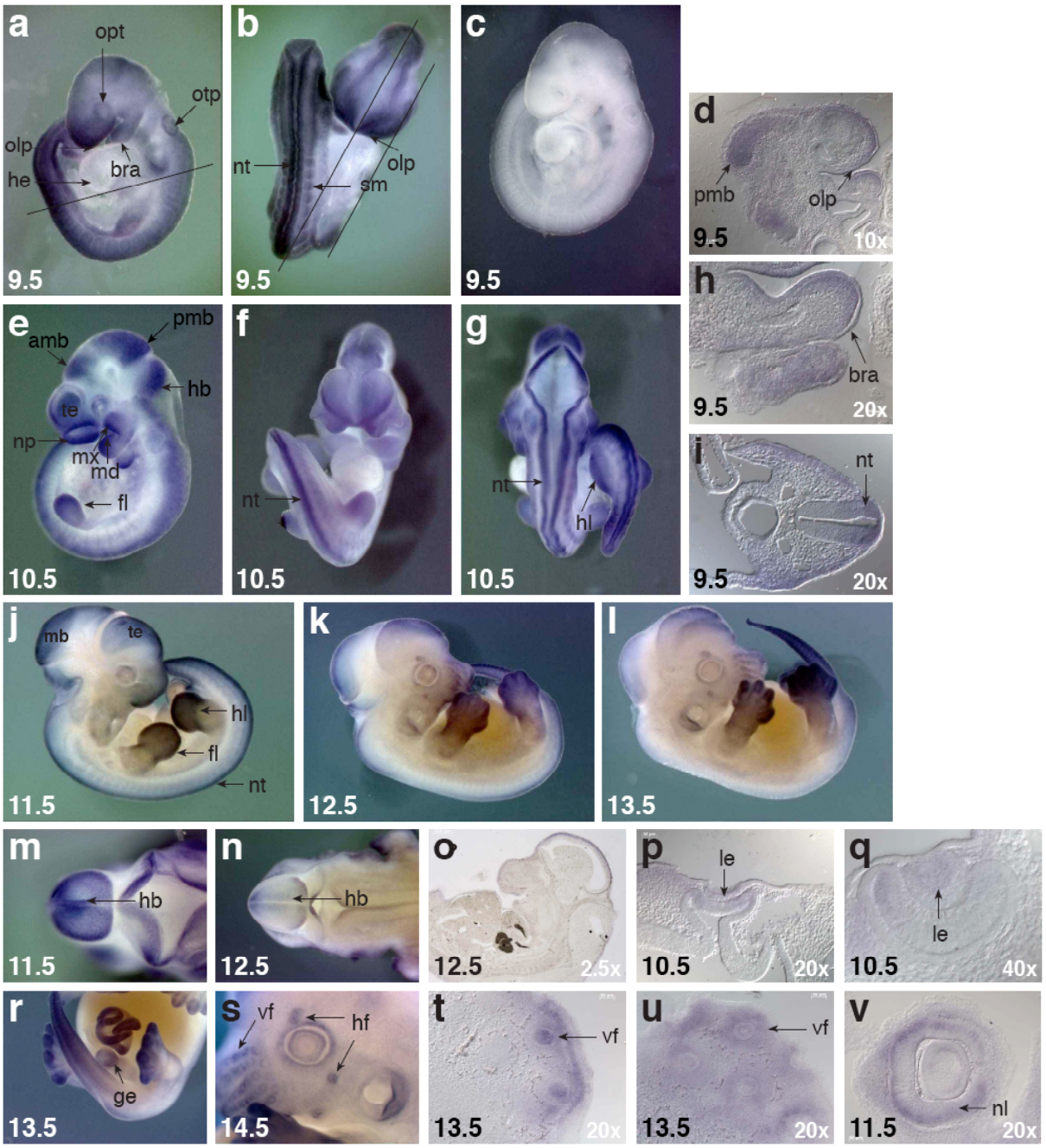


Fig. 3.10: Whole mount *in situ* hybridization analysis during mouse embryonic development. Embryos hybridized with Cats PIII anti-sense RNA probe. (**a, b, d, h, i**) Expression of *Cats* at embryonic stage of 9.5 d.p.c.. Widespread transcript distribution with prominent expression in the neural tube (nt), somites (sm), posterior region of the midbrain (pmb), olfactory placode (olp) and in branchial arches (bra). Note complete absence of *Cats* expression in the primitive heart (he). Otic pit: otp; optic vesicle: opt. Bars indicate the plane of section. (**c**) 9.5 d.p.c embryo hybridized with Cats PI sense RNA probe as a control for staining specificity. (**e-g, p, q**) At 10.5 d.p.c., there is a reduction of widespread *Cats* expression, transcripts are additionally detected in fore- and hindlimbs (fl and hl, respectively), telencephalon (te), nasal process (np), lens vesicle (le), anterior region of the midbrain (amb), hindbrain (hb) and mandibular and maxillary component of first branchial arch (md and mx, respectively). (**j-l**) Right lateral view of embryos from 11.5, 12.5 and 13.5 d.p.c.. Note reduction of *Cats* overall expression and prominent expression in the limbs. (**m, n**) Dorsal view of the cephalic region of 11.5 and 12.5 d.p.c embryos. Note the abrupt reduction of *Cats* expression in the hindbrain and neural tube from stage

11.5 to 12.5 d.p.c.. (**o**) Remaining *Cats* expression in the midbrain of a 12.5 embryo. (**p-v**) Transcripts detected in the lense vesicle (le), genital tubercle (ge), hair follicles (hf), vibrissae follicles (vf) and neural layer of optic cup (nl). (**d, h, i, o-q, t-v**) Sagittal (**d, h, o, t-v**) and transverse (**i, p, q**) sections from whole mount stained embryos. Stage of development and imaging magnification are indicated on the left and right bottom, respectively.

Cats is strongly expressed in limb development. In general, stronger expression is detected in hindlimbs rather than forelimbs and it is slightly more prominent in the posterior region of the limb buds. At 11.5 and 12.5 d.p.c., expression is observed at the distal domain and the underlying mesenchyme but it is not present in the apical ectodermal ridge (AER). Distally, expression of *Cats* becomes confined to the digits in stages 13.5 and 14.5 d.p.c. (Figure 3.11).

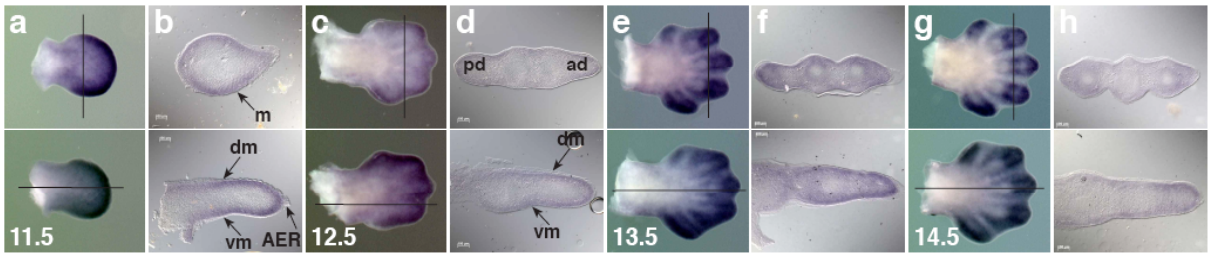


Fig. 3.11: *Cats* expression during limb development. In all panels the dorsal side of the limb is facing, the anterior side is to the top. The upper half of each figure shows a forelimb, the lower a hindlimb. Sections were generated from whole mount stained limb buds with 10x magnification (**b, d, f, h**). Bars indicate the plane of section. Transverse sections (forelimbs) are orientated with posterior side to the left, sagittal sections (hindlimbs) with the proximal side to the left. (**a**) Limb buds of a 11.5 d.p.c. embryo. The expression of *Cats* is more prominent in the distal part of the paddle-shaped fore- and hindlimb buds. (**b**) The transverse section shows staining in the underlying mesenchyme (m) and the sagittal section in the dorsal and ventral mesenchyme (dm and vm, respectively) but not in the apical ectodermal ridge (AER). (**c**) At 12.5 d.p.c. *Cats* expression is still detected at the distal part of the polygonal-shaped fore- and hindlimb buds. (**d**) Note stronger staining in the posterior domain (pd) rather than in the anterior domain (ad). (**e, g**) Distally, expression of *Cats* becomes confined to the digits in stages 13.5 and 14.5 d.p.c..

3.2.4 *Cats* is widely expressed in hematopoietic cell subpopulations

RT-PCR analysis with RNA from FACS sorted cells (2.2.31) was performed to determine *Cats* expression in the hematopoietic compartment. Amplification was performed under stringent condition with the same primer pair used in 3.2.2. The expression analysis revealed wide-spread expression of *Cats* in the different cell subpopulations found in the murine thymus and bone marrow. Transcripts were observed in all 3 tested T-cell subpopulations isolated from the thymus ($CD4^+$, $CD8^+$ and $CD4^+/CD8^+$) although lower levels of transcript are seen in $CD8^+$ cells (Figure 3.12a). Similarly, extremely low levels of *Cats* were observed

in the $CD8^+$ cells isolated from bone marrow. However, except for the pro-B cell subpopulation ($B220^+$) where *Cats* expression was nearly absent, abundant levels of transcript were observed in macrophages ($Mac1^+$), pro-erythrocytes ($Ter119^+$), granulocytes ($Gr\text{-}1^+$), mast cells ($c\text{-}kit^+$) and progenitor cells ($Sca\text{-}1^+/c\text{-}kit^+$) (Figure3.12b).

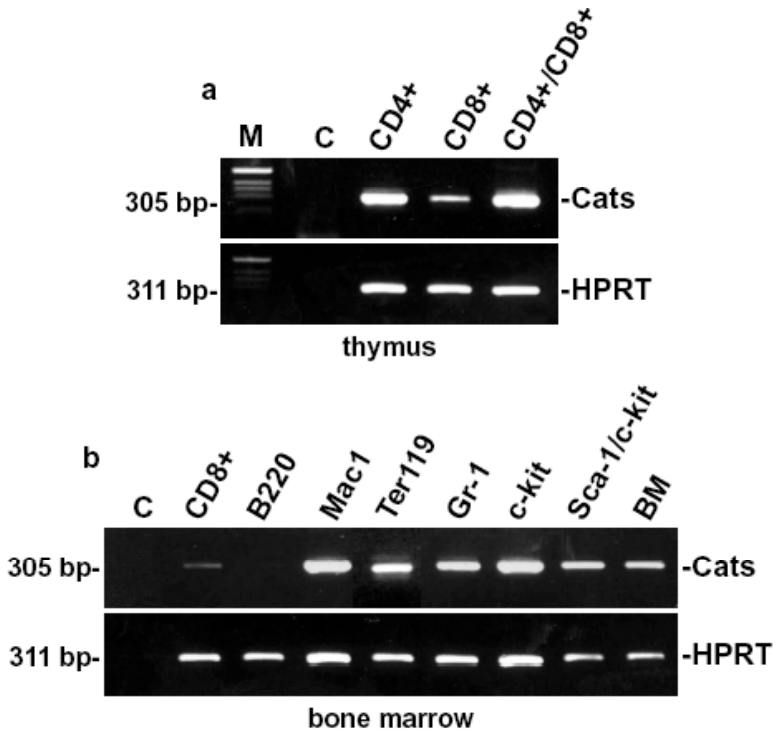


Fig. 3.12: RT-PCR with total RNA from hematopoietic cell subpopulations. (**a**) T-cell subpopulations isolated from murine thymus ($CD4^+$, $CD8^+$ and $CD4^+/CD8^+$). Note lower levels of transcript in $CD8^+$ cells. (**b**) Abundant *Cats* expression was observed in $Mac1^+$, $Ter119^+$, $Gr\text{-}1^+$, $c\text{-}kit^+$ and $Sca\text{-}1^+/c\text{-}kit^+$, low in $CD8^+$ and nearly absent in $B220^+$ cell subpopulations from the murine bone marrow. In the control RT-PCR, a 311 bp murine HPRT fragment was amplified from the same RNA samples. M: DNA size marker. C: negative control PCR reaction with no template. Samples are: T-cells ($CD4^+$, $CD8^+$ and $CD4^+/CD8^+$), pro-B cells ($B220^+$), macrophages ($Mac1^+$), pro-erythrocytes ($Ter119^+$), granulocytes ($Gr\text{-}1^+$), mast cells ($c\text{-}kit^+$), progenitor cells ($Sca\text{-}1^+/c\text{-}kit^+$) and bone marrow (BM).

3.3 Expression analysis of *CATS* at RNA level in leukemia cells

3.3.1 *CATS* is expressed in cells from leukemia patients carrying the CALM/AF10 rearrangement

RT-PCR was performed in order to determine *CATS* expression in leukemic cells carrying the t(10;11)(p13;q14). For this analysis, the U937 cell line as well as cells from 4 different leukemia patients (2.1.17) were used. 3 different *CATS* transcripts could be amplified in both patients carrying the CALM/AF10 fusion (patients 1: T-ALL and patient 2: AML). Similar expression levels of *CATS* were observed in one of the T-ALL patients negative for the

CALM/AF10 fusion (patient 3). However *CATS* was nearly absent in patient 4 (T-ALL). High *CATS* expression was also observed in the CALM/AF10 fusion positive cell line U937 and in 293T. Sequence analysis revealed the 3 amplified fragments to be *CATS* splice variants 1, 2 and 3. Interestingly, the splice variant 3 (775 bp band) is highly expressed in patient 1, whereas it is absent in the other patients and cell lines. Splice variant 1 seems to be more abundant than the splice variant 2, being more prominent in the cell lines than in the patient samples (Figure 3.13).

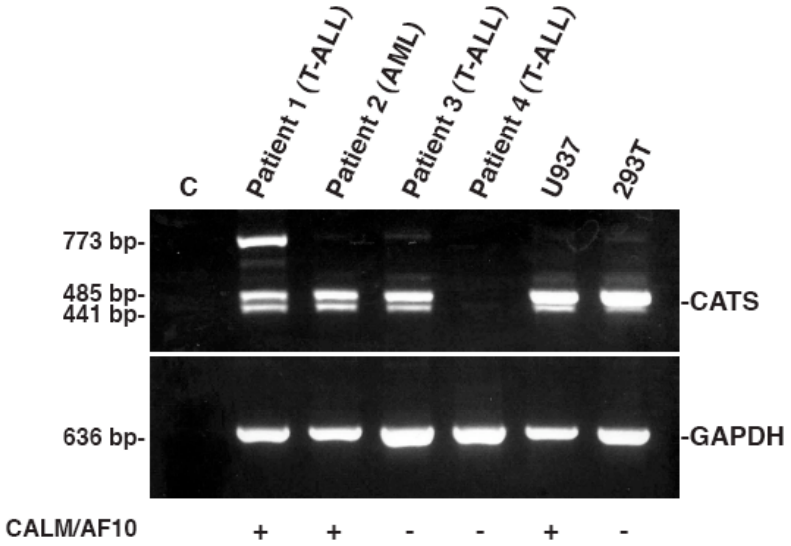


Fig. 3.13: Expression analysis of *CATS* in cells from leukemia patients and cell line carrying the t(10;11)(p13;q14). *CATS* transcripts were amplified using primer pair CATSE1F1(364) and CATSE5R1(828) (2.1.13). Patients 1 and 2 as well as the U937 cell line are positive for CALM/AF10 fusion, whereas patients 3 and 4 are negative. Sequence analysis revealed that the 3 amplified fragments corresponded to *CATS* splices variants 1, 2 and 3 (485, 441 and 775 bp, respectively). Strong expression of *CATS* splice variant 3 was observed only in the CALM/AF10 positive T-ALL patient 1. 293T cell line was used as positive control for *CATS* expression. Amplification of GAPDH fragment from the same RNA samples was used as control for RNA and cDNA integrity. (C) Negative control PCR reaction with no template.

3.3.2 *Cats* is up-regulated in B220$^+$ cells from a CALM/AF10 leukemic mouse

Expression analysis was performed in leukemic cells derived from a CALM/AF10 murine bone marrow transplant model (2.1.17). PCR reactions were performed as in 3.2.2 and 3.2.4. For the analysis, *Cats* expression was determined in B220$^+$/Mac1$^-$, B220$^+$/Mac1$^+$, B220$^-$/Mac1$^+$ populations and whole bone marrow from a CALM/AF10 leukemic mouse. *Cats* was strongly expressed in all 4 samples derived from the leukemic mouse. Moreover, an striking

increase of *Cats* transcripts was observed in B220^{+}/Mac1^{-} population in comparison to the same population from a non-leukemic mouse (Figure 3.14).

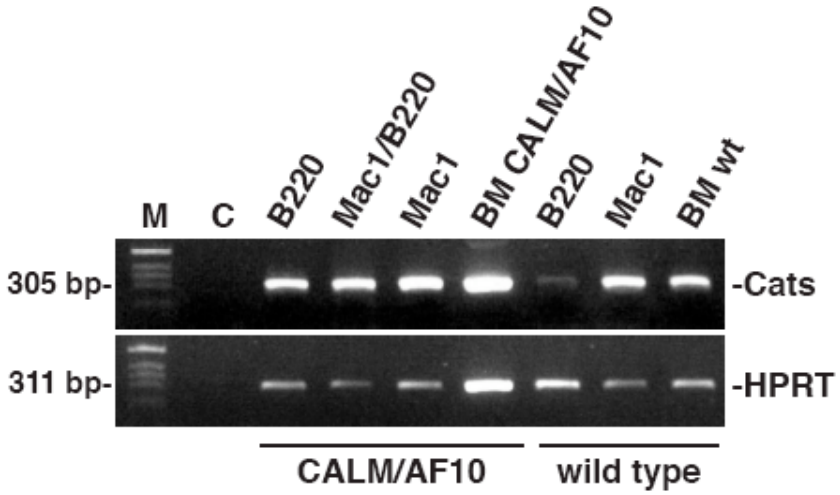


Fig. 3.14: Analysis of *Cats* expression in leukemic cells derived from a CALM/AF10 murine bone marrow transplant model. *Cats* is expressed in the B220^{+}/Mac1^{-}, B220^{+}/Mac1^{+}, B220^{-}/Mac1^{+} populations and whole bone marrow from a CALM/AF10 leukemic mouse. The controls used were B220 positive cells sorted from the spleen, Mac-1 sorted cells from the bone marrow and whole bone marrow from a non-leukemic mouse. Note the different levels of *Cats* expression in B220 positive cells derived from a non-leukemic and a leukemic mouse. Control RT-PCR amplified a 311 bp murine HPRT fragment from the same RNA samples. M: DNA size marker. C: negative control PCR reaction with no template.

3.4 CATS subcellular localization

3.4.1 Construction of plasmids expressing a fluorescent protein fusion of CATS

For the subcellular localization experiments, plasmids expressing green, cyan or yellow fluorescent fusion proteins of CATS were constructed. For GFP-CATSv1 and CFP-CATSv1 constructs, full length CATS was generated by PCR amplification using the CATS clone IMAGp998G039611 (3.1.1) as template and primers containing sites for *Xho*I and *Hin*dIII restriction enzymes (FhCATSXhoI and RhCATSHindIII). The 730 bp PCR product corresponding to CATS isoform 1 was digested with the appropriate restriction enzymes and cloned in frame with the green and cyan fluorescent proteins into the pEGFP-C1 and pECFP-C1 vectors, respectively (2.1.11) (Figure 3.15 a). CFP-CATSv2 was constructed by replacing the 51 amino acids C-terminal of CATS isoform 1 by the corresponding 61 amino acids of CATS isoform 2 in the CFP-CATSv1 clone (described above). The C-terminal portion of the CATS isoform 2 was generated by PCR using the pGADGH-CATSv2CT prey clone (2.1.12) as a template and primers containing sites for the *Sal*I restriction enzyme (hCATSf SalI

635(ex3) and hCATSr SalI 909(ex6)). The PCR product was digested with *Eco*RI (internal site within the sequence) and *Sal*I, the resulting fragment corresponding to amino acids 188 to 248 of isoform 2 was subcloned into the CFP-CATSv1 construct, from which the C-terminus had been excised with the same enzymes (Figure 3.15 b). YFP-CATS constructs were generated by excising the ORF of CATS isoforms from their respective CFP constructs with *Xho*I and *Kpn*I and subcloned into the pEYFP-C1 vector (2.1.11) digested with the same enzymes (Figure 3.15 c; d).

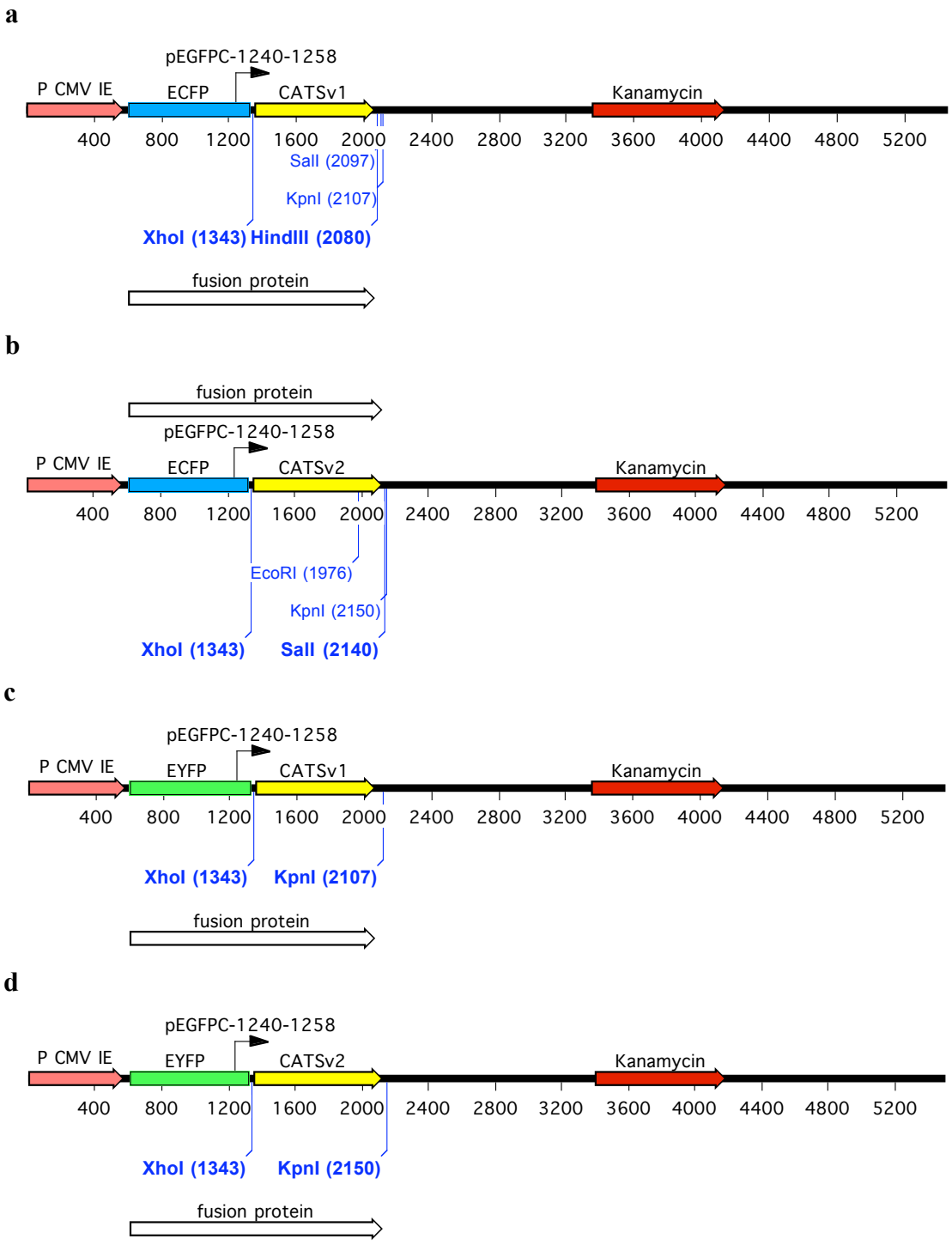


Fig. 3.15: CFP-, GFP- and YFP-CATS constructs. (**a**) The CATSv1 ORF was amplified using primer pair FhCATSXhoI and RhCATSHindIII. The 730 bp product was cloned into pEGFP- and pECFP-C1 vectors digested with *Xho*I and *Hin*dIII. (**b**) CFP-CATSv2 was constructed by replacing the 51 aa C-terminal of CATS isoform 1 by the corresponding 61 amino acids of CATS isoform 2 in the CFP-CATSv1 clone (*Eco*RI and *Sal*I sites were used). (**c;d**) CATS isoforms were excised from their respective CFP constructs with *Xho*I and *Kpn*I and subcloned into the pEYFP-C1 vector. Restriction enzymes used for cloning are in bold. Numbers in brackets indicate nucleotide position, ruler indicates construct size. Arrow indicates binding position of the pEGFP-1240-1258 sequencing primer. Kanamycin: kanamycin resistance gene.

3.4.2 CATS is a nuclear protein with predominant nucleolar localization

To determine the intracellular localization of CATS, NIH3T3 cells were transiently transfected (2.2.21) with plasmids expressing YFP-CATS and CFP-CATS fusion proteins. Expression of fluorescently tagged CATS revealed an identical intracellular distribution for the two CATS isoforms (Figure 3.16 g and h). CATS proteins were localized mainly to the nucleus and to a lesser extent to the cytoplasm (Figure 3.16 a-e). Within the nucleus, CATS was often detected in one or several nodular structures, and at a lower level diffusely in the nucleoplasm. These structures are devoid of DAPI staining (Figure 3.16 c) and were most probably the nucleoli. Coexpression of CFP-CATS with YFP tagged nucleostemin and nucleophosmin, which were used as markers for the nucleolar structures, revealed a colocalization of these proteins with CATS at the nucleoli (Figure 3.16 i-n). Some of the CATS protein detected in the cytoplasm was most probably localized at the plasma membrane (Figure 3.16 e).

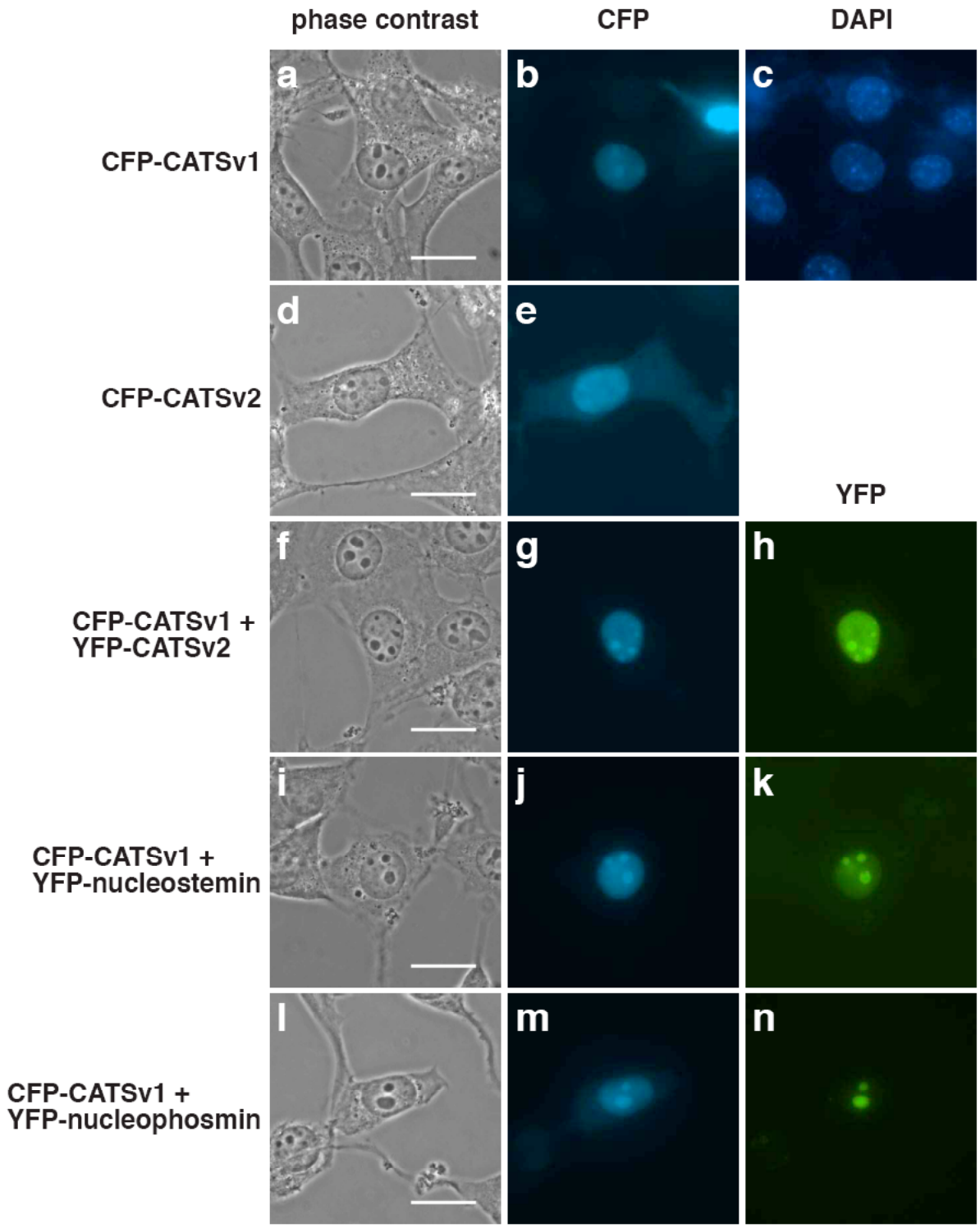


Fig. 3.16: Localization of CATS isoforms and colocalization with nucleostemin and nucleophosmin. (**a-e**) NIH3T3 cells expressing CFP-CATS constructs revealed a predominantly nuclear localization of CATS. (**b, e**) Within the nucleus, the CATS accumulates in nodular structures (**b**), which are devoid of DAPI staining (**c**). Less intense fluorescent signals were also observed in the cytoplasm and at the cell membrane (**e**).
(**f-h**) Coexpression of CFP-CATSv1 and YFP-CATSv2. CATS isoforms colocalize and have an identical subcellular distribution. (**i-n**) CFP-CATS colocalizes with the YFP tagged nucleolar markers nucleostemin and nucleophosmin in the nucleoli. Bar: 20 µm.

3.5 Mapping of CALM-CATS interaction in yeast

3.5.1 Construction of vectors expressing GAL4-AD-CATS fusion proteins

The CATS open reading frames of isoform 1 and 2 were excised from the CFP-CATSv1 and -CATSv2 constructs (3.4.1) using *Xho*I and *Sal*I. The CATSv1 C-terminus was amplified using the CATS clone IMAGp998G039611 as a template and primers containing sites for the *Sal*I restriction enzyme (hCATSf SalI 635(ex3) and hCATSr SalI 909(ex6)). Complete CATS ORF and C-terminal PCR product were inserted into the *Xho*I site of the bacterial/yeast shuttle vector pGADT7 (Figure 3.17) for the expression of yeast GAL4 activation domain CATS fusions.

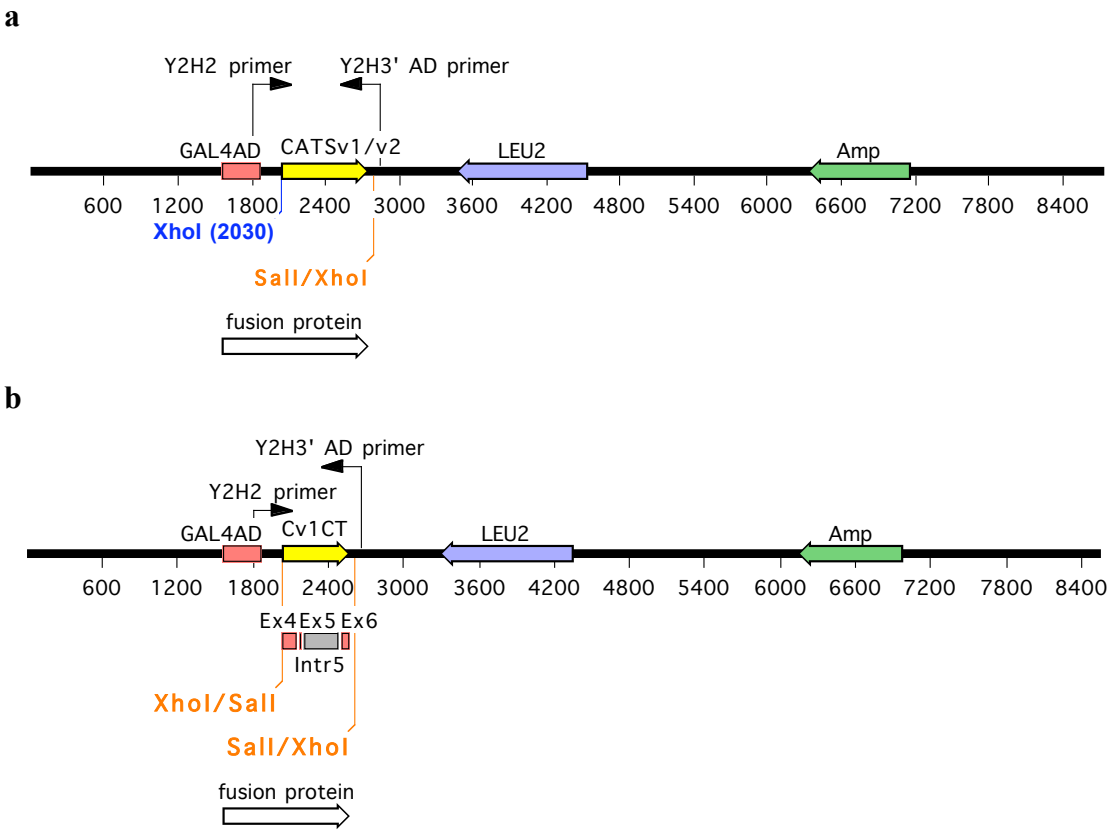
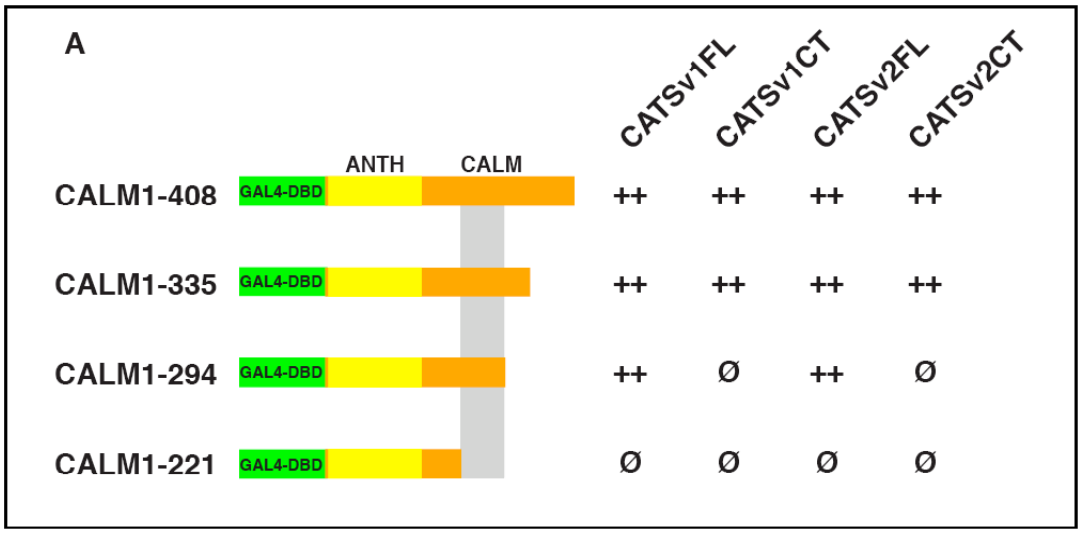


Fig. 3.17: pGADT7-CATS constructs. (**a**) Constructs expressing the GAL4-AD-CATS fusion proteins (either isoform 1 or 2). (**b**) Construct expressing the GAL4-AD fused to the last 51 amino acids of CATS isoform1 (from residue 188 to 238). In bold and blue: restriction enzymes used for cloning. Enzymes in red denote compatible sticky ends used for cloning, which are destroyed upon ligation. Numbers indicate construct size in base pairs. Arrow indicate binding position of the Y2H3'AD and Y2H2-729-751 sequencing primers. LEU2: gene coding for ß-isopropyl malate dehydrogenase an enzyme in the leucine biosynthesis, provides nutritional selection in yeast cells in medium lacking leucine. Amp: ampicillin resistance gene for selection in bacteria.

3.5.2 CATS interacts with amino acids 221 to 294 of CALM

To map the CATS interacting domain of CALM and to test whether this interaction occurs with both CATS isoforms, plasmids encoding CALM deletion mutants fused to the GAL4-DBD (DNA binding domain of the yeast transcription factor GAL4) (2.1.12) were simultaneously transformed (2.2.22.2) with the CATS constructs described above into yeast strain AH109. The cotransformants were assayed for growth on selective plates in the presence of X-α-GAL. CALM(1-408), CALM(1-335), CALM(1-294) but not CALM(1-221) interacted equally with both CATS isoforms. However, there was no interaction of the CALM(1-294) deletion mutant with the C-terminal portions of CATS isoforms 1 (the last 51 amino acids) and 2 (pGADGH-CATSv2CT construct (2.1.12) coding for the last 61 amino acids). These experiments demonstrate that CALM interacts equally with both CATS isoforms and that the N-terminal region of CALM from residues 1-221 does not interact with CATS. Moreover, the CATS interaction domain of CALM could be mapped to amino acids 221 to 294 of CALM for both CATS isoforms. Interestingly, the region comprising amino acids 221 to 294 of CALM does not interact with the CATS C-termini (Figure 3.18 a, b). The data suggest, that the C-termini of CATS require amino acids 294 to 335 of CALM for interaction.



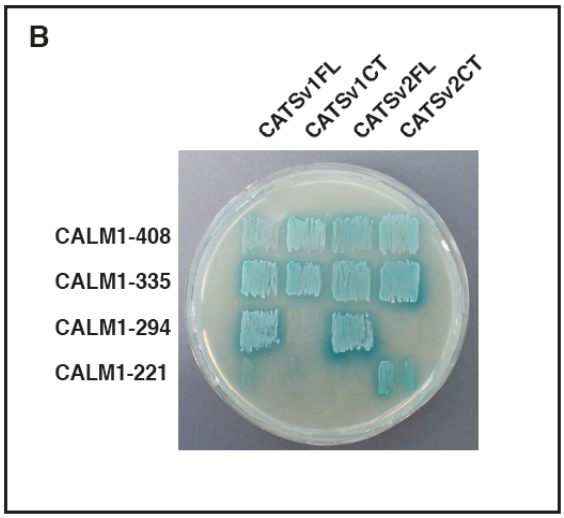


Fig. 3.18: Mapping of the CATS interaction domain of CALM in yeast. Yeast strain AH109 was cotransformed with the following CALM deletion mutants fused to the GAL4-DBD: pGBKT7-CALM(1-408); pGBT9-CALM(1-335); pAS2-CALM(1-294) and pAS2-CALM(1-221) and with the following CATS isoforms (FL) or their respective C-termini (CT) fused to the GAL4-AD: pGADT7-CATSv1FL; pGADT7-CATSv1CT; pGADT7-CATSv2FL and pGADGH-CATSv2CT. (a) Diagram representing the interaction between cotransformed constructs. ++ indicates interaction; Ø indicates no interaction. Gray bar denotes the CATS interaction domain of CALM. (b) Interaction between the proteins was assayed by yeast growth on SD -Trp, -Leu, -His, -Ade plates supplemented with X-α-GAL. ANTH: AP180 N-terminal homology domain.

3.6 CALM interacts with both CATS isoforms *in vivo*

To establish whether endogenous CALM interacts with both CATS isoforms, fluorescently tagged CATS fusion proteins (isoform 1 and 2) were expressed in 293T cells and precipitated with antibody against GFP protein (2.2.28). The GFP antibody detected the immunoprecipitated CATS fusion proteins as shown in figure 3.19, upper lanes 2 and 4 (isoform 1 and 2, respectively). The membrane was stripped and reprobed with goat polyclonal anti-CALM antibodies. The CALM antibody identified two bands of the expected size for endogenous CALM (66 and 72 kDa) in the lanes where immunoprecipitated CATS fusion proteins were present (Figure 3.19, lower lanes 2 and 4). Immunoprecipitation with normal rabbit IgG did not coprecipitate endogenous CALM (Figure 3.19, lanes 3 and 5). Similarly, endogenous CALM could not be coimmunoprecipitated with GFP antibody in 293T cells lacking the GFP-CATS fusion protein (Figure 3.19, lane 1).

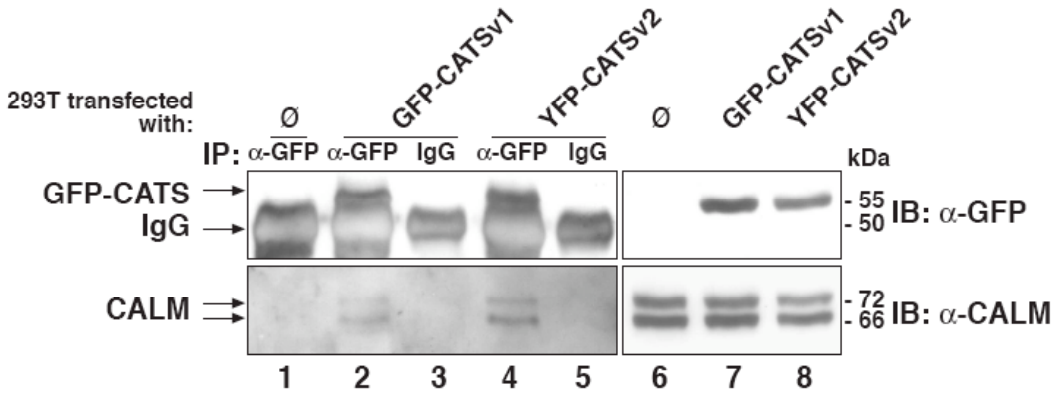


Fig. 3.19: Coimmunoprecipitation of CATS and the endogenous CALM protein. Lysate of cells overexpressing GFP-CATSv1 and YFP-CATSv2 were immunoprecipitated with α-GFP. Immunoprecipitated CATS fusion proteins are detected by immunoblotting using the same antibody (α-GFP), upper lanes 2 and 4 (isoform 1 and 2, respectively). The membrane was stripped and reprobed with goat polyclonal α-CALM, which identified two bands of the expected size for endogenous CALM (66 and 72 kDa) in the CATS precipitates, lower panel lanes 2 and 4. Immunoprecipitates of a lysate from untransfected cells and immunoprecipitates with normal rabbit IgG were used as negative controls, lanes 1, 3 and 5. 50 µg cell extract was used as input control in lanes 6, 7 and 8.

3.7 CATS influences the subcellular localization of CALM and CALM/AF10

To further validate the CALM-CATS interaction and to study a possible influence that the expression of one protein might have on the subcellular localization of the other protein, a yellow fluorescent protein tagged version of CALM (YFP-CALM) and the CFP-CATS proteins were coexpressed in murine fibroblasts (NIH3T3). The construct expressing YFP-CALM fusion protein was generated by digesting the CALM full length cDNA (clone pQE31-CALM) with *Xho*I and *Bam*HI and subcloning the fragment into the pEYFP-C1 vector digested with the same enzymes (Figure 3.20).

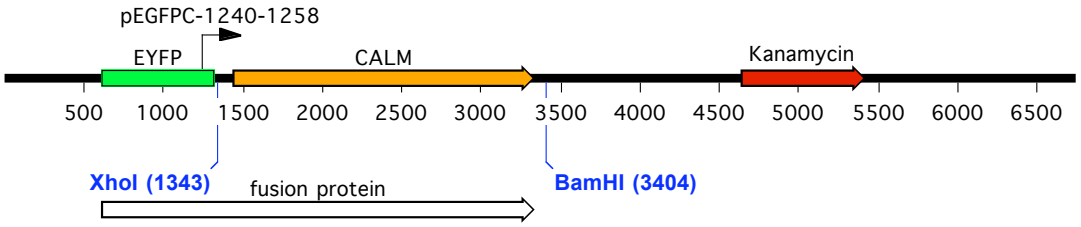


Fig. 3.20: YFP-CALM construct. The CALM ORF was cloned into the *Xho*I and *Bam*HI sites of the EYFP-C1 vector. Restriction enzymes used for cloning are in bold. Numbers in brackets indicate nucleotide position. The arrow indicates the binding position of the pEGFP-1240-1258 sequencing primer.

Colocalization experiments revealed that CATS (both isoforms) was able to influence the subcellular localization of CALM. CALM alone is predominantly localized in the cytoplasm and at the plasma membrane (Figure 3.21 b). However, in some cells, where CATS is coexpressed, CALM localization was observed both at the cytoplasm/membrane and in the nucleus at almost equal levels. On one hand the cytoplasmic portion of CATS colocalizes with CALM in the cytoplasm and at the membrane and on the other hand a portion of CALM is withdrawn from the cytoplasm towards the CATS-containing nucleus (Figure 3.21 d; e). Interestingly, the effect of CATS on the CALM protein is not found in every cotransfected cell. In the majority of cells coexpressing YFP-CALM and CFP-CATS, CALM still localizes only to the cytoplasm and at the membrane (data not shown). In order to address this varying effect of CATS on the subcellular localization of CALM, the transfected cells were examined at different time points after transfection. This experiment showed that the effect of CATS on CALM localization is partially dependent on the time after transfection. No effect of CATS on CALM localization was observed up to 20 hrs after transfection. The effect became only apparent after 22 hrs, 28 hrs and 48 hrs. However, among the time points 22, 28 and 48 hrs there was no significant difference in the proportion of cells in which CATS changed the subcellular localization of CALM.

In order to test if CATS interacts also with the CALM portion of the leukemic fusion protein CALM/AF10 and if this interaction has an effect on the subcellular distribution of CALM/AF10, the same experiments as described above were performed using a YFP-tagged version of CALM/AF10 (2.1.12). Like CALM, CALM/AF10 alone is predominantly found in the cytoplasm (Figure 3.21 g). In the presence of CFP-CATS, most of the YFP-CALM/AF10 fusion protein is localized in the nucleus and even colocalizes with CATS at the nucleolus in some cells (Figure 3.21 i; j). The nuclear localization effect seems to be stronger for CALM/AF10 than for CALM alone. However, similarly to CALM, in some cells CALM/AF10 can also maintain its normal localization in the cytoplasm or be just partially relocalized to the nucleus in the presence of CATS (data not shown).

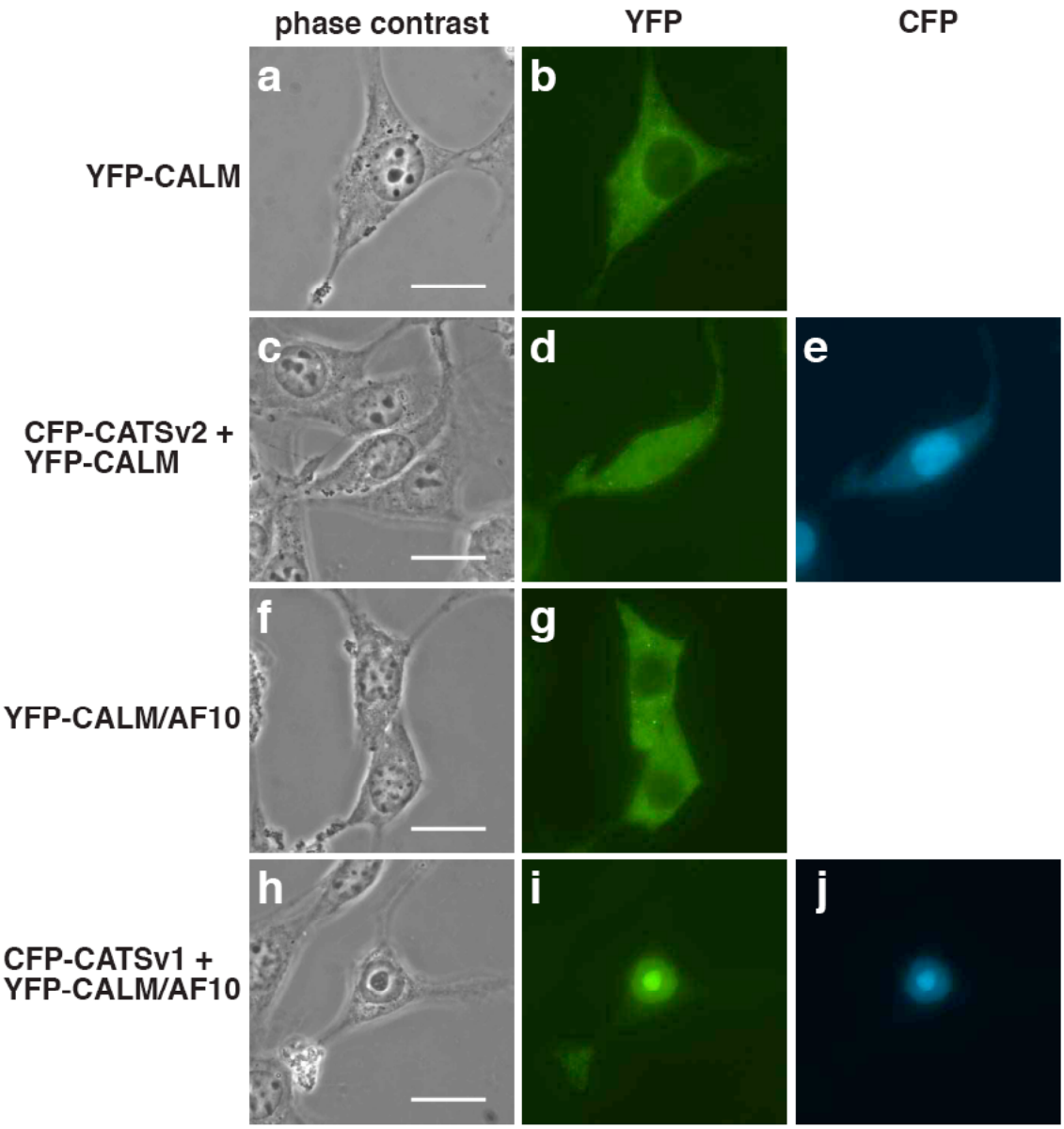
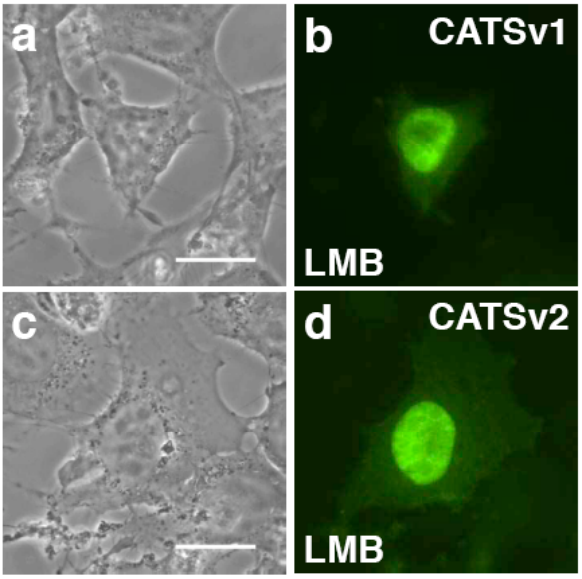


Fig. 3.21: Colocalization of CATS with CALM and the leukemic fusion protein CALM/AF10. (**a-e**) Colocalization of CFP-CATS and YFP-CALM. CALM alone is predominantly found in the cytoplasm (**b**). In cells expressing both YFP-CALM and CFP-CATS, the presence of CATS (**e**) influences the subcellular localization of YFP-CALM, which is then also present in the nucleus (**d**). Note that cytoplasmic portion of CATS also colocalizes with CALM at the plasma membrane (**d, e**). **(f-j)** Colocalization of CFP-CATS and the leukemic YFP-CALM/AF10 fusion protein. In cells expressing the YFP-CALM/AF10 fusion protein alone, the CALM/AF10 fusion protein localizes to the cytoplasm (**g**). In cells expressing both YFP-CALM/AF10 and CFP-CATS, the YFP-CALM/AF10 fusion protein is nearly completely relocalized to the nucleus where it colocalizes with CATS both in the nucleoplasm and at the nucleoli (**i, j**). Bar: 20 µm.

3.8 Nuclear-cytoplasmic shuttling of CALM and CALM/AF10 but not of CATS

The fact that CALM and CALM/AF10 accumulate in the nucleus of some CATS expressing cells together with the cytoplasmic and nuclear localization of CATS prompted us to investigate whether this is the result of a nuclear-cytoplasmic shuttling of the CATS protein. Leptomycin B (LMB) was used to inhibit CREM-mediated nuclear export (2.2.21.1) in NIH3T3 cells that had been transiently transfected with YFP-CATS (both isoforms), YFP-CALM, or YFP-CALM/AF10. The relative distribution of nuclear and cytoplasmic CATS was not affected by LMB treatment, indicating that CATS does not shuttle between nucleus and cytoplasm. However, this treatment result in a much lower accumulation of YFP-CATS in nucleoli as evidenced in figure 3.22 d and c. In contrast to CATS, both CALM and CALM/AF10 strongly accumulated in the nucleus upon LMB treatment. Interestingly, the leukemic fusion protein was nearly completely absent from the cytoplasm and often revealed a speckled nuclear staining in LMB treated cells (Figure 3.22). These results show that in contrast to CATS, both CALM and CALM/AF10 proteins shuttle between nucleus and cytoplasm.



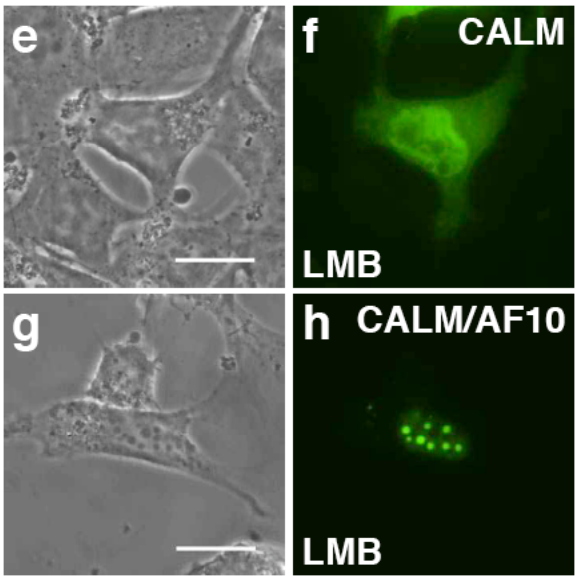


Fig. 3.22: Nuclear export inhibition by LMB. NIH3T3 cells were transiently transfected with YFP-CATS, YFP-CALM or YFP-CALM/AF10 and treated with 100 nM LMB for 4 hours. Both CALM and CALM/AF10, which normally localize almost exclusively to the cytoplasm, accumulate in the nucleus upon LMB treatment (**f, h**). Neither CATSv1 nor Catsv2 relative nuclear-cytoplasmic distribution was affected by the nuclear export inhibitor LMB however, the protein seems to be translocated from the nucleolus to the nucleoplasm (**b, d**). Bar: 20 μm.

3.9 Production of CATS monoclonal antibodies

A panel of monoclonal antibodies raised against the CATS protein was generated in cooperation with Dr. Elizabeth Kremmer from the Institute of Molecular Immunology, National Research Center for Environment and Health, GSF, Munich. The GST gene fusion system was used to express a GST-CATS fusion protein, which was used to immunize rats.

3.9.1 Expression of GST-CATS fusion proteins

The GST fusion of CATS isoform 1 and the C-terminal portion of CATS isoform 2 (61 amino acids) were expressed and purified as described in 2.2.30. The resulting GST-CATSv1 fusion protein revealed a different size from expected. The reason for this is unknown. Possibly due to degradation or cleavage of the GST-CATSv1 fusion protein. Nevertheless, high expression of the pGEX4T-2-CATSv2CT construct (2.1.12) was achieved and the purified GST-CATSv2CT fusion protein showed the expected size of approximately 36 kDa (28 kDa from the GST protein plus approx. 6 kDa from the C-terminus of CATSv2) (Figure 3.23). The 61 amino acids of CATS from this fusion protein correspond to residue 188-228, common to both CATS isoforms, plus the last 20 amino acids specific to isoform 2.

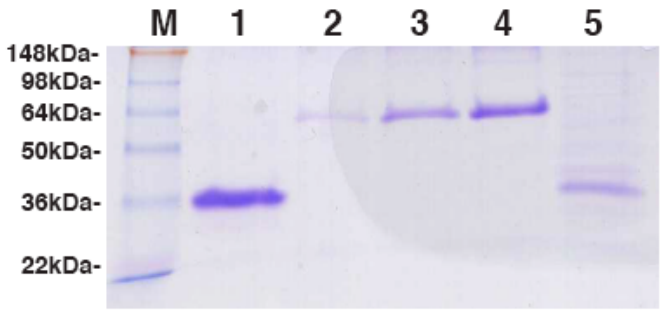


Fig. 3.23: GST-CATS fusion proteins in Coomassie stained PAGE gel. (**1**) 10 µl of GST-CATSv2CT fusion protein expressed in BL21 cells and glutathione agarose purified. (**2-4**) BSA as a standard for protein quantification (0.2 µg, 0.5 µg and 1 µg). (**5**) 10 µl of glutathione agarose purified GST-CATSv1 fusion protein. Note that the GST-CATSv1 fusion does not correspond to the expected size (51 kDa) for the CATS full length fused to GST protein. (**M**) protein molecular weight standard.

3.9.2 Monoclonal antibodies against CATS recognize specifically the CATS proteins

50 µg of purified GST-CATSv2CT fusion protein was injected both i.p. and subcutaneously into Lou/C rats (Dr. Kremmer, IMI). Fusion of myeloma cell line (P3X63Ag8.653, mouse, BALB/c) with cells from the spleen of immunized rats was performed according to standard procedures. Hybridoma supernatants were tested in a solid phase immunoassay.

Sixteen hybridomas designated CATS-4D2, -2F12, -6F10, -6E2, -4D8, -7F6, -4D10, -3G2, -3H8, -2B5, -7H2, -2C4, -2C6, -4B7, -3A6 and -2A2 were obtained. Western blotting was used to test the specificity of the hybridoma supernatants against CATS proteins. Incubation with CATS hybridoma supernatants diluted 1:5 was carried out for 1 hr at RT, followed by incubation with the secondary antibody (goat or mouse α-rat HRP diluted 1:3000 and 1:2000, respectively) for 50 min at RT. The Western blot analyses revealed that 9 hybridomas detected the fluorescently tagged CATS protein (approx. 55 kDa) and 2 out of the 9 specifically recognized CATS isoform 2 (CATS-7H2 and -3H8) (Figure 3.24).

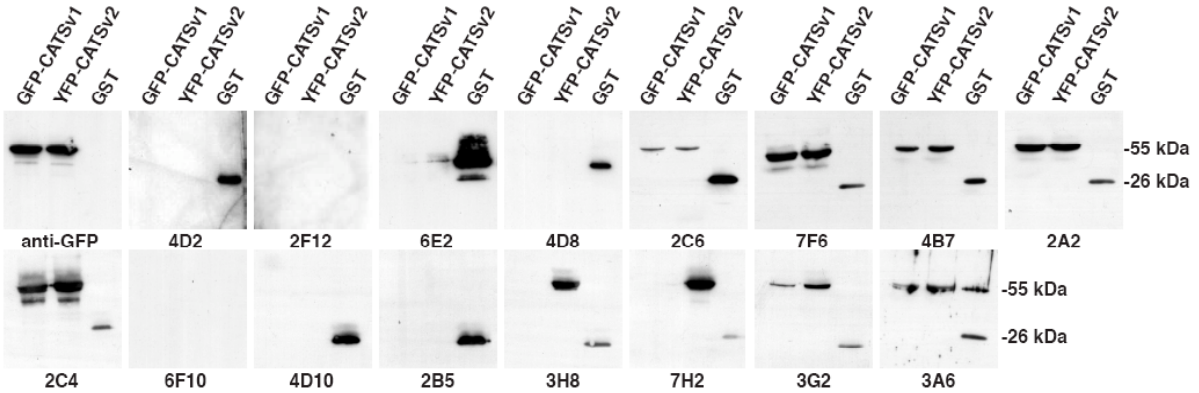


Fig. 3.24: Western blot analysis testing CATS antibodies. Extracts of 293T cells (6 µg) overexpressing either the GFP-CATSv1 or YFP-CATSv2 fusion protein and lysate from bacteria expressing GST protein alone revealed that the antibodies CATS-7F6, -3G2, -3A6, -2A2, -2C4, -2C6, -4B7 recognize both CATS isoforms (approx. 55 kDa) while antibodies CATS-3H8 and -7H2 recognize exclusively CATS isoform 2. CATS-2F12 and -6F10 recognize neither the CATS isoforms nor GST. CATS-4D2, -6E2, -4D8, -4D10 and -2B5 recognize specifically the GST protein (approx. 26 kDa). An α-GFP antibody was used as control for the detection of the CATS fusion proteins in the 293T cell extracts.

Hybridomas producing antibodies which detected the tagged CATS proteins or specifically detected CATS isoform 2, were stably subcloned and used for further analysis (CATS-2A2, -7F6, -2C4, -3H8 and -7H2). The immunoglobulin isotype was determined using antibodies against the rat IgG heavy and light chains. The CATS-2C4 antibody has the IgG subclass IgG2a while CATS-2A2, -7F6, -3H8 and -7H2 antibodies have the IgG subclass IgG1. The resulting monoclonal rat antibodies were tested for sensitivity on Western blots of 293T cells overexpressing either GFP-CATSv1 or YFP-CATSv2. These antibodies showed specific recognition of tagged CATS at much lower levels than in the Western blot described above (down to 1 µg of cell lysate) and no background detection of the GST protein. Antibody 2C4 recognized both CATS isoforms most efficiently followed by 7F6, 2A2 and 7H2 (specific for isoform 2). 3H8 weakly recognized the overexpressed CATS isoform 2 (Figure 3.25).

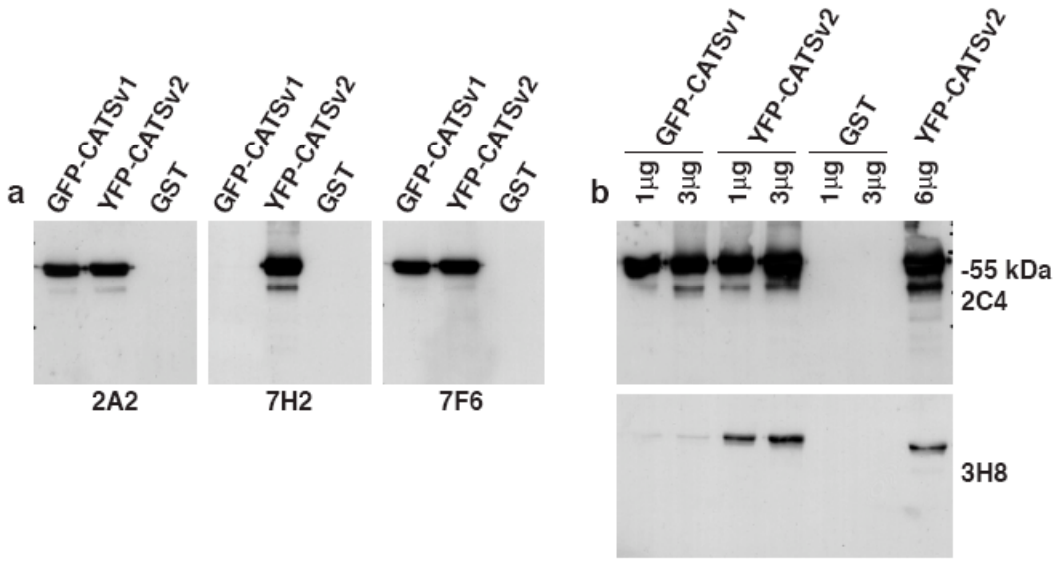


Fig. 3.25: Western blot analysis using monoclonal CATS anti-sera. Extract of 293T cells (1-6 µg) overexpressing either GFP-CATSv1 or YFP-CATSv2 fusion protein and from bacteria expressing GST protein was blotted and probed with the recloned CATS monoclonal antibodies 7F6, 2A2, 2C4 (recognizing both tagged CATS isoforms of approx. 55 kDa), 7H2 and 3H8 (recognizing exclusively the CATS isoform 2). Antibodies are specific for CATS protein and do not recognize GST protein. **(a)** Blots with 3 µg cell lysate. **(b)** Blot was probed for 2C4, striped and reprobed with 3H8. Note the difference in the signal strength. The film was exposed for 10 sec for 2C4 and 30 sec for 3H8.

Further expression analysis revealed that CATS antibodies recognize both endogenous and overexpressed CATS proteins. Western blot with lysate from transfected 293T cells overexpressing the pM1-CATSv1 construct showed that CATS-2C4 antibody strongly recognizes both the overexpressed GAL4-DBD-CATS fusion protein (approx. 42 kDa) and an additional band of the expected size for the wild type CATS protein (28 kDa). A lysate of non transfected 293T cells was used as control for the expression of endogenous CATS. Similarly to what was observed by the detection of the overexpressed CATS proteins, antibody 2C4 recognized the endogenous CATS most efficiently followed by 7F6 and 2A2. Both 7H2 and 3H8 (specific for isoform 2) do not seem to detect the endogenous CATS isoform 2. However, we do not know whether these antibodies are not able to recognize the endogenous isoform 2 of CATS or if the endogenous expression levels of the isoform 2 are too low for detection (Figure 3.26).

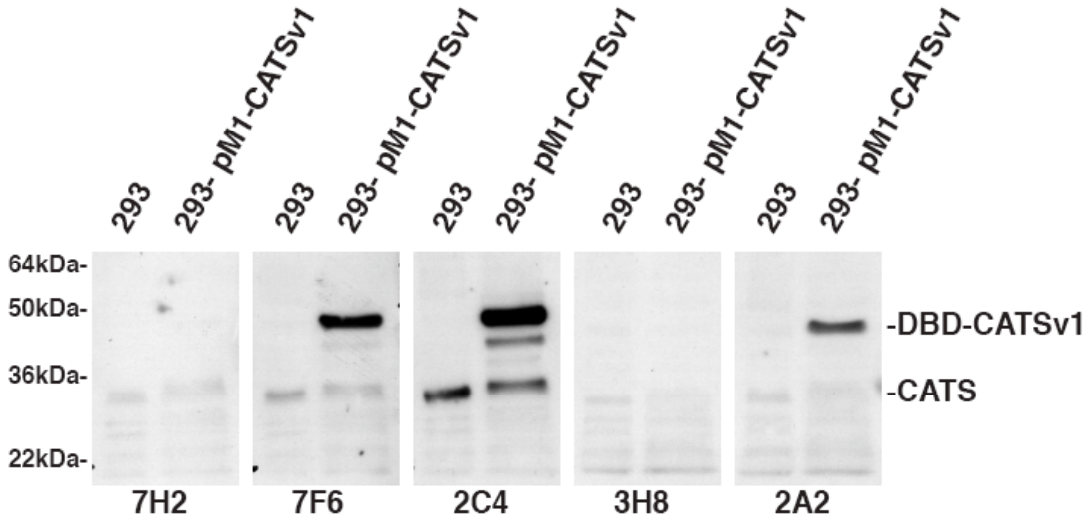


Fig. 3.26: CATS antibodies recognize both endogenous and overexpressed CATS proteins in 293T cells. 30 µg protein of transfected (pM1-CATSv1 construct) and non-transfected 293T cells were blotted and probed with the α-CATS 7F6, 2A2, 2C4, 7H2 and 3H8 antibodies (the last two recognize exclusively CATS isoform 2). α-2C4 antibody strongly detected both the overexpressed GAL4-DBD-CATSv1 (approx. 42 kDa) and the endogenous protein (28 kDa). α-CATS 7F6 and 2A2 clearly detected the overexpressed CATS however detection of the endogenous protein was weak. Note that no detection of GAL4-DBD-CATSv1 protein was observed with α-CATS 7H2 and 3H8 since these antibodies are specific for CATS isoform 2. The faint bands observed in blots probed with α-CATS 7H2 and 3H8 are most likely background of the secondary antibody.

3.9.3 CATS antibodies raised against human CATS also detect the murine Cats protein

CATS antibodies generated against the human protein were tested for crossreactivity with Cats proteins from other species. Lysates from the murine cell lines NIH3T3 and BA/F3 were blotted and probed with α-CATS 7H2, 7F6, 2C4, 3H8 and 2A2 antibodies. Simultaneously, the 293T cell extract was used as a positive control for antibody detection. α-CATS 7H2, 7F6, 2C4 and A2 clearly detected the murine protein, which has the same size (28 kDa) as the human protein. Similarly to what we observed on extract from human cell lines, the α-CATS 2C4 antibody shows the strongest detection of Cats followed by 7F6 and then by 2A2. In order to select a secondary antibody which gives the best results in combination with CATS antibodies, blots probed for CATS were incubated with either goat or mouse anti-rat secondary antibody. As negative controls (ø) to test the specificity of the secondary antibodies, blots were incubated with either mouse or goat anti-rat alone. The best results were obtained with the mouse anti-rat secondary antibody since lower background was observed and the position of unspecific bands did not overlap with the Cats band. Using

mouse anti-rat as a secondary antibody, good detection of CATS was also achieved with α-CATS 7F6 and 2A2 antibodies, which had previously given much weaker signals (Figure 3.27).

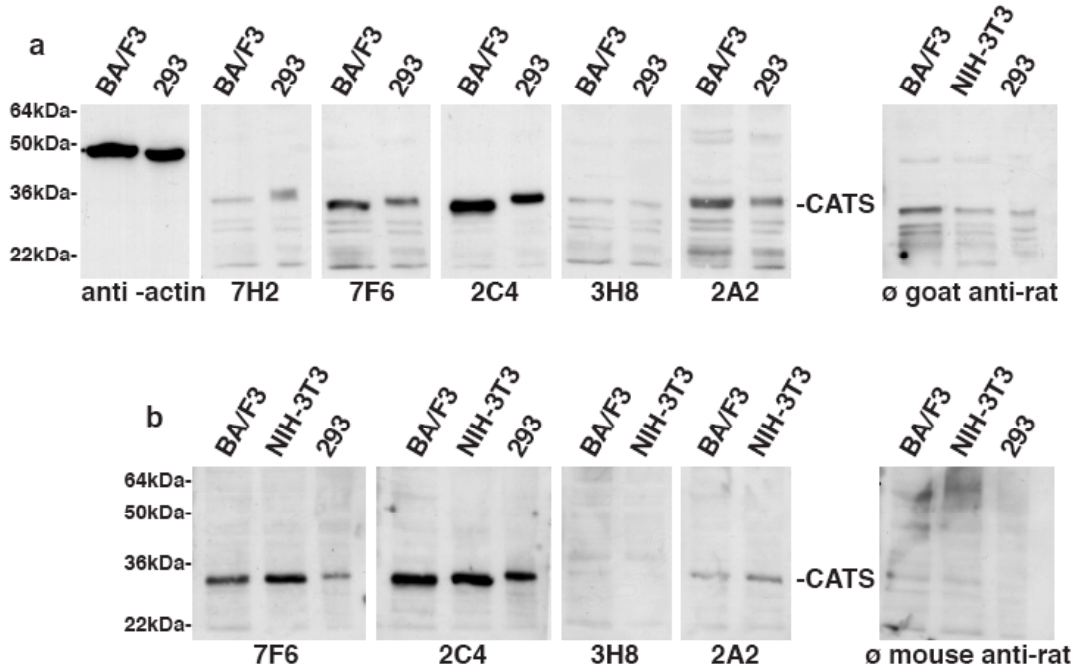


Fig. 3.27: CATS antibodies recognize the murine protein. 30 µl total cell lysate from murine cell lines NIH3T3 and BA/F3 was blotted and probed with α-CATS antibodies (7H2, 7F6, 2C4, 3H8 and 2A2). 293T cell extract was used as a positive control for antibody detection. (**a**) Blots with 50 µg of cell extract were probed with CATS antibodies followed by incubation with goat anti-rat secondary antibody. An α-beta actin antibody was used as a positive control to demonstrate the integrity of proteins in the cell lysates. (**b**) Blots with 30 µg of cell extract were probed with α-CATS antibodies followed by incubation with mouse α-rat secondary antibody. (**ø**) Negative control. Blots, which were not probed with α-CATS primary antibodies were incubated with either mouse or goat anti-rat secondary antibodies. Note that incubation with the mouse anti-rat antibody gives lower background resulting in a more specific detection of the weak Cats signals (as observed with α-CATS 7F6 and 2A2).

3.9.4 Expression of Cats protein in murine cell lines

Western blot analysis performed with lysate from mouse (BA/F3, NIH3T3, MEF null for p53 and ARF genes) and rat (TGR) cell lines revealed a high expression of Cats in murine pro B-cells (BA/F3) and mouse embryonic fibroblasts (NIH3T3 and MEF). Similar levels of Cats expression was observed in MEF cells lacking either the p53 or ARF protein or when these cells, had been treated with Uv radiation (40 Joule). No detection of Cats protein was observed in the rat fibroblast cell line (TGR), most probably because our CATS antibody does not recognize the rat protein and not because this cell line lacks Cats expression. CATS is highly expressed in both human and mouse fibroblast cell lines (Figure 3.28).

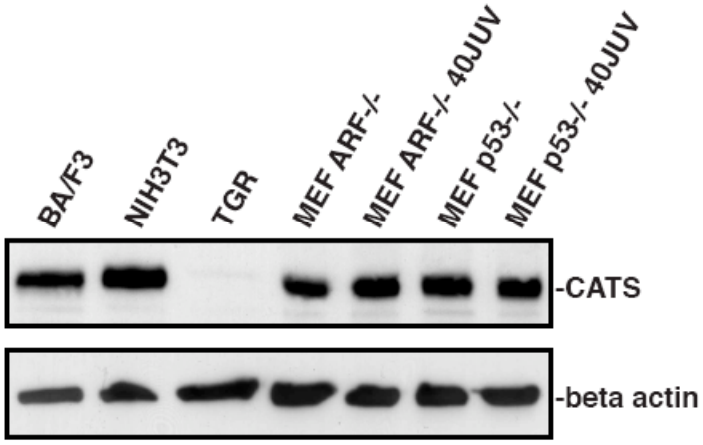


Fig. 3.28: Expression analysis of Cats at protein level. Western blot with 30 µg lysate from BA/F3, NIH3T3, TGR and MEF cell lines probed with α-CATS 2C4. The CATS antibody recognizes the murine but not the rat protein. MEF $p53^{-/-}$: MEF cell line with a targeted disruption of the *TP53* gene. MEF $ARF^{-/-}$: MEF cell line with a targeted disruption of the *ARF* gene. MEF cell lines were treated with 40 Joules of UV radiation.

3.10 Expression analysis of CATS at the protein level

3.10.1 Expression of CATS in human thymus

Western blots with cellular extract from human thymus (2.2.24.2) were probed with α-CATS 2C4 antibody. The antibody clearly recognized the 28 kDa CATS protein, confirming CATS expression in the human thymus (Figure 3.29).

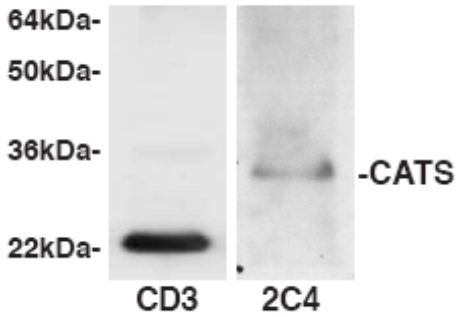


Fig. 3.29: CATS is expressed in the human thymus. Western blot with 20 µl cell lysate from human thymus was probed with α-CATS 2C4 antibody. α-CATS 2C4 detected the 28 kDa CATS protein. α-CD3 12, an antibody which detects CD3, was used as a positive control to demonstrate the integrity of proteins in the thymus lysate.

3.10.2 Expression analysis of CATS in hematopoietic cell lines

A Western blot with 15 µg lysates from hematopoietic cell lines was probed with the α-CATS 2C4 antibody. The expression analysis revealed that CATS is highly expressed in all cell lines tested. These cell lines represent different leukemia and lymphoma subtypes (B- and T-ALL, B- and T-lymphoma and AML FAB M2, M4 and M5). The levels of CATS expression did not seem to correlate with the differentiation stage of the cells as expression were similar for immature and mature T and B cell lines. Slightly lower expression was observed in myeloid leukemia cell lines than in the lymphoblastic leukemia cell lines. No CATS protein could be detected in the HL-60 cell line (AML FAB M2) although CATS expression was detected in another cell line of the same leukemia subtype (KASUMI-1). Interestingly, HL-60 was the only tumor cell line tested which did not show CATS expression. Additionally, in the CALM/AF10 fusion positive cell line U937, an additional band of lower molecular weight was detected with the α-CATS 2C4 antibody (Figure 3.30).

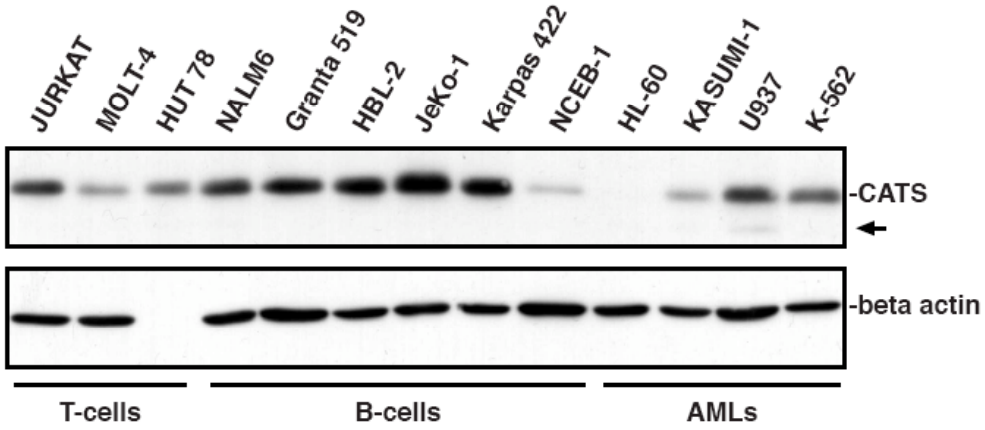


Fig. 3.30: CATS is highly expressed in leukemia and lymphoma cell lines. Cellular extracts (15 µg) from human leukemia and lymphoma cell lines were blotted and probed with α-CATS 2C4 antibody. CATS is highly expressed in both immature (JURKAT) and mature T-cell lines (HUT 78) as well as in pre B (NALM6) and mature B cell lines (Granta 519, HBL-2, JeKo-1, Karpas 422). Lower expression is observed in MOLT-4 (immature T-cell line) and NCEB-1 (mature B-cell line) which is comparable to the level of expression observed in cellular extract from myeloid leukemias of FAB subtypes M2, M4 and M5 (KASUMI-1, U937 and K-562, respectively). No CATS expression was observed in the HL-60 extract (AML M2). The arrow indicates the position of the smaller band present in the cellular extract of the CALM/AF10 fusion positive cell line U937. The blot was stripped and reprobed with α-beta actin antibody, which served as a control for the loading and integrity of the protein extracts. Note that beta actin is not present in extracts from the HUT 78 cell line.

3.10.3 Expression analysis of CATS in normal and tumor cell lines

Additional Western blot analysis with the α-CATS 2C4 antibody revealed high expression of the CATS protein in solid tumor cell lines (U2OS, SaOS, and HeLa), as well as in normal proliferating cell lines (293T) but not in normal non-proliferating T-cell lines (TYRF8 and JB4). Lower expression of CATS was observed in WI-38, a normal human fibroblasts cell line, which have a finite life-span of approx. 50 population doubling and display a lower proliferative rate (Figure 3.31). The fact that CATS expression was not observed in non-proliferating cells but strong expression was seen in highly-proliferating cells, indicates that CATS might play a role in cell proliferation. Interestingly, similar to what we observed in U937, an additional band was also detected in protein extracts of HeLa cells. This band may correspond to an additional CATS isoform resulting from alternative splicing.

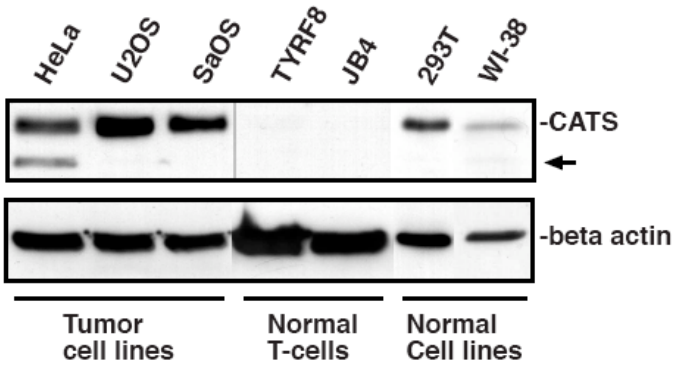


Fig. 3.31: CATS expression in normal and tumor cell lines. Cellular extracts (30 µg) from human normal and tumor cell lines were blotted and probed with α-CATS 2C4 antibody. CATS is highly expressed in solid tumor cell lines (HeLa, U2OS and SaOS) and in the transformed embryonal kidney cell line 293T. Lower expression of CATS was observed in the normal human fibroblast cell line WI-38, a cell line with low proliferative rate. No CATS expression was detected in the non-proliferating T-cell lines TYRF8 and JB4. The arrow indicate the additional band in the cellular extract of the HeLa cell line. Blots were stripped and reprobed with α-beta actin antibody, which served as a control for the loading and integrity of the protein extracts.

3.10.4 CATS is a marker for proliferation

Expression analysis of CATS in lysates of serum starved and serum stimulated T98G (glioblastoma cell line) cells (2.1.18) revealed a strong correlation between CATS expression and proliferation. Low levels of CATS were observed in lysate from quiescent cells as well as from cells shortly after release from serum starvation. Gradual and progressive upregulation of CATS expression is seen after serum stimulation, especially from hour 12 onwards (Figure 3.32). These results indicate that CATS is a marker for proliferation and is regulated in a cell cycle dependent manner (see also below).

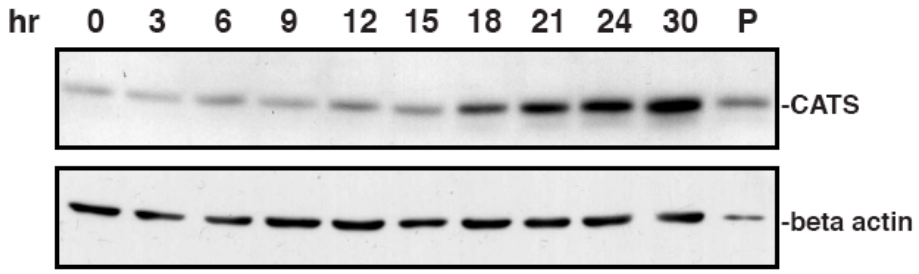


Fig. 3.32: CATS expression in serum stimulated T98G cells. After 48 hrs of serum starvation, T98G cells were serum stimulated. Cell extracts, prepared every 3 hrs for up to 30 hrs after stimulation were blotted and probed with α-CATS 2C4 antibody. CATS is strongly upregulated in proliferating cells. Low levels of CATS expression were observed in quiescent cells (0) and in cells which, had shortly been released from serum starvation (3-9 hrs). Gradual increase in CATS expression is clearly seen from hour 12 onwards, with strongest expression detected 30 hrs after serum stimulation. Numbers indicate hours after serum stimulation. **(0)** starved cells. **(P)** proliferating cells, which have not gone through starvation. The blot was stripped and reprobed with the α-beta actin antibody, which served as a control for the loading and integrity of the protein extracts. Note that the low intensity of CATS signal in extracts from proliferating cells (P) is due to lower amount of protein as shown by the weak signal obtained with the beta actin control.

3.10.5 CATS is regulated in a cell cycle dependent manner

Lysates from U2OS and HeLa cells (2.1.18), which had undergone synchronization through a double thymidine block, were blotted and probed with the α-CATS 2C4 antibody. FACS analysis of the corresponding cell populations was used to confirm the quality of the cell cycle synchronization. Analysis of CATS expression in synchronized cells revealed that CATS expression varies throughout the cell cycle and is regulated in a cell cycle dependent manner. CATS is upregulated in samples corresponding to S-, G_2- and G_2/M- phases. Samples in which most of the cells were found in G_1-phase showed lower expression of CATS (Figure 3.33).

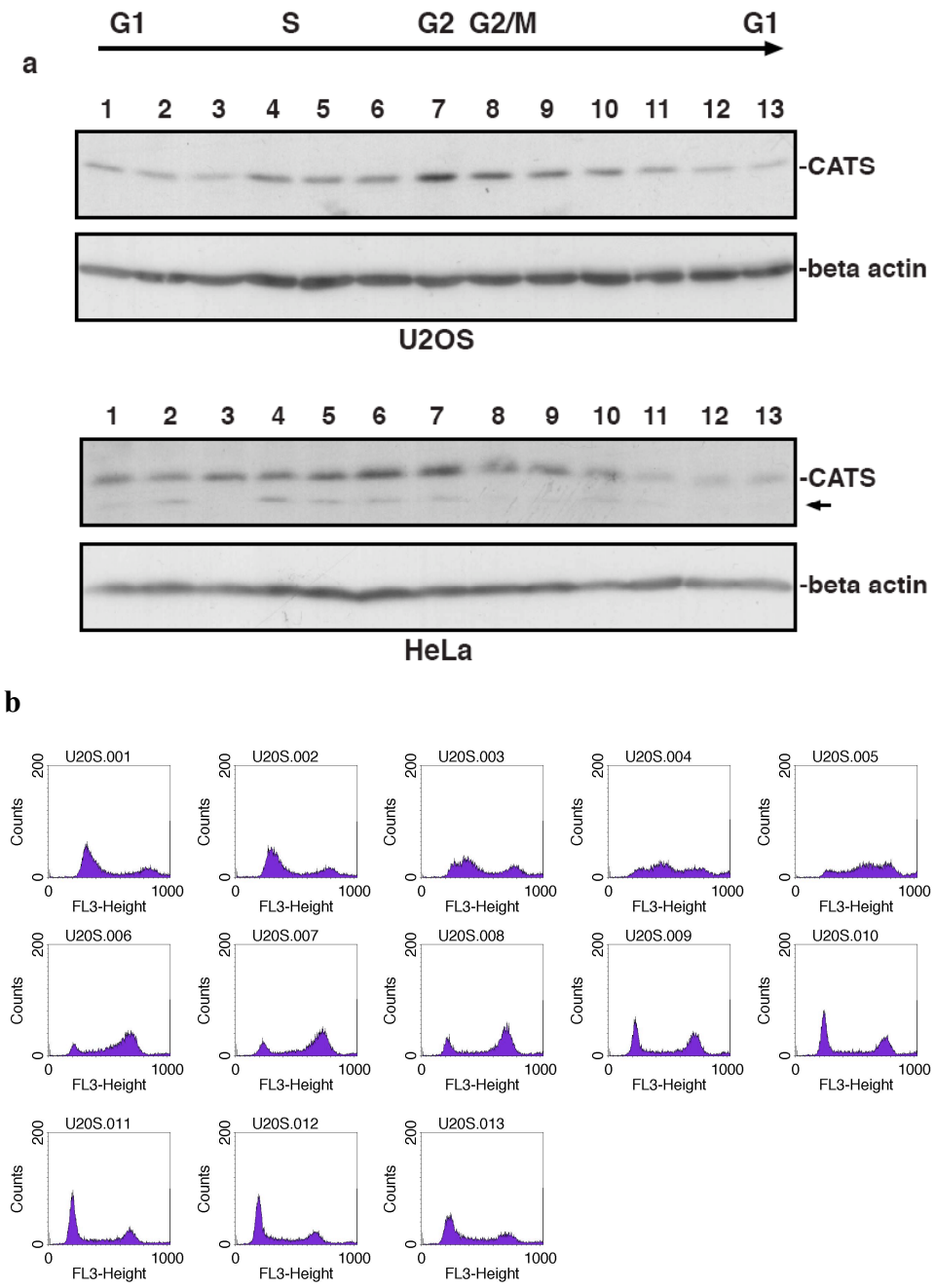


Fig. 3.33: CATS expression during the cell cycle. U2OS and HeLa cell were synchronized by a double thymidine block. Synchronization was confirmed by FACS. (**a**) Western blot with extracts from the same cell samples analyzed by FACS. CATS expression varies in the different stages of the cell cycle. Upregulation of CATS protein was observed in cell extracts corresponding the S, G_2 and G_2/M phases of the cell cycle (sample 4 to 11 from U2OS and samples 5 to 10 from HeLa). Sample 1 from HeLa lysates is from a non-synchronized cell population, the arrow indicates the smaller CATS isoform expressed also in U937 cells (3.10.2). Blots were stripped and reprobed with α-beta actin antibody, which served as a control for the loading and integrity of the protein extracts. **(b)** FACS analysis of U2OS samples (1-13).

3.11 HL-60 cell line produces CATS mRNA but lacks CATS protein

Among all the 17 cancer cell lines tested for CATS expression, only the HL-60 cell line did not express the protein. Since HL-60 has a deletion of part of chromosome 17 and lacks the *TP53* gene (*TP53* is located proximal to CATS on 17p13), we used PCR to examine whether the CATS genomic locus is deleted in this cell line. A deletion of the CATS locus would explain the lack of its protein in this highly proliferative cell line. However, PCR amplification of a 697 bp *CATS* fragment from HL-60 genomic DNA revealed the presence of an intact CATS locus. Moreover, *CATS* transcript could be amplified by RT-PCR from HL-60 RNA. (Figure 3.34). These results suggest a posttranscriptional regulation of CATS in HL-60.

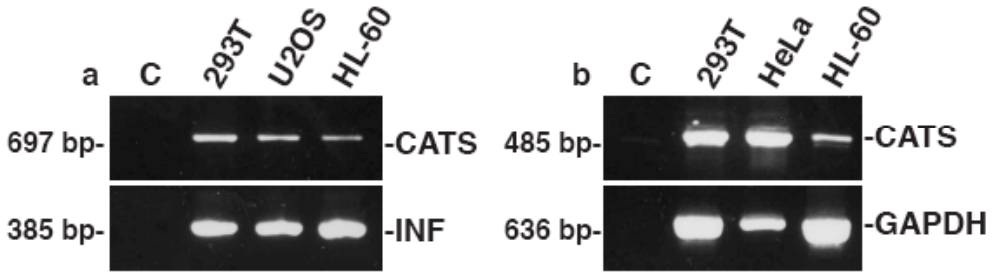


Fig. 3.34: Amplification of the CATS genomic locus and of *CATS* transcript from the HL-60 cell line. (a) PCR was performed using primer pair CATSE1F1(364) and CATSE5R1(828) and genomic DNA of 293T, U2OS and HL-60 cell lines as template. Amplification of HL-60 DNA reveals an intact genomic locus of the CATS gene. DNA of 293T and U2OS were used as normal and tumor cell line control for CATS amplification. Amplification with primers for the interferon genes (INF 5' and INF 3', see in 2.1.13) was used as control for DNA integrity. **(b)** RT-PCR with total RNA from 293T, HeLa and HL-60 cell lines. Amplification from HL-60 cDNA (primer pair CATSE1F1(364) and CATSE5R1(828)) reveals clear expression of *CATS* transcripts in this cell line. Note that the CATS protein cannot be detected in HL-60. RT-PCR with 293T and HeLa was used as control for *CATS* expression in normal and tumor cell line. Amplification of GAPDH fragment from the same RNA samples was used as control for RNA and cDNA integrity. (C) Negative control: PCR reaction with no template shows no amplification

3.12 Subcellular localization of endogenous CATS

Immunofluorescence (2.2.29) was performed in U2OS cells in order to determine the cellular localization of endogenous CATS. α-CATS 2C4 antibody detected the endogenous protein only in the nucleus where different levels CATS expression was observed from cell to cell. Strong accumulation of endogenous CATS was also observed in the nucleolus of some U2OS cells. However, the nucleolar expression of CATS was not a constant feature, since in some cells with evident nucleolar structure either CATS was not expressed or did not accumulate in the nucleolus (Figure 3.35).

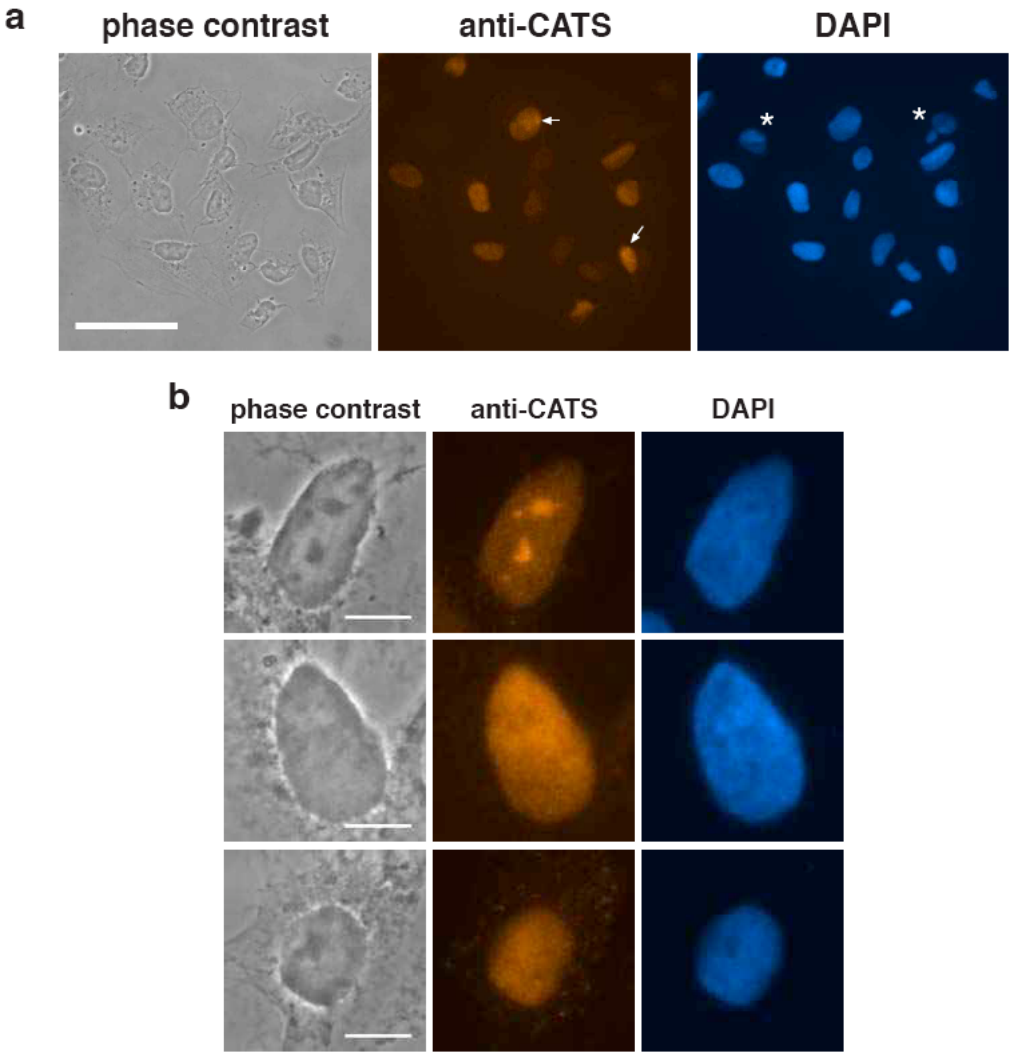


Fig. 3.35: Subcellular localization of endogenous CATS. Endogenous CATS was visualized by immunofluorescence in non-synchronized U2OS cells. (**a**) CATS is a nuclear protein. Note the different levels of CATS expression from cell to cell. Asterisks indicate DAPI stained cells, which are free from CATS expression. Arrows indicate cells with nucleolar accumulation of CATS. (**b**) Upper panel: strong CATS expression at the nucleoli. Middle panel: strong CATS nuclear expression. Lower panel: despite visible nucleoli, CATS does not accumulate to a great extent in the nucleoli. Bars: 25 µm (a); 10 µm (b).

3.13 CALM does not activate transcription in a GAL4-based transactivation assay

It has previously been reported that CALM is able to activate transcription when fused to a heterologous DNA-binding domain (GAL4-DBD) in a mammalian cell reporter gene assay (Vecchi et al., 2001). Here we show that CATS physically interacts with CALM and that CATS can affect the subcellular localization of CALM. We thus asked the question whether CATS might affect the transcriptional activity of CALM either as a co-activator or co-repressor of transcription (Figure 3.36).

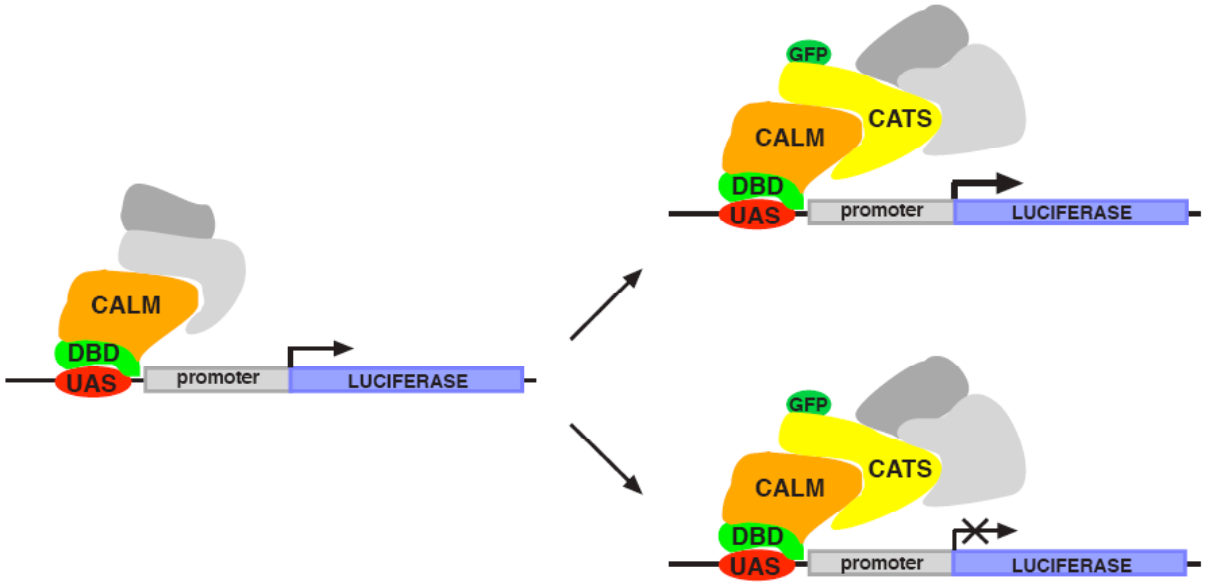


Fig. 3.36: **Model of transactivation in GAL4-based transcription assay.** Mammalian cells are cotrasfected with the GAL4-DBD-CALM and CFP-CATSv1 expressing plasmids and a reporter construct. GAL4-DBD-CALM binds to the GAL4-UAS in the promoter region of the reporter gene. CALM recruits proteins to form a transcription activation complex and induce transcription of the luciferase reporter gene. Through the CALM-CATS interaction, CATS would be part of the transcription complex. This assay was used to examine whether CATS can enhance or repress the tanscriptional activity of the GAL4-DBD-CALM fusion protein.

293T cells were cotransfected with 1.5 µg of the GAL4-CALM (1-652) construct (encoding the GAL4-DBD fused to CALM), 0.3 µg of CFP-CATS constructs (both isoforms), 0.3 µg of the reporter plasmid pGAL4LUC (encoding the luciferase gene under the transcriptional control of the GAL4-binding sites) or pGAL4$_5$tkLUC (encoding the luciferase gene under the transcriptional control of the Herpes simplex virus thymidine kinase promoter and 5 GAL4-binding sites) and 6 ng of pRL-null co-reporter vector (encoding the *Renilla* luciferase protein) (Promega). Alternatively, the experiment was performed with a ratio of 1:1 of GAL4-DBD construct to the reporter plasmid (Figure 3.37 b and d). Expression of GAL4-DBD-

CALM fusion protein was confirmed by Western blot using antibody against the GAL4-DBD (2.1.14) (Figure 3.37 e). However, expression of GAL4-DBD-CALM protein either alone or together with CATS had no effect on the transcription of the reporter genes in all four duplicate transfection experiments. The luciferase activity stayed at basal levels, similar to the control experiments, in which cells expressed only the GAL4-DBD (Figure 3.37 a-d). Positive control experiments, in which the GAL4-DBD-STAT2 fusion was expressed, showed a 92 fold activation of the luciferase activity over basal levels (data not shown). Surprisingly, the previously reported function of GAL4-DBD-CALM as a transcriptional activator could not be reproduced.

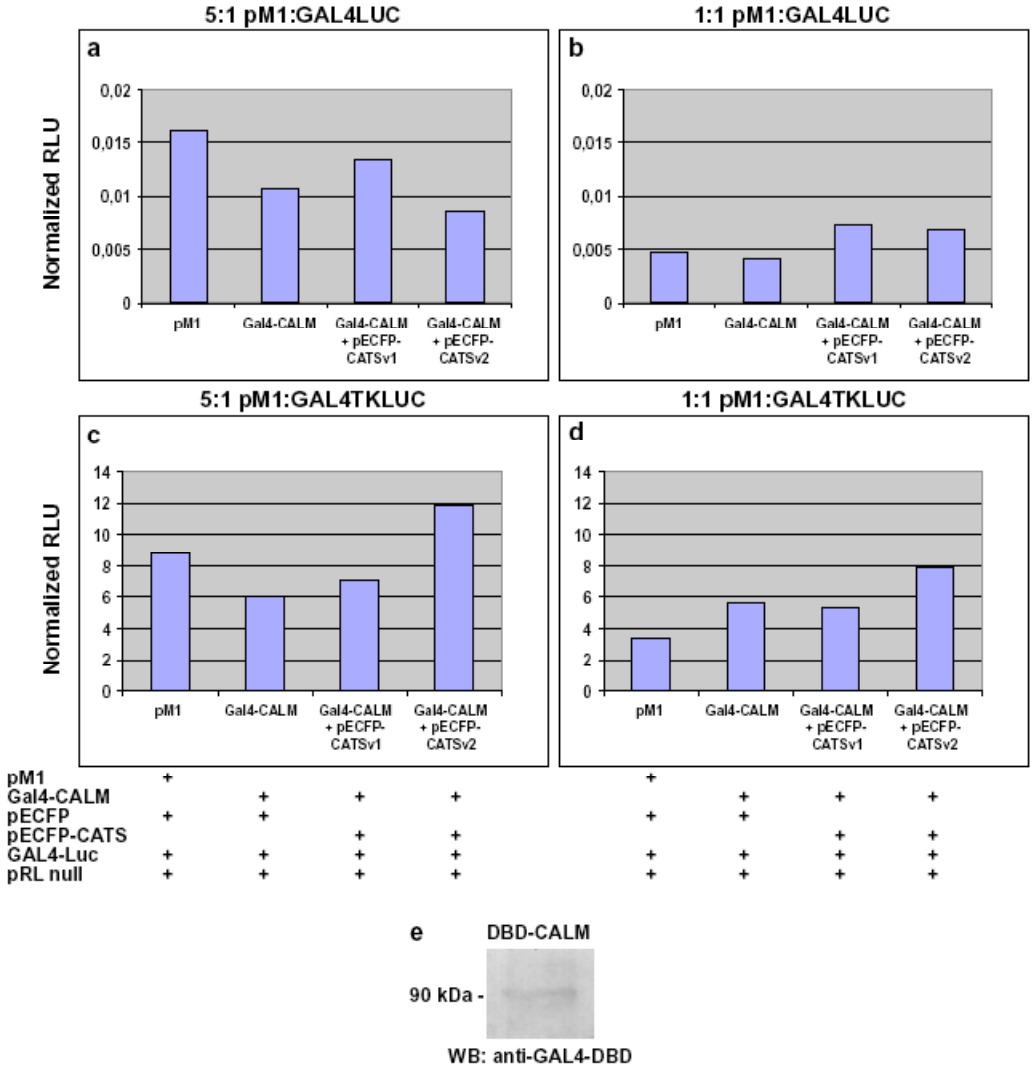


Fig. 3.37: CALM does not activate transcription when fused to a heterologous DNA binding domain. 293T cells were transiently transfected with a GAL4-regulated luciferase reporter construct (pGAL4LUC or pGAL4$_5$tkLUC), GAL4-DBD-CALM and CFP-CATS expressing plasmids. pM1 (GAL4-DBD) and pECFP-C1 empty vectors were used as negative controls. The GAL4-DBD-CALM fusion protein did not activate transcription of the luciferase reporter genes when compared to the GAL4-DBD empty vector (**a-d**). The expression of CATS (both isoforms) did not change level of luciferase expression, neither activating nor repressing transcription of the reporter gene. The luciferase activity was normalized for transfection efficiency

using the activity levels of the *Renilla* luciferase. Experiments were performed in duplicate. (**e**) Expression of GAL4-DBD-CALM was confirmed by Western blotting.

3.14 CATS isoform 1 acts a transcriptional repressor in a GAL4-based transactivation assay

Since CATS localizes to the nucleus, a GAL-4-based transcription assay (2.2.23) was used to investigate a possible function of CATS as a transcriptional regulator. For that purpose, CATS was cloned into a mammalian expression vector to be expressed as GAL4-DBD fusion protein. The complete CATS ORF was excised from the CFP-CATSv1 clone (3.4.1 a) with *Xho*I and *Hin*dIII and cloned into the pM1 vector (2.1.11) digested with *Sal*I and *Hin*dIII (Figure 3.38).

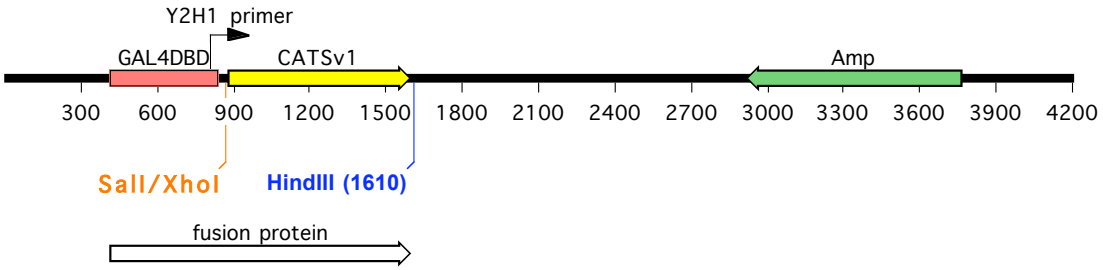


Fig. 3.38: pM1-CATSv1 construct. CATSv1 ORF was cloned into the *Sal*I and *Hin*dIII sites of the pM1 vector to be expressed as GAL4-DBD-CATS protein in the mammalian system. In bold and blue: restriction enzymes used for cloning. Enzymes in red demonstrate compatible sticky ends used for cloning, which are destroyed upon ligation. Ruler indicates construct size. The arrow indicates the binding position of the Y2H1 sequencing primer. Amp: ampicillin resistance gene.

293T cells were cotransfected (as described in 2.2.21) with 0.66 µg of pM1-CATS construct, 0.33 µg of pGAL4$_5$tkLUC reporter plasmid and 6.6 ng of pRL-null co-reporter vector. Transfected cells were disrupted with 1 ml PLB lysis buffer and 8 µl of the lysate was used for measurement of the luciferase activity (2.2.23.1). Expression of GAL4-DBD-CATS repressed the luciferase expression about 4.6 fold in comparison to GAL4-DBD alone (Figure 3.39).

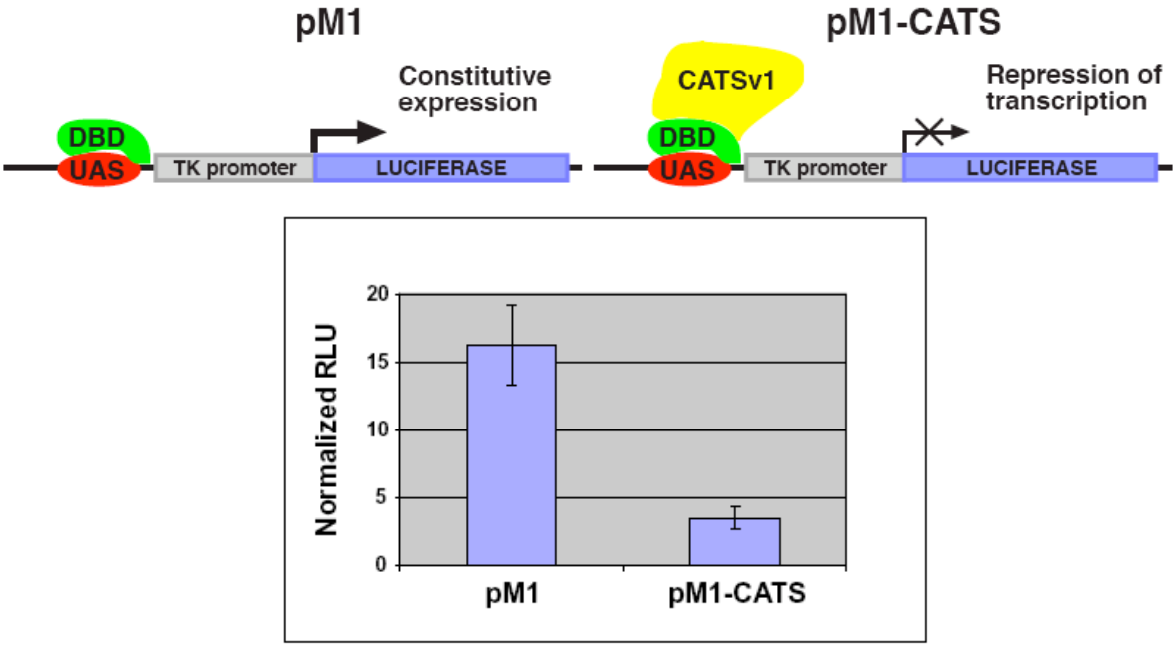


Fig. 3.39: **CATS acts as a repressor of transcription when fused to a heterologous DNA binding domain (GAL4-DBD).** The upper diagram is a model of repression of transcription by the DBD-CATS protein. 293T cells were transiently transfected with a GAL4-regulated luciferase reporter plasmid and pM1-CATSv1 or empty pM1 vector. CATS represses luciferase activity about 4.6 fold in comparison to GAL4-DBD alone. The bar graph represents the average of normalized firefly luciferase values obtained from four independent triplicate experiments. Error bars show standard deviation.

3.15 CATS interacting proteins

3.15.1 Construction of the bait plasmid

In order to gain further insights into CATS function, a yeast two-hybrid screen was performed to identify interaction partners of CATS. For this purpose the full length coding region of CATS isoform 1 (residues 1-238) was cloned into the pGBKT7 vector (2.1.11) to be expressed as GAL4-DBD fusion protein. The complete CATS ORF was excised from the CFP-CATSv1 construct (3.4.1) with *Xho*I and *Sal*I and cloned into the yeast expression vector pGBKT7 digested with *Sal*I (Figure 3.40).

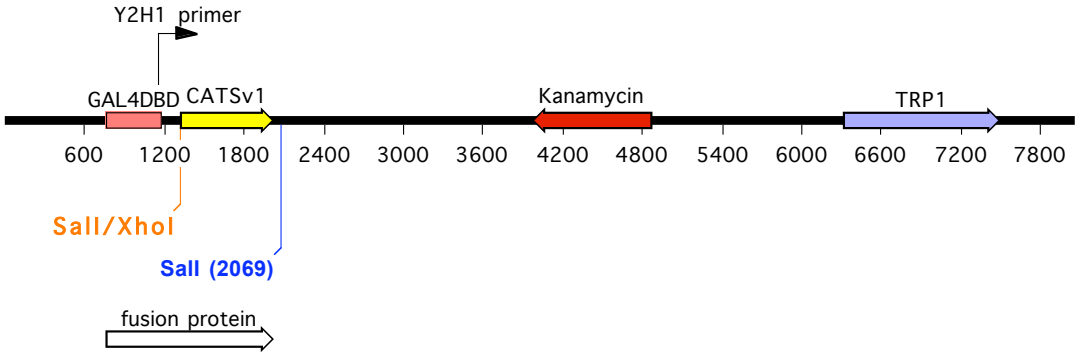


Fig. 3.40: pGBKT7-CATSv1 bait plasmid. CATSv1 ORF was cloned into the pGBKT7 vector (at the *Sal*I site) to be expressed as GAL4-DBD-CATS fusion protein in yeast. Restriction enzymes used for cloning are in bold. Enzymes in red demonstrate compatible stick ends used for cloning, which are destroyed upon ligation. Numbers indicate nucleotide position. The arrow indicates the binding position of the Y2H1 sequencing primer. TRP1: gene coding for phosphoribosylanthranilate isomerase, an enzyme in the tryptophan biosynthesis. Kanamycin: kanamycin resistance gene for selection in bacterial cells.

3.15.2 Two-hybrid screen

Test of the pGBKT7-CATS bait plasmid for autonomous activation (2.2.22.1) showed that the CATS isoform 1 does not activate the reporter genes of the yeast two-hybrid system when fused to GAL4-DBD. Therefore, the CATS full length ORF was used as a bait in a Y2H screen of a S3 HeLa cDNA library (2.2.22). A middle stringency screen of $1x10^6$ transformants yielded 230 independent clones containing potentially interacting prey plasmids. Forty-seven (20 %) of these clones were sequenced with vector primer Y2H2-729-751 (2.1.13). BLAST analyses with these sequences against Genbank showed that these sequences matched to 12 different cDNAs. Ten of them (21.2 %) were found to code for CATSv2 protein, five (10.6 %) were found to code for PCBP1 and five for SIVA. Three clones (6.3 %) were found to code for CSNK2B, 2 (4.2 %) were found to code for HAX1 and two for UXT. Finally, single clones coding for KRT17, KIS, MCM3AP, MKRN3, RCN1 and ATP60C were found (2.1 %). Table 3.4 summarizes the potential CATS interacting proteins.

CATS interacting protein	Frequency of the interacting clones
hypothetical protein FLJ10491 (CATSv2)	10x
poly(rC) binding protein 1 (PCBP1)	5x
CD27-binding (Siva) protein (SIVA)	5x
casein kinase type II beta subunit (CSNK2B)	3x
HS1 binding protein (HAX1)	2x
ubiquitously-expressed transcript (UXT), transcript variant 2	2x
keratin 17 (KRT17)	1x
kinase interacting with leukemia-associated gene (stathmin) (KIS)	1x
minichromosome maintenance deficient 3 associated protein (MCM3AP)	1x
makorin, ring finger protein, 3 (MKRN3)	1x
reticulocalbin1 (RCN1)	1x
ATPase, H+ transporting, lysosomal 16 kDa, V0 subunit c (ATP60C)	1x

3.15.3 First confirmation of potential protein interaction

A initial test to confirm that the proteins from the screen are indeed CATS interacting partners was performed by co-transforming the individual prey plasmids containing the potential interacting partners and the CATS bait plasmid into yeast strain AH109. Except for CATSv2, all the prey proteins showed interaction with CATSv1 as assayed by cell growth on selective plates (Figure 3.41). The fact that the CATS prey clones did not activate the yeast reporter genes together with the CATS bait indicates that the CATS-CATS interaction is either very weak or that the result of the yeast two-hybrid screen was an artifact.

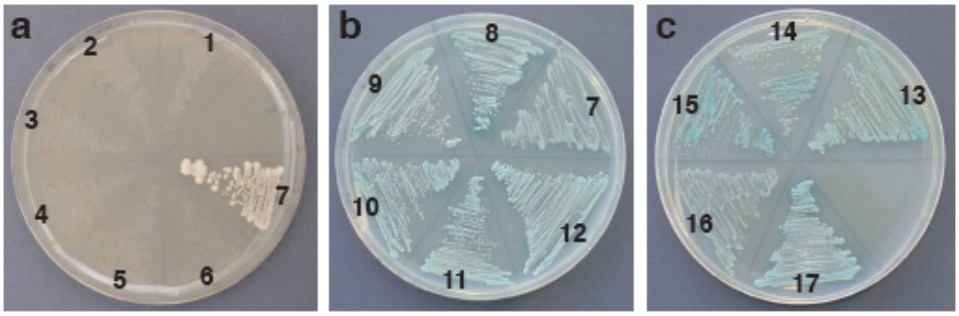


Fig. 3.41: Interaction of CATS with 11 different proteins. Yeast strain AH109 was cotransformed with pGBKT7-CATSv1 and GAL4-AD-CATS clones (full length of both isoforms plus deletion mutants cloned into the pGADT7 vector; see in 3.5.1) or the prey clones isolated from the Y2H screen (HeLa cDNA library cloned into the pGADGH vector). Transformed cells were streaked out on high stringent SD -Trp, -Leu, -His, -Ade selection plates (a) supplemented with X-α-GAL (b, c). (**a**) CATS-CATS interaction assay. (1-7) Yeast cells expressing the GAL4-DBD-CATSv1 bait protein and the prey proteins (**1**): GAL4-AD-CATSv1. (**2**): GAL4-

AD-CATSv1CT. (**3**): GAL4-AD-CATSv2. (**4-6**): independent GAL4-AD-CATSv2CT prey clones from the HeLa cDNA screen. (**7**): pGADGH-KRT17 used as positive control for CATS interaction. Note that yeast clones containing both CATS bait and prey plasmids did not activate the reporter genes indicating that CATS does not interact with itself. (**b, c**) Yeast clones containing the CATS bait plasmid and the prey clones isolated from the yeast two-hybrid screening. All the isolated proteins are potential CATS interacting proteins as shown by cell growth on SD -Trp, -Leu, -His, -Ade + X-α-GAL plates. (**7**): KRT17. (**8**): KIS. (**9**): PCBP1. (**10**): MCM3AP. (**11**): UXT. (**12**): MKRN3. (**13**): SIVA. (**14**): CSNK2B. (**15**): RCN1. (**16**): HAX1. (**17**): ATP6V0C.

The potential interaction of CATS with proteins involved in so many different cellular pathways indicates that CATS might play a role in many different cellular processes. The most frequently found and most interesting CATS interacting partners were chosen for further analysis. Some interactions were further confirmed by colocalization experiments and/or co-immunoprecipitation.

3.15.4 CATS interacting protein PCBP1

The poly r-C binding protein 1 (PCBP1) was identified among the most frequent CATS interacting protein in the Y2H screen. This protein is of particular interest because it also interacts with CALM (Pasalic, Z., CCG Leukemia, GSF, Munich; personal communication). PCBP1 is a ribonucleoprotein involved in post-transcriptional control pathways, such as mRNA stabilization and regulation of translation from both cellular and viral RNAs (Makeyev & Liebhaber, 2002; Ostareck-Lederer & Ostareck, 2004).

None of the 5 independent PCBP1 clones isolated in the yeast two-hybrid screen, coded for the complete PCBP1 ORF (these clones started at nt position 287, 311 and 464, or amino acid residues 37, 45 and 96 of the PCBP1 coding region) (Figure 3.42).

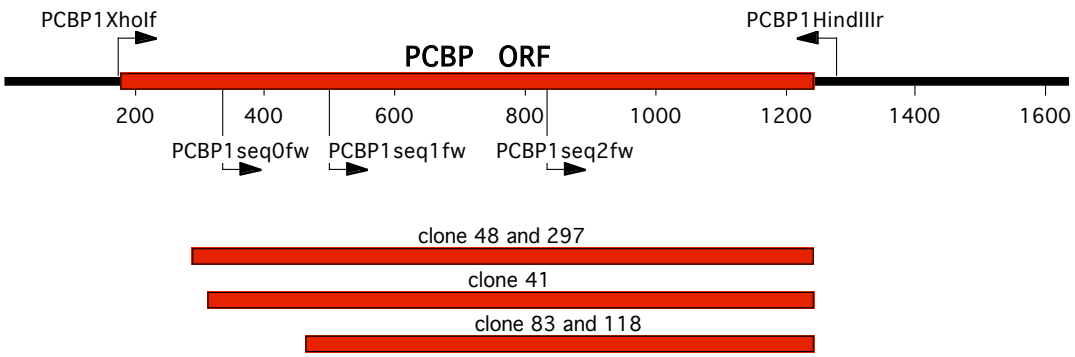


Fig. 3.42: PCBP1 clones isolated in the Y2H screen. Diagram representing PCBP1 nucleotide and amino acid sequences. Red bars underneath represent amino acids coded by the cDNA clones 48/297 (aa residues 35-356), 41 (aa residues 45-356) and 83/118 (aa residues 96-356) and its position related to the complete ORF. The arrows indicate binding position of sequencing primers.

The full length cDNA sequence deposited in Genbank (accession NM_006196) was used to design primers flanking the PCBP1 ORF. A 1106 bp cDNA fragments was amplified by RT-PCR from HeLa RNA (2.2.11 and 2.2.12) using PCBP1 specific primers containing sites for the *Xho*I and *Hin*dIII restriction enzymes (PCBP1XhoIf and PCBP1HindIIIr). The PCR product was digested with the appropriate enzymes, cloned into the pECFP-C1 vector (2.1.11) and sequenced (Figure 3.43).

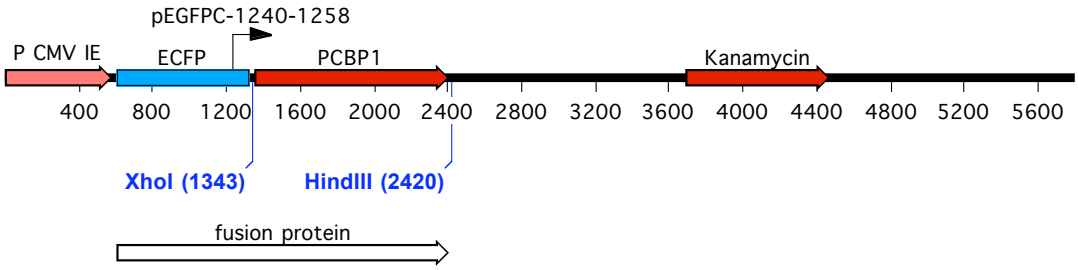


Fig. 3.43: CFP-PCBP1 construct. The PCBP1 ORF was amplified by RT-PCR from HeLa RNA using primer pair PCBP1XhoIf and PCBP1HindIIIr. The 1106 bp product was cloned into the pECFP-C1 vector digested with *Xho*I and *Hin*dIII. Restriction enzymes used for cloning are in bold. Numbers in brackets indicate nucleotide position. The numbers are position in base pairs. The arrow indicates the binding position of the pEGFP-1240-1258 sequencing primer.

To further validate the CATS-PCBP1 interaction, YFP-CATS (both isoforms) and the CFP-PCBP1 protein were coexpressed in murine fibroblasts (NIH3T3). These experiments revealed a colocalization of CFP-PCBP1 and YFP-CATS mainly in the nucleus but also to a lesser extent in the cytoplasm (Figure 3.44).

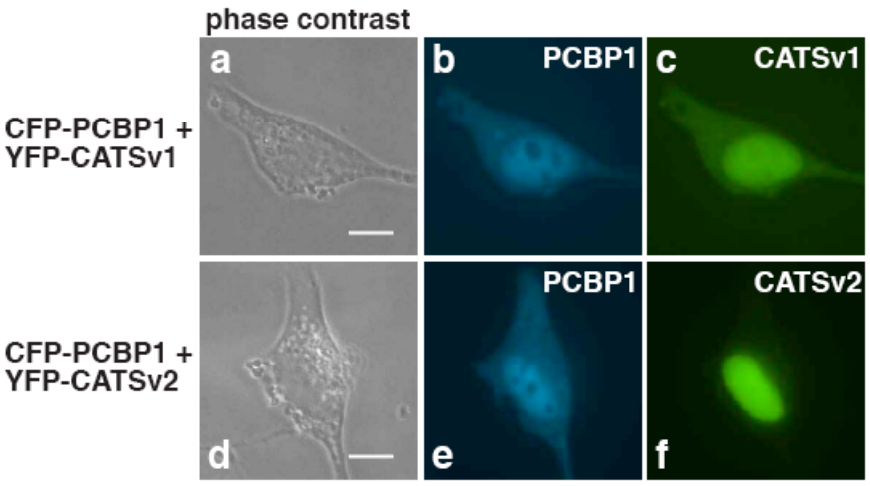


Fig. 3.44: Colocalization of YFP-CATS with CFP-PCBP1. NIH3T3 cells transiently transfected with YFP-CATS and CFP-PCBP1 expressing plasmids revealed a predominantly nuclear co-localization of these proteins. Bar: 10 µm.

Cloning of PCBP1 ORF and detailed analysis of CATS-PCBP1 interaction were performed together with Moritz Middeke (CCG Leukemia, GSF, Munich), an MD student who is working under the supervision of Leticia Fröhlich Archangelo.

3.15.5 The CATS interacting protein KIS

The kinase interacting with stathmin (KIS or UHMK1) is a serine/threonine kinase that positively regulates cell cycle progression through phosphorylation of $p27^{KIP}$ on S10, thereby promoting nuclear export of $p27^{KIP}$ and reducing its inhibitory effect on cell cycle (Boehm et al., 2002; Maucuer et al., 1995).

In the yeast two-hybrid screen described above (3.15) a single clone coding for KIS was identified as CATS interacting partner. The isolated clone 31 did not correspond to the complete *KIS* ORF as its sequence started at nucleotide position 243 of the *KIS* cDNA sequence (GenBank acc. Nr.: NM_175866/AJ536197), which corresponds to amino acid residue 33 of the coding region (Figure 3.45).

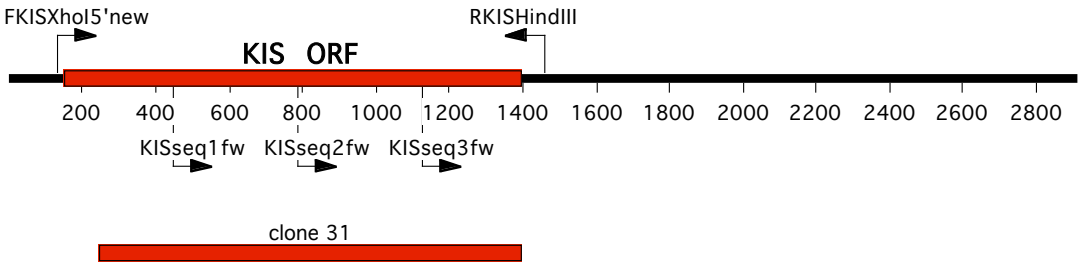


Fig. 3.45: KIS clone isolated in the Y2H screen. Red bar underneath represents the amino acid residues coded by the cDNA clone 31 (aa residues 33-419 of KIS ORF) and its position related to the complete ORF. The arrows indicate binding position of sequencing primers.

The cDNA sequence from Genbank was used to design primers flanking the KIS ORF. A 1328 bp cDNA fragments was amplified by RT-PCR from HeLa RNA (2.2.11 and 2.2.12) using KIS specific primers containing restriction enzyme sites for the *Xho*I and *Hin*dIII (KISXhoI5'new and RKISHindIII). The PCR product was digested with the appropriate enzymes, cloned into the pEYFP-C1 vector (2.1.11) and sequenced (Figure 3.46).

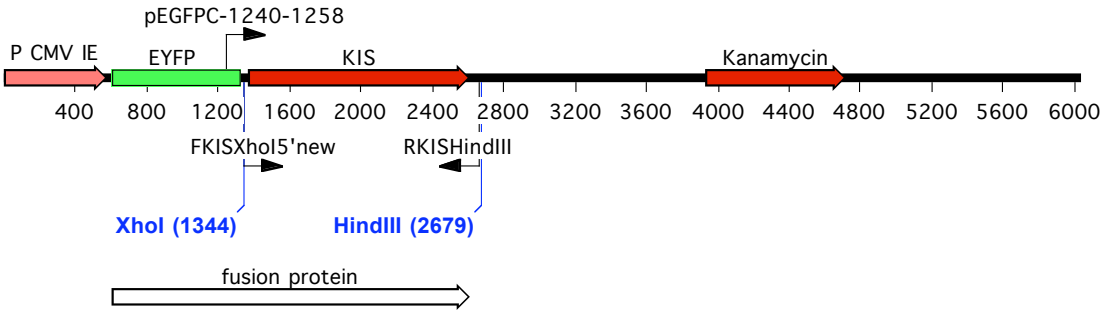


Fig. 3.46: YFP-KIS construct. The KIS ORF was amplified by RT-PCR from HeLa RNA using primer pair KISXhoI5'new and RKISHindIII. The 1328 bp product was cloned into the pEYFP-C1 vector digested with *Xho*I and *Hin*dIII. Restriction enzymes used for cloning are in bold. Numbers in brackets indicate nucleotide position. The numbers indicate the base pair positions. The arrows indicate primer binding positions.

3.15.6 CATS interacting protein HAX1

HAX1 has been found in anti-apoptotic signaling counteracting the pro-apoptotic effects of BAX. HAX1 was identified as a protein interacting partner of HS1 (hematopoietic lyn substrate 1), which is part of the B-cell and T-cell receptor signaling cascade (Sharp et al., 2002; Suzuki et al., 1997).

Two independent clones coding for HAX1 were identified in the Y2H screen. The longer clone (clone 12) started at nucleotide position 559 and the shorter (clone 305a) at nucleotide position 622 (GenBank acc. #: NM_006118), corresponding to residues 133-279 and 154-279 of the HAX1 ORF, respectively (Figure 3.47). The results show that CATS interacts with the C-terminal region of HAX1 (residues 154-279) and the N-terminal residues 1 to 153 of the HAX1 ORF are not necessary for the interaction.

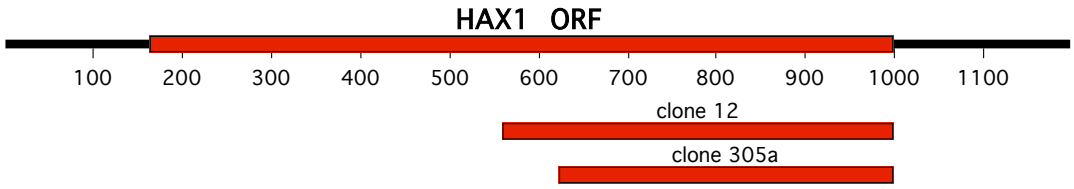


Fig. 3.47: HAX1 clones isolated in the Y2H screen. Diagram represents HAX1 nucleotide and amino acid sequences The red bars underneath represent amino acids coded by the cDNA clones 12 and 305a (residues 133-279 and 154-279, respectively) and its position related to the complete ORF. Note that N-terminal residues 1-153 are not necessary for the interaction with CATS.

To establish whether CATS interacts with HAX1 *in vivo*, fluorescently tagged CATS and HAX1 (2.1.12) fusion proteins were coexpressed in 293T cells and precipitated in CoIP buffer I (2.1.5) with an antibody against the HAX1 protein (2.2.28). The α-HAX1 antibody detected the immunoprecipitated HAX1 fusion protein as shown in figure 3.48, upper lanes 1

and 2. The membrane was stripped and reprobed with α-CATS 2C4 antibody. The CATS antibody identified the expected sized band for overexpressed CFP-CATS (approx. 55 kDa) in the lanes where immunoprecipitated HAX1 fusion protein was present (Figure 3.48, lower lane 2). Immunoprecipitation with control mouse IgG did not coprecipitate overexpressed CFP-CATS (Figure 3.48, lane 3). Similarly, a CFP-CATS band was not detected in immunoprecipitate from cells which had been transfected with YFP-HAX1 plus the empty CFP vector (Figure 3.48, lane 1). These results confirm that HAX1 is indeed a CATS interacting protein.

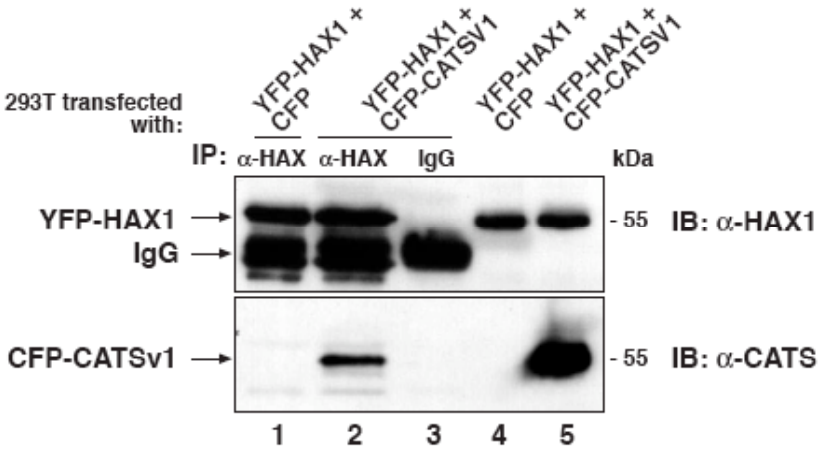


Fig. 3.48: Coimmunoprecipitation of CATS and HAX1 proteins. Lysates of cells overexpressing CFP-CATSv1 and YFP-HAX1 were immunoprecipitated with α-HAX1 in CoIP buffer I. Immunoprecipitated HAX1 fusion protein is detected by immunoblotting using the same antibody, upper lanes 1 and 2. The blot was stripped and reprobed with α-CATS 2C4 antibody, which identified a band of the expected size for the overexpressed CFP-CATS (approx. 55 kDa) in the HAX1 precipitate, lower lane 2. Immunoprecipitate of a lysate from cells transfected with YFP-HAX1 plus the empty CFP vector or immunoprecipitates with normal mouse IgG were used as negative controls, lanes 1 and 3, respectively. 10 µg cell extract was used as input control, lanes 4 and 5.

3.15.7 CATS interacting protein SIVA

Together with PCBP1, SIVA is the most frequent protein identified in the Y2H screen as CATS interacting partner. All five independent clones coded for the complete ORF of SIVA transcript variant 1 (GenBank acc. #: NM_006427) (Figure 3.49).

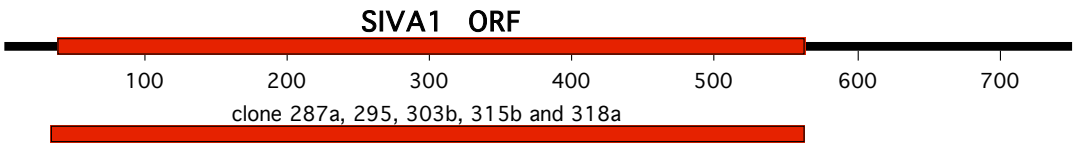


Fig. 3.49: SIVA clones isolated in the Y2H screen. All cDNA clones coded for the complete SIVA ORF (175 aa). Red bars represent the coding region.

SIVA (from Shiva = the Hindu god of destruction) is a pro-apoptotic protein originally identified through its association with the cytoplasmic tail of CD27 (TNFRSF7), a member of the tumor necrosis factor receptor superfamily (Prasad et al., 1997). It is an important intracellular signaling molecule that transduces CD27-, GITR- and TCR-mediated apoptotic response (Gudi et al., 2006; Py et al., 2004; Spinicelli et al., 2002).

To establish whether CATS interacts with SIVA *in vivo*, fluorescently tagged CATS and SIVA (2.1.12) fusion proteins were coexpressed in 293T cells and precipitated in CoIP buffer I (2.1.5) with antibody against the CATS protein (2.2.28). The α-CATS 2C4 antibody detected the immunoprecipitated CATS fusion protein as shown in figure 3.50, upper lanes 1 and 2. The membrane was stripped and reprobed with the α-SIVA antibody. The SIVA antibody identified the expected sized band for overexpressed YFP-SIVA (approx. 43 kDa) protein in the lanes where immunoprecipitated CATS fusion protein was present (Figure 3.50, lower lane 2). Immunoprecipitation with normal rat IgG did not coprecipitate overexpressed YFP-SIVA (Figure 3.48, lane 3). Similarly, the YFP-SIVA band was not detected in the immunoprecipitate from cells which had been trasfected with CFP-CATS plus the empty YFP vector (Figure 3.48, lane 1). These results confirm that SIVA is indeed a CATS interacting protein.

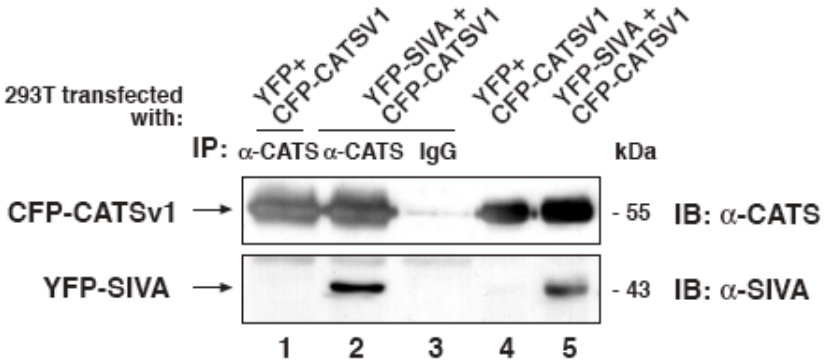


Fig. 3.50: Coimmunoprecipitation of CATS and SIVA proteins. Lysate of cells overexpressing CFP-CATS and YFP-SIVA were immunoprecipitated with 100 µl α-CATS 2C4 in CoIP buffer I. Immunoprecipitated CFP-CATS fusion protein is detected by immunoblotting using the same antibody, upper lanes 1 and 2. The blot was stripped and reprobed with the α-SIVA antibody, which identified a band of the expected size for the overexpressed YFP-SIVA protein (approx. 43 kDa) in the CATS precipitate, lower lane 2. Immunoprecipitate of a lysate from cells transfected with CFP-CATS plus the empty YFP vector and immunoprecipitates with normal rat IgG were used as negative controls, lanes 1 and 3, respectively. 10 µg cell extract was used as input control, lanes 4 and 5.

3.16 Conditional expression of the CATS protein

Conditional expression systems are an important tool for dissecting the function and relevance of poorly characterized proteins. In order to further characterize CATS and obtain insights into its biological function we choose a tetracycline-inducible gene expression system (Tet system). Of particular interest is to examine the impact of CATS expression on cell proliferation. For that purpose both CATS isoforms were cloned into the *reverse transactivator silencer-1* (pRTS-1) vector (2.1.11). In this Doxycyclin-regulatable (Dox) episomal "all-in-one" vector, the gene of interest is expressed from the bidirectional promoter P_{tet}bi-1 that allows simultaneous expression of two genes, of which one may be used as marker for the expression of the gene of interest. In addition, this vector constitutively expresses from a bicistronic expression cassette the Dox-sensitive reverse tetracycline controlled transactivator rtTA2^S-M2 and the Tet repressor-KRAB fusion protein (tTS^{KRAB}). Tight down regulation is achieved through binding of the silencer tTS^{KRAB} to P_{tet}bi-1 in the absence of Dox. Addition of Dox releases repression and via binding of rtTA2^S-M2 activates the P_{tet}bi-1 (Bornkamm et al., 2005) (Figure 3.51).

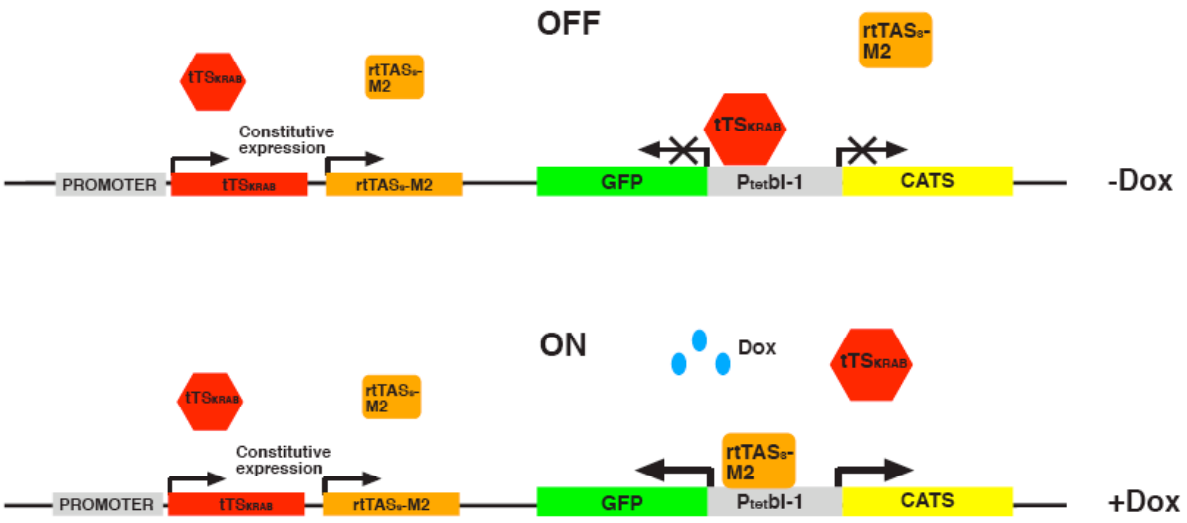


Fig. 3.51: Principle of Dox-inducible gene expression.

3.16.1 Generation of an HA-tagged CATS fusion genes

Human *CATS* cDNAs were amplified with the *Pfu* proof reading DNA polymerase and primer pair hCATS ATGf(PUC18) - hCATSΔTGAv1r(PUC18) or - hCATSΔTGAv2r(PUC18) in which a consensus Kozak sequence was added 5' of the start codon of the forward primer. The CFP-CATSv1 and YFP-CATSv2 constructs were used as templates for the amplification of CATS ORF isoform 1 and 2, respectively. The blunt ended PCR products were cloned in frame with the 3' HA tag from the pUC18-HA vector (2.1.11) which had been linearised with the *Eco*RV restriction enzyme. The orientation of the insert was determined by colony PCR using the pUC18-M13rev vector primer and CATSE1F1(364) primer internal to CATS sequences (Figure 3.52).

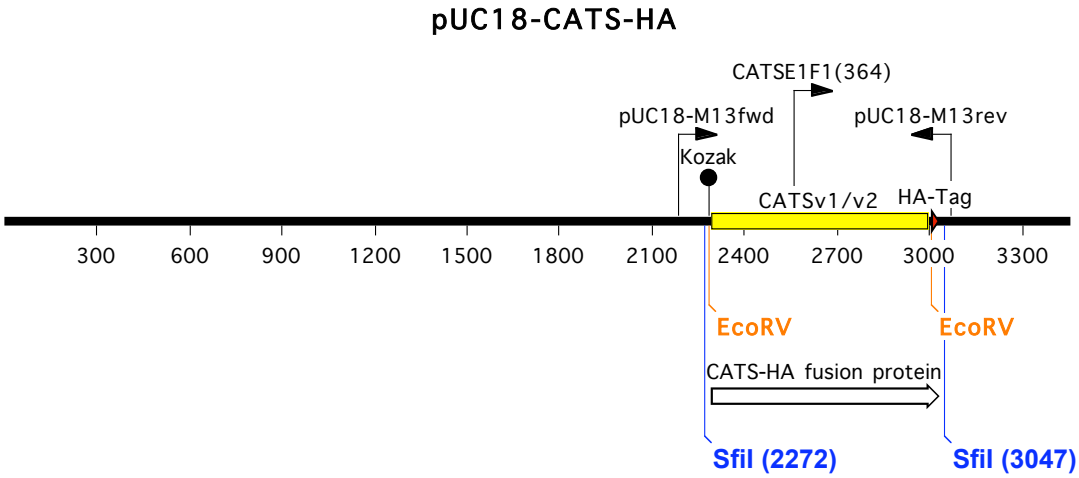


Fig. 3.52: pUC18-CATSv1/v2-HA tagged constructs. CATS ORF was amplified with *Pfu* polymerase and primer pair hCATSATGf(PUC18) - hCATSΔTGAv1r(PUC18) or - hCATSΔTGAv2r(PUC18). A consensus Kozak sequence was added 5' to the start codon from the forward primer. Both 714 bp and 789 bp products (coding for isoform 1 and 2, respectively) were cloned into the *Eco*RV site of the pUC18-HA vector. In red is the enzyme used for cloning, the enzyme site is destroyed upon ligation. In bold and blue is the *Sfi*I restriction sites used to excise the HA-tagged CATS in order to transfer the insert to the pRTS-1 vector. Numbers in brackets indicate nucleotide positions. The arrows indicate the binding position of the sequencing primers. The HA tag is shown in red.

3.16.2 Cloning of the HA-tagged CATS into pRTS-1 vector

The HA-tagged version of both CATS isoforms were excised with *Sfi*I from the constructs described above (pUC18-CATSv1/v2-HA) and cloned into the Dox-inducible episomal vector pRTS-1 digested with *Sfi*I (Figure 3.53). The orientation of the insert was determined by colony PCR using the pEBNA_Sfi+55bp (rev) vector primer and the CATSE1F1(364) primer.

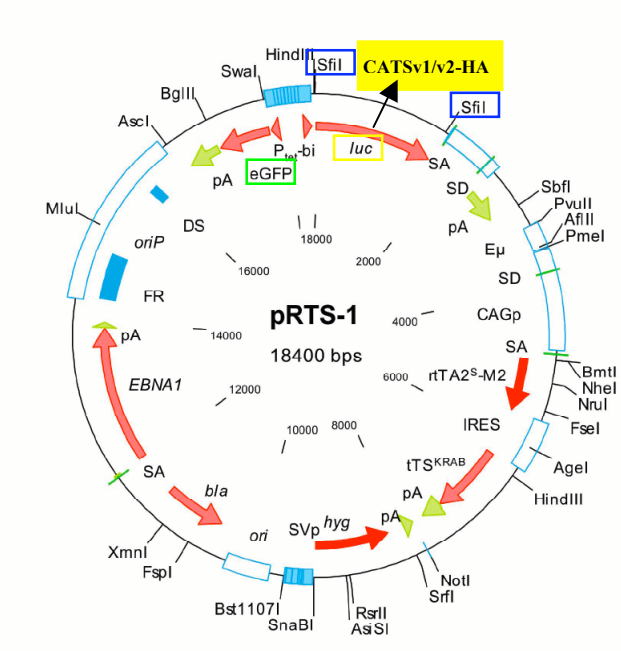


Fig. 3.53: Schematic map of pRTS-1. pRTS-CATSv1/v2-HA tagged constructs were generated by excising both HA-tagged versions of CATS from the pUC18-CATSv1/v2-HA with *Sfi*I restriction enzyme and subcloning into the pRTS-1 vector digested with the same enzyme. The *Sfi*I restriction sites used for cloning are in blue boxes. Yellow boxes indicate genes of interest, either HA-tagged CATS or luciferase (Luc) from the control plasmid. The green box indicates the GFP gene, used as marker for CATS expression. Note that the bidirectional promoter P_{tet}bi-1 allows simultaneous expression of both CATS-HA (or Luc for control experiments) and GFP genes. pRTS-1 also contains the ampicillin resistance gene for bacterial selection and the hygromycin B resistance gene (*hyg*) for selection in mammalian cells.

4 Discussion

4.1 The novel CALM interactor CATS and the significance of the CALM-CATS interaction for CALM/AF10-mediated leukemogenesis

4.1.1 CATS is a novel protein which interact with CALM and influence the subcellular localization of CALM/AF10

The t(10;11)(p13;q14) translocation is found in patients with acute myeloid as well as acute lymphoblastic leukemias and in malignant lymphoma. This translocation results in the fusion of *CALM* on chromosome 11 band q14 to *AF10* on chromosome 10 band p13. The CALM/AF10 fusion protein, which encompasses nearly all the protein domains of the two proteins, rather than the reciprocal AF10/CALM fusion protein, is critical for the malignant transformation. Desphande et al. have recently shown that the expression of CALM/AF10 is sufficient to induce leukemia in a murine bone marrow transplant model (Deshpande, 2003). CALM has been described as an important endocytic protein, which plays a pivotal role in clathrin-mediated endocytosis (De Camilli et al., 2002; Evans & Owen, 2002; Ford et al., 2001; Kalthoff et al., 2002; Meyerholz et al., 2005; Tebar et al., 1999). Recently, an MLL/CALM fusion has been described in a case of childhood AML (Wechsler et al., 2003).
The involvement of *CALM* in two separate translocations with both *MLL* and *AF10* as well as the fact that a number of other *MLL* and non-*MLL* fusion partners found in leukemia are proteins which play a role in clathrin-mediated endocytosis (CME) suggests that disturbing CME may be important in leukemogenesis (Bohlander, 2000; Di Fiore & De Camilli, 2001; Floyd & De Camilli, 1998; Polo et al., 2004; Wechsler et al., 2003). In order to further characterize *CALM* function and understand its contribution in *CALM/AF10*-mediated leukemogenesis we used the N-terminal portion of CALM (1-335) as a bait in a yeast 2 hybrid screen. The C-terminal end of a novel protein was identified. The corresponding gene was named *CATS* (CALM interacting protein expressed in thymus and spleen) because multiple tissue Northern blot analysis revealed a 1.6 kb transcript which is expressed predominantly in thymus, spleen and colon. The *CATS* gene is located on chromosome 17 band p13. The genomic locus spans approximately 7 kb and contains 6 exons. At least three alternatively

spliced versions of *CATS* could be identified which code for two protein isoforms of 238 or 248 amino acids in length.

Expression of fluorescent-tagged CATS proteins in NIH 3T3 cells showed that CATS localizes mainly to the nucleus where an accumulation of CATS is seen at the nucleoli (see below). However, a subfraction of CATS is also observed in the cytoplasm and at the cell membrane.

The CATS interaction region of CALM was mapped to amino acids 221 to 294 of CALM. The interaction between CATS and CALM seems to be quite complicated since in contrast to the full length CATS the C-termini of CATS do not interact with CALM1-294 (which includes the interaction domain of for full length CATS) but need amino acids 1-335 of CALM for interaction.

The N-terminal portion of CALM (150 aa) comprises the ANTH domain, which binds specifically to lipids (PtdIns(4,5)P_2) in the membrane (De Camilli et al., 2002; Evans & Owen, 2002; Ford et al., 2002; Ford et al., 2001; Stahelin et al., 2003). In contrast to the N-terminal domain of CALM, which is composed of α-helices, the C-terminal two thirds of CALM contains poorly ordered protein domains containing multiple copies of various short protein interaction motifs (e.g. NPF, DFP, DIF, DLL and FESVF; (Meyerholz et al., 2005)) and behaves like a flexible arm (Kalthoff et al., 2002). This organization of CALM is thought to permit the establishment of complex networks of interactions. Evan and Owen compared this structure to a long fishing line anchored to the membrane by the ANTH domain, with multiple hooks that can interact with various partners during clathrin-coated pit assembly (Evans & Owen, 2002). We were able confirm the CALM-CATS interaction (by cotransforming both the CALM bait and the CATS prey plasmids into yeast as well as by coimmunoprecipitation experiments) and determine the CATS interaction domain of CALM. The fact that CATS interacts within the central domain of CALM suggests an involvement of CATS in this complex network of interactions in the endocytic machinery. CATS might function as an adaptor protein which mediates the interaction of CALM with other endocytic proteins. The idea of CATS functioning in the cytoplasm as member of the endocytic machinery together with CALM is supported by the fact that expression of CFP-CATS and YFP-CALM resulted in the colocalization of both proteins in the cytoplasm and at the plasma membrane of transfected NIH3T3 cells. However, the majority of the CATS protein localizes to the nucleus, implying also a nuclear function for this protein. In reporter gene assays CATS functions as transcriptional repressor when it is fused to a DNA binding domain. Whether this observation is relevant for the physiological function of CATS needs to be explored further.

Interestingly, the CATS interaction domain of CALM is present in the CALM portion of both CALM/AF10 and the MLL/CALM fusion proteins. CATS not only colocalizes with CALM/AF10 in transient transfection experiments, but in some cells the subcellular localization of both CALM and CALM/AF10 can be affected by the presence of CATS. CATS was able to increase the nuclear fraction of both CALM and CALM/AF10 and this effect seems to be stronger for CALM/AF10. In contrast to CALM, which only partially shifted to the nucleus, CALM/AF10 was nearly completely relocalized to the nucleus and even accumulated at the nucleoli when CATS was coexpressed. Since we showed that not just CALM (as previously described; Vecchi et al., 2001) but also the CALM/AF10 fusion protein shuttle between nucleus and cytoplasm, it is tempting to speculate that the interaction between CATS and CALM or CALM/AF10 takes place in the nucleus, resulting in the accumulation of these proteins in the nucleus. These findings imply that in cells with high CATS expression a greater portion of CALM and CALM/AF10 might be present in the nucleus. Considering the high expression of CATS in lymphoid tissues, the CATS-CALM interaction might play an important role in CALM/AF10 mediated leukemogenesis. These findings have to be interpreted with caution at the present time, because the change in CALM and CALM/AF10 localization was not observed in all cell coexpressing CATS and the two proteins. However, an analogous situation has been observed in a group of MALT lymphomas harboring the t(1;14)(p22;q32) translocation which results in the API2/MALT1 fusion protein. BCL10 interacts with the non-rearranged MALT1 and some of the API2/MALT1 fusion proteins and MALT1 can regulate the subcellular localization of BCL10. Association of BCL10 with MALT1 and API2/MALT1 is thought to synergize the activation of NF-κB, the pathway underlying the anti-apoptotic effect of API2/MALT1 fusion protein (Nakagawa et al., 2006; Sanchez-Beato et al., 2003).

4.1.2 Possible mechanisms underlying CALM/AF10-mediated leukemogenesis

The mechanisms by which the CALM/AF10 fusion protein causes leukemia are not known. One possibility would be a gain of function mechanism by which the CALM/AF10 fusion functions as a CALM protein with additional functions or as an AF10 protein with additional functions. Alternatively, the CALM/AF10 fusion protein might exerts its effect through a dominant negative mode of action, i.e. disrupting normal AF10 and/or normal CALM function. Of course, a combination of both mechanisms could also be the case.

It is known that the localization of a protein (especially nuclear versus cytoplasmatic) is critical for its function (e.g. nuclear translocation of NF-κB causes transcription of target genes). It is tempting to speculate that the CALM/AF10 fusion resides predominantly in the cytoplasm and thereby causes mislocalization of the wildtype AF10 protein from the nucleus into the cytoplasm through AF10 homo-dimerization (Linder et al., 2000). In the light of the CATS–CALM interaction, which is able to change the subcellular localization of CALM/AF10 to the nucleus, this scenario appears less likely. However, it could still be that through its interaction with CATS, the CALM/AF10 fusion protein attains an abnormal subnuclear localization (e.g. a more nucleolar localization). In this context, it should be noted that CATS is highly expressed in lymphoid cells. Thus it can be hypothesized that the CALM/AF10 fusion exerts its leukemogenic effect through a disruption of AF10 function. Since AF10 is a putative transcription factor and the *Drosophila* AF10 homologue *alhambra* has been shown to affect transcriptional regulation through *Polycomb* group-responsive elements (PREs), disruption of AF10 function could lead to transcriptional deregulation of *polycomb* and *trithorax* target genes (Perrin et al., 2003). Indeed, leukemias involving CALM/AF10 fusion have been recently characterized by overexpression of HOXA genes and its co-factor MEIS1, and by overexpression of BMI1, a member of the polycomb (PcG) family (Dik et al., 2005; Krause, 2004; Soulier et al., 2005).

On the other hand, it is conceivable that the CALM/AF10 fusion protein might disrupt the function of CALM through the mechanism of protein mislocalization. An altered CALM function might affect clathrin mediated endocytosis (CME) and protein trafficking in the trans-Golgi network. Endocytosis is well established mechanism of signal attenuation via receptor clearance from the plasma membrane. Tightly connected with signaling processes, endocytosis can itself be regarded as a signaling pathway, triggered by the activation of surface receptors and leading to their internalization. CME is involved then in propagating the intracellular signals, which continues within the cell until activated receptors have reached the end point of the endocytic journey where they undergo inactivation. For receptors whose fate is the lysosome, there is evidence that regulation of these steps may affect the duration of the stimulus (Di Fiore & De Camilli, 2001; Polo et al., 2004). Thus impairment of endocytosis will affect the down-regulation of activated transmembrane receptors resulting in prolonged growth factor signaling leading to an increase in proliferative stimuli to the cell (Di Fiore & De Camilli, 2001; Polo et al., 2004). There are several endocytic proteins which have been identified as parts of leukemic fusion proteins or which have been reported as mutated in various forms of cancer (Floyd & De Camilli, 1998). In this respect, a recent report that a

mutation in the murine *CALM* homologue *Picalm* results in altered hematopoiesis is interesting (Klebig et al., 2003).

Indeed, Chao and coworkers have recently reported perturbed endocytosis by CALM-containing fusion proteins, associated with prolonged growth factor signaling and enhanced cellular proliferation (Chao, 2005; Chao, 2004). Interestingly, the TSG101 tumor susceptibility gene (Li & Cohen, 1996) was found to interact with CALM at the same domain of CALM used for CATS interaction (Gläsner, 1999). TSG101 has an important role in ubiquitin-mediated endosomal sorting pathways and impairment of its function perturbs endosomal trafficking which is thought to contribute for the tumorigenic phenotype exhibited by suppressed expression of TSG101 in fibroblasts (Babst et al., 2000; Bishop et al., 2002). Similarly, increased expression of TSG101 in cells results in abnormal cell growth possibly mediated by the property of this protein to act as a dominant-negative inhibitor of the ubiquitination pathway controlling MDM2/p53 levels (Li et al., 2001; Ruland et al., 2001).

Whether CATS and TSG101 are part of a complex with CALM or whether they compete for CALM interaction is not known. Nevertheless it is tempting to speculate that the mislocalization of CALM or CALM/AF10 through the interaction with CATS could interfere with the normal function of TSG101. On one side enhancing the effects of disrupted down regulation of activated growth factor receptors. On the other hand a greater portion of TSG101 protein would be free in the cytoplasm mimicking the overexpression situation where TSG101 inhibits MDM2 ubiquitination and degradation and consequently decreases the cellular level of p53. The hypothesis of an involvement of both CATS and TSG101 in CALM/AF10-mediated leukemogenesis is supported by the fact that TSG101, like the CATS interactor KIS (kinase interacting with stathmin; see bellow), interacts with stathmin, a phosphoprotein phosphorylation of which in important in diverse regulatory pathways including T-cell activation and cell cycle progression. Stathmin expression is also increased in acute leukemia and in malignant lymphoma (Li & Cohen, 1996; Maucuer et al., 1995 and references therein).

As mentioned above, an alternative mode of action of the CALM/AF10 fusion protein would be a gain of function. In this scenario CATS, which could be shown to have transcriptional properties when fused to a heterologous DNA binding motif, would be recruited to AF10 target genes through CALM thereby deregulating AF10 target gene expression. This transcriptional deregulation could also be caused directly by the CALM portion of the CALM/AF10 fusion protein. A transcriptional activation domain (TAD) could be delineated in CALM in the yeast system (Archangelo et al., 2006). It has also been reported that CALM

is able to upregulate transcription when fused to a GAL4-DBD in a mammalian reporter gene assay (Vecchi et al., 2001). However, we were unable to reproduce CALM-mediated transcriptional activation in a similar system. It should be noted that the CATS interaction domain of CALM and the CALM TAD map to distinct regions of the CALM protein. It remains to be tested which of these regions is critical for the transformation potential of the CALM/AF10 fusion protein.

Structure function analyses of the MLL/AF10 fusion protein have shown that the critical region for transformation in AF10 is the octapeptide motif (OM) and the leucine zipper (LZ) of AF10 (DiMartino et al., 2002). The OM/LZ motif of AF10 was shown to interact with GAS41 a component of a chromatin remodeling complex and with the histone methyl transferase hDOT1L (Debernardi et al., 2002; Okada et al., 2005). The OM/LZ motif is also present in the CALM/AF10 fusion protein and it is very likely that this motif is critical for the transformation potential of the CALM/AF10 fusion protein. Since we could show that CATS is capable of recruiting the CALM/AF10 fusion protein to the nucleolus, there is the possibility that the AF10 portion of CALM/AF10 can affect the function of nucleolar proteins. Nucleolar proteins have been shown to regulate cell proliferation and growth by controlling ribosome biogenesis and p53 function and deregulation of these finely balanced mechanisms is a key event in the initiation and progression of malignant transformation (Maggi & Weber, 2005; Olson, 2004; Rubbi & Milner, 2003; Ruggero & Pandolfi, 2003). The significance of CATS nucleolar localization will be discussed below in more detail.

In summary, we propose that CATS is a critical modulator of normal CALM function in lymphoid cells and that the interaction of CATS with CALM could be very important for understanding leukemogenic potential of the CALM/AF10 fusion protein.

4.2 CATS is a nucleolar protein involved in proliferation control

Nucleoli have been long known as the center of ribosome biogenesis, a major metabolic activity within the cell. This dynamic organelle dissociates during mitosis and assembles as cells renter cell cycle underlining the important relationship between cell cycle and ribosome production and the tight link between nucleolar function, cell growth and proliferation (Bernardi & Pandolfi, 2003; Du & Stillman, 2002; Schlosser et al., 2005; Schlosser et al., 2003; Tsai & McKay, 2002). Recently, reports have suggested additional functions for the nucleolus in cell cycle control and cellular response to stress coupling nucleoplasmic levels of p53 to the functional state of ribosome biogenesis (Holzel et al., 2005; Kamijo et al., 1998; Kurki et al., 2004a; Kurki et al., 2004b; Olson, 2004; Rubbi & Milner, 2003; Tao & Levine,

1999). Not surprisingly subversion of nucleolar function has been associated with cancer progression (Maggi & Weber, 2005; Ruggero & Pandolfi, 2003) and much attention has been paid to this organelle as a potential target for anti-cancer drugs (Yao & Yang, 2005). Schlosser and colleges have indeed shown that many target genes of the proto-oncogene MYC are nucleolar proteins and conclude that MYC regulates cell growth and proliferation by the coordinated induction of cdk activity and ribosomal processing (Schlosser et al., 2005; Schlosser et al., 2003). Interestingly, proteomic analysis of the human nucleolus revealed that 30 % of the identified proteins are encoded by previously uncharacterized genes (Andersen et al., 2005; Andersen et al., 2002).

CATS is an example of this growing list of novel nucleolar proteins placing CATS in a defined molecular network. Consistent with the function as a nucleolar protein regulating cell proliferation, *Cats* is highly expressed throughout murine embryogenesis but not in differentiated cells of most adult tissues. *Cats* expression decreased gradually and proportionally similarly to the proliferative marker PCNA and the nucleolar nucleophosmin (Tsai & McKay, 2002), reflecting the increase in cells exiting cell cycle. Western blot analyses using CATS antibody have shown strong endogenous CATS expression in leukemia, lymphoma and tumor cell lines where CATS protein levels seems to be inversely correlated to cell doubling time, which in turn is known to be directly related to nucleolar function (Derenzini et al., 2000). In line with these findings, no expression of the CATS protein was observed in resting T-cells (JB4 and TYRF8). Furthermore, in cells induced to proliferate CATS showed an increasing and progressive upregulation with accumulation of CATS when cells entered S phase and maximum protein levels in G_2 phase. Similarly, expression analysis on synchronized cells revealed an evident cell cycle dependent accumulation of CATS protein in the S and G_2 phase of the cell cycle. These data clearly correlate accumulation of CATS protein with high nucleolar activity and thus a proliferative state of the cell. Thus CATS is a marker for proliferation and the amount of the protein is correlated to the cell cycle phases, being low in G_1 and high in the S-G_2 phase.

Surprisingly, we failed to detect CATS protein in the leukemic cell line HL60. The fact that we found abundant amounts of *CATS* transcript in HL60 cells but not protein, suggests a post-transcriptionally regulation of CATS and that CATS is not essential for proliferation in this cell line.

Nevertheless, a function of CATS as a marker for proliferation was nicely demonstrated by *Cats* up-regulation in $B220^+$ cells from a CALM/AF10 leukemic mouse population in comparison to the same population from a non-leukemic mouse. In this murine leukemia

model, the $B220^+$ cells represent the leukemia propagating cells (Deshpande, 2005). These findings not only confirm CATS as a marker for proliferation but also suggest that CATS is an early player in CALM/AF10 mediated transformation.

In this work, we described several monoclonal antibodies generated against the C-termini of CATS protein. The monoclonal antibody CATS 2C4 recognized the endogenous protein most efficiently followed by 7F6 and 2A2, all detecting both CATS isoforms. Differently from these antibodies, the antibodies specific for isoform 2 (7H2 and 3H8) did not seem to detect the endogenous CATS isoform 2 (only the overexpressed protein). Whether these antibodies are not able to recognize the endogenous isoform 2 of CATS or if the endogenous expression levels of the isoform 2 are too low for detection has to be determined. The observation that the transcript coding for isoform 2 are lower expressed than the ones coding for CATS isoform 1 supports the idea that the endogenous isoform 2 of CATS is less abundant. Immunofluorescence experiments of non-synchronized cells using the antibodies mentioned above showed levels of endogenous CATS expression, which were variable from cell to cell. There was also cell to cell variability of the nucleolar localization. To rule out that these observations were due to methodological problems different fixation protocols and experimental conditions were employed. In line with the results from the Western analysis, CATS failed to accumulate in the nucleoli of cells which grew at high density, i.e. growth arrested cells. These cell completely lacked nucleolar CATS staining, indicating that the nucleolar localization of CATS is associated with cell proliferation. Consistent with these observations, transiently expressed YFP-CATS did also not accumulate in the nucleoli of every cell expressing the plasmid. Moreover, YFP-CATS was not found in the nucleoli of cells which had been treated with the nuclear export inhibitor leptomycin B (LMB). LMB is one of the 13 agents described to disrupts nucleolar function and induce p53 (Rubbi & Milner, 2003). Consistent with the nucleolar export model proposed by Rubbi and Milner, disruption of nucleolar function/structure caused by cellular stress promotes translocation of many nucleolar proteins to the nucleoplasm where they are able to interfere with p53 degradation and consequently with its stabilization and activation (Kamijo et al., 1998; Kurki et al., 2004a; Kurki et al., 2004b; Maggi & Weber, 2005; Olson, 2004; Rubbi & Milner, 2003; Tao & Levine, 1999). Interestingly, we identified two CATS interacting proteins, PCBP1and CSNK2 in the nucleolar proteome database (Leung et al., 2006). The nucleolar localization of these proteins also decreases following the disruption of nucleolar function (upon treatment with the proteasome inhibitor MG132) (Andersen et al., 2005; Leung et al., 2006). These results strongly suggest that the proliferative function of CATS resides in the nucleolus while its

nucleoplasm localization could possibly be related stabilization of p53. However, this link to p53 has to be further investigated.
Another question raised by our observations is whether CATS in one of the several MYC nucleolar targets that regulate cell proliferation. The fact that we found KIS as a CATS interacting partner further confirms this hypothesis. KIS positively regulates cell cycle progression by inhibiting $p27^{KIP}$ function, an important downstream target of MYC. It would be very important to address this question.

4.3 CATS interacting proteins

In order to gain further insights into CATS function we performed a yeast two-hybrid screen to identify interaction partners of CATS. Determining protein interactions we were able to indentify potential pathways that CATS might be involved thus placing CATS in a functional context within the living cell.

4.3.1 PCBP1

The poly(C)-binding protein 1 (PCBP1, also referred to as αCP1 or hnRNP-E1) is a RNA-binding protein characterized by its triple KH structure and by its poly(c) binding specificity (Kiledjian et al., 1995; Leffers et al., 1995). It is a member of the PCBP family of proteins, which are involved in a wide spectrum of post-transcriptional control pathways. As for other PCBPs, the function of PCBP1 in mRNA stabilization, translation activation and translational silencing from both cellular and viral RNAs has been well characterized (for review; Makeyev & Liebhaber, 2002; Ostareck-Lederer & Ostareck, 2004; Ostareck-Lederer et al., 1998). For example, PCBP1 is a component of a ribonucleoprotein complex that assembles on the 3'-UTR of a subset of long-lived mRNA to promote their stabilization. On the other hand, PCBP1/ hnRNP K-DICE complex formation at the 3'-UTR of the r15-LOX mRNA silences translation by blocking ribosome assembly at the translation initiation codon. PCBP1 not only affects the fate of cellular mRNA but it also stabilizes and enhance translation of poliovirus RNA via interaction with stem-loop IV of the internal ribosome entry site (IRES) (Gamarnik & Andino, 1997) and silences the translation of papillomavirus L2 (Collier et al., 1998). Most recently, several reports have described PCBP1-mediated enhancement of translation through stimulation of internal IRES element within cellular mRNAs, such c-myc, X-linked inhibitor of apoptosis (XIAP) and of the anti-apoptotic Bag-1 (Bcl-2 associated athanogene-1) (Evans et al., 2003; Nishimura et al., 2004; Pickering et al., 2003). Besides its function on mRNA

stability and translation, additional roles for PCBP1 in transcriptional control and apoptotic pathways have been proposed (Kim et al., 2005; Ko & Loh, 2005; Nishimura et al., 2004).

We identified PCBP1 as a CATS interacting partner in a yeast two-hybrid screen. None of the 5 independent PCBP1 cDNA clones isolated in the screen coded for the complete PCBP1 ORF, but rather for PCBP1 proteins starting from different residues located within the first KH domain (KHI) or downstream of this motif, just 5' of the KHII domain. The fact that two clones coded for PCBP1 lacking the complete first KH domain indicates that this motif is not necessary for the CATS-PCBP1 interaction. It has been described that both KHI and KHIII domain of PCBP1 confer the poly(c)-binding activity of the protein whereas all three KH domains exhibit binding activity towards other nucleic acids, albeit at significantly lower levels (Dejgaard & Leffers, 1996). Phosphorylation of PCBP1 results in a marked decrease in poly(c)-binding activity (Leffers et al., 1995), which for hnRNP K (member of the PCBP family and interacting partner of PCBP1 in the hnRNP K/PCBP1-DICE complex) results in the association with transcriptional repressors such as Eed and protein kinases such as Src, Erk and Lck and in the cytoplasmatic accumulation of the protein. The cellular distribution of the hnRNP K between nucleus and cytoplasm can be controlled by the MAP kinase signaling pathway. Serine phosporylation of hnRNP K by ERK kinase leads to its accumulation in the cytoplasm and enhances hnRNP K/PCBP1-DICE- dependent inhibition of mRNA translation (for review; Perrotti & Calabretta, 2004). This hnRNP K/PCBP1-DICE- dependent inhibition of mRNA can be abolished by tyrosine phosphorylation of hnRNP K by c-Src (PCBP1 is not an activator or substrate for c-Src) (Ostareck-Lederer et al., 2002). Although PCBP1 is itself a shuttling protein (Chkheidze & Liebhaber, 2003; Makeyev & Liebhaber, 2002), localization experiments have shown that CATS colocalizes with PCBP1 mainly in the nucleus and to a lesser extent in the cytoplasm. Interestingly, directed proteomic analysis of the human nucleolus has identified PCBP1 as one not previously described nucleolar protein (Andersen et al., 2005). It is tempting to speculate that the PCBP1-CATS interaction might take place in the nucleus or even in the nucleolus where most of CATS is localized. In the nucleus, CATS might function as regulator of transcription so one could postulate a role for the PCBP1-CATS interaction in transcription control. Nevertheless, we cannot exclude a possible involvement of CATS in protein complexes controlling post-transcriptional pathways.

In line with this idea, we have identified two protein kinases as CATS interacting partners, the kinase interacting stathmin (KIS) and casein kinase type II beta subunit (CSNK2B). KIS is a protein kinase with RNP-type recognition motif and is involved in regulating RNA associated factors and enhances formation of protein-RNA complexes. Thus suggesting that post-

translational modifications on CATS or mediated by CATS could, for example, be involved in the activity of PCBP1. It would be of great value to investigate a possible kinase activity within the CATS protein and whether PCBP1 is a substrate for CATS. The involvement of CATS in RNA metabolism is further supported by the fact that the anti-apoptotic protein HAX1, also identified as CATS interacting partner is itself a RNA-binding protein suggesting its role in mRNA translation (Al-Maghrebi et al., 2002). Interestingly, PCBP1 is also a CALM interacting partner (Zlatana Pasalic, personal communication). The interaction between PCBP1 and CATS and these both proteins with CALM suggests that they form a protein complex. However, the biological significance of the interaction between these proteins, which function apparently in completely different cellular processes, has to be investigated.

Intriguing is the implication that the CATS-CALM-PCBP1 interaction has in the leukemic context of CALM/AF10. By investigating the mechanism of leukemic transformation of hematopoietic cells, Perroti and coworkers (Perrotti & Calabretta, 2004 and references therein) have indeed found that expression and activity of several RNA-binding proteins was altered by the BCR/ABL fusion protein. Moreover, they identified MDM2 and CEBPA mRNAs as targets for these PCBPs, being directly relevant for the altered differentiation and survival of leukemic cells.

A recent report by Giles et al. has described PCBP1 to bind and to regulate $p21^{WAF1}$ mRNA stability. Moreover, multiple reports show that EGF induces $p21^{WAF1}$ expression via stabilization of $p21^{WAF1}$ mRNA (Giles et al., 2003 and references therein). Since clathrin mediated endocytosis (CME) is the major mechanism of down regulation of activated EGFR it is tempting to speculate that altered CALM function in CALM/AF10 leukemic cells would influence the normal function of PCBP1 and the post-transcriptional regulation of its target mRNAs. Supporting this idea is the fact that a down regulation of normal CALM mRNA is observed in leukemic cells from T-ALL patients carrying a CALM/AF10 rearrangement (Dik et al., 2005) and the observation that CALM/AF10 including the CALM portion of the fusion protein is mislocalized to the nucleus through the CATS-CALM/AF10 interaction (Archangelo et al., 2006).

4.3.2 KIS

We have identified the kinase interacting with Stathmin (KIS) as a CATS interacting partner in the yeast two-hybrid screen. A first confirmation of this interaction was performed by cotransforming both CATS bait and KIS prey plasmids into yeast and assaying for growth on selective plates. The single KIS cDNA clone isolated in the screening coded for residues 33 to 419 of the KIS ORF. Although, the first 10 aa N-terminal of the kinase core domain of KIS (Maucuer et al., 1997) were missing in the cDNA clone most of the core domain including the K54 kinase active site was still retained, indicating that CATS could be a substrate for KIS. The fact that KIS is a serine/threonine kinase suggests that CATS might be a substrate for KIS on these residues. In fact, CATS has three serine (S131, S185 and S195) and one threonine (T74) proline-directed residues, which are preferentially phosphorylated by KIS (Maucuer et al., 2000), with the S131 being a very good candidate site, since it lies within the SGSP consensus sequence for KIS phosphorylation. CATS seems to be involved in several regulatory pathways (such as signaling and control of apoptosis) controlled by post-translational modifications implicating a possible role for CATS as a kinase substrate. Whether CATS is a substrate for KIS has to be further investigated. Nevertheless, the fact that both CATS and KIS share similar expression pattern and subcellular localization strengthens the biological relevance of this interaction *in vivo*.

KIS was first identified as one of the proteins interacting with stathmin, a phosphoprotein phosphorylation of which correlates with diverse regulatory pathways including T-cell activation and cell cycle progression (Maucuer et al., 1995). The 46.5 kDa KIS protein localizes, like CATS, mainly to the nucleus and to a lesser extent to the cytoplasm. KIS has an N-terminal kinase core (282 aa) and a C-terminal domain that contains characteristic RPN-type RNA recognition motif (RRM) with high sequence identity to U2AF65, a 65 kDa subunit of the splicing factor U2AF (Alam et al., 1996; Caldwell et al., 1999; Maucuer et al., 1995; Maucuer et al., 1997). Because of its RNA recognition motif, KIS is thought to phosphorylate and regulate RNA associated factors (Maucuer et al., 1997). Recently, Manceau and colleges have indeed described a interaction between KIS and the splice factor 1 (SF1). They showed that phosphorylation of SF1 by KIS increases binding of SF1 to U2AF65 and enhances formation of ternary SF1-U2AF65-RNA complex (Manceau et al., 2006). The involvement of CATS in an interaction with KIS, a RNA-binding kinase which regulates the formation of protein-RNA complex, suggests a role for CATS in RNA metabolism. In line with this idea we have found PCBP1 (also known as hnRNPE1), a RNA binding protein

involved in translational regulation and/or mRNA stabilization in hematopoietic cells (Ostareck-Lederer & Ostareck, 2004) as CATS interacting partner (see above).

The most well characterized function of KIS is however its ability to positively regulate cell cycle progression (Boehm et al., 2002; Le et al., 2005). KIS is the major kinase responsible for S10 phosphorylation on the cyclin-dependent kinase inhibitor $p27^{Kip}$ and phosphorylation on S10 by KIS promotes nuclear export of $p27^{Kip}$ reducing its inhibitory effect on cell cycle (Boehm et al., 2002). Thus abnormally elevated KIS activity, which is expected to relieve cells from growth inhibition dependent on $p27^{Kip}$, might be involved in some aspects of tumor development. It was indeed shown that depletion of KIS significantly lowered the phosphorylation of $p27^{Kip}$ and enhanced growth arrest in leukemic cells suggesting that KIS promotes cell cycle progression in those cells (Nakamura, 2005). Moreover, elevated levels of KIS expression were observed in different types of neurological tumors (Bieche et al., 2003) and KIS expression has been shown to be targeted by the anti-HER2 antibody trastuzumab, used in treatment of human metastic breast cancer with HER2-overexpression (Le et al., 2005). The KIS kinase activity is induced by mitogens during G_0/G_1, where it promotes cell cycle progression in G_1 phase of the cell cycle. Interestingly, like KIS, CATS expression is also induced by mitogens with low levels of protein found in G_0 cells and increased levels upon serum stimulation. Moreover, CATS is highly expressed in proliferating and tumor cells in a cell cycle dependent manner. Thus it is tempting to hypothesize that CATS is involved in the same cell cycle control pathway regulated by KIS and that CATS might modulate or be modulated by KIS kinase activity. It would be very interesting to investigate whether CATS overexpression or down regulation would mimic the effect of KIS and whether it has an effect on $p27^{Kip}$.

In this work we have shown that CATS function in the nucleus as a repressor of transcription, thus we cannot rule out that through the CATS-KIS interaction KIS can also have an additional role in the nucleus of modulating the transcriptional activity of CATS.

Although KIS is a ubiquitous protein it displays a preferentially neural expression. (Bieche et al., 2003; Caldwell et al., 1999; Maucuer et al., 1995; Maucuer et al., 1997). Interestingly, KIS expression in the central nervous system (CNS) and dorsal ganglia of rat embryos has suggested a role for KIS in developing nervous system (Maucuer et al., 1997). Like KIS, CATS expression was detected enriched in the murine CNS suggesting a similar role for both proteins during embryogenesis. In the nervous system, a role for the cytoplasmic portion of KIS has also been described. Alam and coworkers have independently identified KIS as a PAM cytosolic interactor protein (they named KIS as P-CIP2) (Alam et al., 1996).

Phosphorylation of the cytosolic domain (CD) of PAM by KIS allows PAM to affect trafficking in the regulated secretory pathway it is involved (Alam et al., 1996; Alam et al., 2001; Caldwell et al., 1999). CATS is not expressed in the adult neural cells, however this does not rule out a role for CATS through CATS-KIS interaction in post-translational modification of membrane protein, affecting signaling and protein trafficking in lymphoid cells where CATS is expressed. We have shown that CATS interacts with CALM, another membrane protein involved in clathrin mediated endocytosis and trafficking. Moreover, we have proposed that CATS is a critical modulator of normal CALM function in lymphoid cells and that CATS could be very important for understanding the leukemic potential of CALM/AF10 fusion protein (Archangelo et al., 2006). The fact that the KIS kinase activity modulates trafficking of membrane protein links CATS function with post-translational modification and protein internalization/trafficking in CATS expressing cells supporting a role for CATS in normal CALM function.

All in all, we have shown strong evidences for the involvement of CATS in the 3 different nuclear as well as cytoplasmic cellular pathways modulated by KIS suggesting a close functional relationship between these proteins in all these cellular processes.

4.3.3 HAX1

We identified HAX1 as a CATS interacting partner in the yeast two-hybrid screen. This interaction was confirmed *in vivo* by cotransforming both the CATS bait and the HAX1 prey plasmids into yeast as well as by coimmunoprecipitation experiments in mammalian cells. Both HAX1 cDNA clones isolated in the yeast two-hybrid screen lacked sequences coding for the N-terminal portion of the protein (residues 1-132 or 1-153), indicating that the association between CATS and HAX1 is mediated through the C-terminal half of HAX1 (residues 153-279).

Although only a small proportion of the total cellular CATS protein was found in the cytoplasm, the fact that CATS was found to interact with two unrelated proteins that have a cytoplasmic localization and are known to function in the cytoplasm, such as CALM and HAX1, suggests a role for CATS in the cytoplasm.

HAX1 was first identified as an interacting partner of the hematopoietic specific protein 1 (HS1), a substrate of the Src family tyrosine kinase LYN which is part of the B-cell and T-cell receptor signaling cascade playing an important role in apoptotic and proliferative responses. HAX1 is a 35 kDa ubiquitously expressed protein that localizes to the mitochondria, endoplasmic reticulum and nuclear envelope and shows sequence similarity to the apoptosis

related protein NIP3 and BCL2 (Suzuki et al., 1997). To date, several other cellular and viral proteins have been described to interact with HAX1 including cortactin (the non-hematopoietic homolog of HS1), polycystic kidney disease protein PKD2, EBNA-LP, interleukin-1α (IL-1α), Kaposi's sarcoma-associated herpesvirus (KSHV) K15, IQGAP2, anti-apoptotic protein BCL2 and its Epstein-Barr virus homolog BHRF1, alpha-subunit of the G13 protein, HTRA2 (Omi) protease and the Human deficiency virus type 1 (HIV-1) viral protein R (Vpr). Thus HAX1 is implicated in modulating apoptosis and in actin cytoskeleton binding and motility functions (Cilenti et al., 2004; Dufva et al., 2001; Gallagher et al., 2000; Kawaguchi et al., 2000; Matsuda et al., 2003; Radhika et al., 2004; Sharp et al., 2002; Weissbach et al., 1998; Yedavalli et al., 2005; Yin et al., 2001). Among the different functions proposed for HAX1, its role in modulating apoptosis has been studied extensively. HAX1 was found in anti-apoptotic signaling counteracting the pro-apoptotic effects of BAX (Sharp et al., 2002) and down regulation of HAX1 was shown to induce apoptosis in HaCat cells (Mirmohammadsadegh et al., 2003). Recently, Cilenti et al. reported a mechanism of HAX1 regulation by the HTRA2 (Omi) serine protease. Evidence was presented that the anti-apoptotic function of HAX1 might be mediated through its ability to regulate the mitochondrial membrane potential and that the cleavage of HAX1 by HTRA2 in mitochondria would allow the depolarization of the mitochondrial membrane leading to the release of pro-apoptotic proteins to the cytoplasm. In such a way, HAX1 and its regulation by HTRA2 would play a central role in mammalian cell death (Cilenti et al., 2004).

The interaction between CATS and the anti-apoptotic protein HAX1 suggests a possible role for CATS in B-cell receptor mediated signaling leading to apoptotic and proliferative responses. Like *HS1*, *CATS* is also expressed specifically in lymphoid tissues and hematopoietic cells. Furthermore, expression of the CATS protein strongly correlates with the proliferative state of the cells. Thus it is tempting to speculate that CATS and HAX1 are part of the same cellular circuit and that the ability of HAX1 to promote cell survival might correlate with CATS expression in proliferating cells. The fact that CATS was found to interact with SIVA, another apoptosis-related protein, further supports the idea of CATS functioning in the control of apoptotic pathways.

Nevertheless it is also interesting to consider a function for the CATS-HAX1 interaction other than in regulating apoptosis. We showed that CATS interacts with the RNA-binding protein PCBP1, a protein involved in stabilization and translational control of mRNAs suggesting a role for CATS in this processes. Interestingly, Al-Maghrebi et al. have recently reported HAX1 as a new RNA-binding protein which can strongly and specifically bind to the 3'

untranslated region of the human vimetin mRNA suggesting a role for HAX1 in post-transcriptional control pathways (Al-Maghrebi et al., 2002). In summary, the interaction between CATS and HAX1 suggests a role for CATS in important regulatory pathways, but the biological significance of this interaction and the possible effect that one protein might have on the function of the other has to be further investigated.

4.3.4 SIVA

We identified the pro-apoptotic protein SIVA1 together with PCBP1 as the most frequent CATS interacting partner in the yeast two-hybrid screen. Interaction between CATS and SIVA1 was confirmed *in vivo* by cotransforming both the CATS bait and the SIVA1 prey plasmids into yeast as well as by coimmunoprecipitation experiments in mammalian cells.
SIVA1 is a pro-apoptotic protein which was initially identified using the cytoplasmic tail of CD27 (TNFRSF7), a member of the tumor necrosis factor receptor (TNFR) superfamily, as the bait in a yeast two-hybrid screen (Prasad et al., 1997). Since then a role for SIVA in various receptor (extrinsic) and nonreceptor (intrinsic) mediated apoptosis pathways has been described (Cao et al., 2001; Chu et al., 2005; Chu et al., 2004; Gudi et al., 2006; Henke et al., 2000; Henke et al., 2001; Lin & Ying, 1999; Padanilam et al., 1998; Py et al., 2004; Qin et al., 2002; Spinicelli et al., 2002; Xiao et al., 2000; Xue et al., 2002; Yoon et al., 1999). The full length main isoform of SIVA (SIVA1) is a 175 aa protein with a putative 20 aa amphipathic helical region (SAH) at the NH_2 terminus and cysteine zinc-finger towards the C-terminus. The the minor isoform (SIVA2) lacks exon 2 that codes for the SAH region and is much less pro-apoptotic (Chu et al., 2004; Prasad et al., 1997; Yoon et al., 1999). Recently, several reports have looked into the mechanism of SIVA-mediated apoptosis. SIVA1 was shown to be a downstream target for post-translational modification by the ABL2 (ARG) tyrosine kinase signaling in the apoptotic response to oxidative stress (Cao et al., 2001) and to be a direct transcriptional target of the tumor suppressor gene *TP53* and the transcription factor *E2F1* (Fortin et al., 2004). Prasad, and co-workers showed that SIVA1 binds to the anti-apoptotic members of the BCL2 family (Bcl2/Bcl-X_L) through the SAH domain and that this interaction abrogates the Bcl2/Bcl-X_L cell survival function (Chu et al., 2004; Xue et al., 2002). Moreover, the SIVA1-mediated apoptotic pathway results in the activation of both initiator and effector caspases, such as caspase-8, -9 and -3, it involves loss of mitochondrial integrity through the activation of BID and cytochrome *c* release and this activity can be modulate by overexpression of BCL2 or BCL2L1 (Bcl-X_L) (Py et al., 2004).

Additionally to its expression in normal tissues, several studies demonstrated SIVA overexpression in various pathologic circumstances, such as acute ischemic (Padanilam et al., 1998) or neuronal injury (Fortin et al., 2004), virus infection (Henke et al., 2000; Henke et al., 2001), and tumor cells exposed to genotoxic agents that induce a TP53 death response (Daoud et al., 2003; Qin et al., 2002). However, the main role of SIVA induced apoptosis has been characterized in response to TNFR signaling cascade in the immune system (Chu et al., 2004; Gudi et al., 2006; Prasad et al., 1997; Py et al., 2004; Spinicelli et al., 2002; Xue et al., 2002; Yoon et al., 1999). SIVA is the intracellular signaling molecule that associates with CD27 (TNFRSF7) and GITR (TNFRSF18), two TNFR transmembrane receptors expressed on lymphoid cells. CD27 is known to provide important co-stimulatory signals for T- and B-cell growth and B-cell Ig production and the GITR signal transduction pathway counteracts T cell apoptosis. Through interaction with SIVA1 these receptors are able to transduce their apoptotic response (Py et al., 2004; Spinicelli et al., 2002). SIVA association with these receptors and its participation in their receptor-mediated apoptosis pathways suggests an important role for SIVA in the regulation of T cell homeostasis. In line with these findings, is the fact that natural SIVA1/Bcl-X_L complexes are found in murine thymocytes and HUT78 cells. Moreover, Gudi et al. have very recently demonstrated a significant role for SIVA1 in TCR-mediated AICD (activation-induced cell death), through an inhibitory effect on NF-κB activity further underpinning to the role of SIVA1 in T-cell homeostasis (Gudi et al., 2006; Py et al., 2004; Xue et al., 2002).

The fact that CATS is specifically expressed in lymphoid tissues and interacts with SIVA, a pro-apoptotic protein involved in receptor-mediated apoptotic pathways and T-cell homeostasis, suggests a role for CATS in TNF receptor signaling and regulation of apoptotic response in lymphoid cells. However, we also showed that CATS is strongly expressed in proliferating cells and that CATS is likely to play a role in the regulation of cell proliferation. In line with these findings, we identified HAX1, PCBP1 and CSNK2 (involved in anti-apoptotic function, enhancement of translation of anti-apoptotic genes, and cell growth/proliferation and suppression of apoptosis, respectively) as CATS interacting proteins. Thus it is intriguing that CATS should be involved in a protein–protein interaction with a pro-apoptotic protein. Although the biological relevance to this interaction has to be further investigated it is tempting to speculate that through the interaction with SIVA CATS is able to sequester SIVA and modulate its pro-apoptotic function for example by inhibiting its association with the BCL2 members or by abrogating the inhibitory function of SIVA on NF-κB activation. Similar mechanisms have been reported for other apoptotic proteins, for

instance the apoptotic protein BIM is prevented from associating with BAX after phosporylation and thereby has a pro-apoptotic function (Harada et al., 2004). Whether CATS is able to modulate SIVA function just by forming complex or by posttranslational modification has to be investigated.

4.4 CATS can be placed in key pathways involved in tumorigenesis

In summary, CATS is a novel protein which was identified to interact with and influence the subcellular localization of CALM/AF10. However, the involvement of CATS in malignant transformation seems to go beyond its interaction with the leukemogenic fusion protein CALM/AF10. CATS is highly expressed in nearly all tumor cell lines examined and its nucleolar localization connects CATS function with proliferation and possibly to TP53 regulation. Moreover, evidence presented in this work suggests an involvement of CATS in signaling, apoptosis and cell cycle control. Thus, CATS function might be tightly linked to cancer initiation and/or progression. It will be very interesting to investigate to which extent CATS contributes to or determines cell proliferation and what effect its depletion might have on tumor cells.

5 Summary

The Clathrin Assembly Lymphoid Myeloid leukemia gene (*CALM*) was first identified as the fusion partner of *AF10* in the t(10;11)(p13;q14) translocation. The CALM/AF10 fusion protein plays a crucial role in t(10;11)(p13;q14) associated leukemogenesis. Using the N-terminal half of CALM as a bait in a yeast two-hybrid screen a novel protein named CATS (CALM interacting protein expressed in thymus and spleen) was identified as CALM interacting partner. Multiple tissue Northern blot analysis showed predominant expression of *CATS* in lymphoid tissues. *CATS* codes for two protein isoforms of 238 and 248 amino acids. The interaction between CALM and CATS was confirmed by co-immunoprecipitation and colocalization experiments. The CATS interaction domain of CALM was mapped to amino acids 221 to 294 of CALM. This domain is contained in the CALM/AF10 fusion protein. CATS localizes to the nucleus and shows a preference for nucleoli. Expression of CATS was able to markedly increase the nuclear localization of CALM and of the leukemogenic fusion protein CALM/AF10. This effect of CATS seems to be stronger on CALM/AF10 than on CALM. Several monoclonal antibodies against the C-terminus of human CATS were generated. These antibodies recognize both the human and the murine CATS protein. Western blot analyses showed that CATS is strongly expressed in different human leukemia, lymphoma and tumor cell lines but not in resting T-cells. High CATS expression in proliferating cells as well as its nucleolar localization suggest a role of CATS in the control of cell proliferation.

In order to gain further insight into CATS function we used CATS as a bait in a yeast two-hybrid screen. Several CATS interacting proteins with apparently unrelated function were identified. Interestingly, on closer scrutiny these proteins could be associated with three key regulatory pathways: signaling, apoptosis and cell cycle control. We discuss in detail the biological relevance of the CATS interaction with the two apoptosis-associated proteins HAX1 and SIVA, the cell cycle regulator KIS and the CALM interacting ribonucleoprotein PCBP1.

Our results indicate that the subcellular localization of CALM and CALM/AF10 could depend in part on the presence of CATS with a greater fraction of CALM or CALM/AF10 being present in the nucleus of cells with high CATS expression (e.g. lymphoid cells have high CATS expression). Moreover we provide evidences that CATS function might be tightly linked to cancer initiation and/or progression. The CALM-CATS interaction might thus play an important role in CALM/AF10 mediated leukemogenesis.

6 Zusammenfassung

Das Clathrin Assembly Lymphoid Myeloid leukemia gene (*CALM*) wurde zuerst als Fusionspartner von AF10 bei der t(10;11)(p13;q14) Translokation identifiziert. Das CALM/AF10 Fusionsprotein spielt eine entscheidende Rolle bei der t(10;11)(p13;q14) assoziierten Leukämieentstehung. Mit der N-terminalen Hälfte von CALM als Köder in einem Yeast-Two-Hybrid-Screen, wurde ein neues Protein namens CATS (CALM interacting protein expressed in thymus and spleen) als CALM Interaktionspartner indentifiziert. Multiple-Tissue-Northern-Blot-Analysen zeigten, dass *CATS* überwiegend in lymphatischen Geweben exprimiert wird. Das *CATS*-Gen kodiert für zwei Proteinisoformen, die aus 238 bzw. 248 Aminosäuren bestehen. Die Interaktion zwischen CALM und CATS wurde durch Koimmunoprezipitations- und Kolokalisationsexperimente bestätigt. Die CATS-Interaktiosdomäne von CALM konnte den Aminosäuren 221 bis 294 von CALM zugeordnet werden. Diese Domäne ist auch Bestandteil des CALM/AF10-Fusionsproteins. Das CATS-Protein ist im Zellkern, überwiegend in den Nucleoli, lokalisiert. Die Überexpression von CATS führte zu einer deutlichen Anreicherung von CALM und dem leukämischen Fusionsprotein CALM/AF10 im Zellkern. Dieser Effekt von CATS ist stärker bei CALM/AF10 als bei CALM. Es wurden mehrere monoklonale Antikörper gegen den C-terminus von CATS hergestellt. Diese Antikörper erkennen spezifisch sowohl das humane als auch das murine CATS-Protein. Western-Blot Analysen zeigten, dass CATS in verschiedenen Leukämie-, Lymphom- und Tumorzelllinien, allerdings nicht in ruhenden T-Zellen exprimiert wird. Die hohe Expression von CATS in proliferierenden Zellen sowie seine nucleoläre Lokalization sprechen für eine regulierende Rolle von CATS bei der Zellproliferation.

Um weitere Einblicke in die Funktion von CATS zu erlangen, haben wir CATS als Köder in einem Yeast-Two-Hybrid-Screen verwendet. Dabei haben wir mehrere interagierende Proteine mit unterschiedlichen Funktionen gefunden. Bei genauerer Betrachtung dieser Proteine, konnten wir sie nach ihren Funktionen in drei Gruppen einteilen, nämlich Signaltransduktion, Apotose und Regulation des Zellzyklus. Die Interaktionen zwischen CATS und den Apoptose-assoziierten Proteinen HAX1 und SIVA, dem Zellzyklusregulator KIS sowie dem Ribonucleoprotein PCBP1 werden auführlich diskutiert.

Unser Ergebnisse zeigen, dass die zelluläre Lokalisation von CALM und CALM/AF10 teilweise von der Anwesenheit von CATS abhängt, da größere Mengen CALM oder CALM/AF10 im Zellkern von Zellen mit hoher CATS-Expression (z.B. haben lymphatische Zellen eine hohe CATS-Expression) vorhanden sind. Darüber hinaus liefern wir Anhaltspunkte dafür, dass die Funktion von CATS mit der Entstehung bzw. dem

Fortschreiten von Krebserkrankungen eng zusammenhängen könnte. Die CALM-CATS Interaktion könnte also eine wichtige Rolle bei der CALM/AF10 vermittelten Leukämieentstehung spielen.

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Publications:

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