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The four and a half LIM domain protein 2 (FHL2) interacts with CALM and is highly expressed in acute myeloid leukemia (AML) with complex aberrant karyotypes

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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2008

Aus der Medizinischen Klinik und Poliklinik III am Klinikum Großhadern

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der Ludwig-Maximilians-Universität München,
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Das Four and a half LIM Domänen Protein 2 (FHL2) interagiert mit CALM und ist hoch experimiert in akuten myeloischen Leukämien (AML) mit komplex aberranten Karyotypen.

Dissertation zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der Ludwig-Maximilians-
Universität zu München, Deutschland

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2008

Mit Genehmigung der Medizinischen Fakultät der Universität München

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Dekan:	Prof. Dr. med. Dr. h.c. M. Reiser, FACR
Tag der mündlichen Prüfung:	16.12.2008

To my family

Acknowledgments

I would like to thank,

Prof. Stefan Bohlander for giving me the opportunity to work in this completely new field for me. I would like to express my deep gratitude for his support and understanding that were essential to complete this work.

all the colleagues from the CCG leukemia, the AG Bohlander members Belay Tizazu, Alexandre Krause, Deepak Bararia, Purvi Kakadia, Luciana Fontanari-Krause, Leticia Fröhlich Archangelo, especially Philipp Greif and Medhanie Mulaw for helpful and fruitful discussions as well as Marc Weinkauf from Dr. Dreylings group for the hematopoietic cell lines

my colleague, my close friend and neighbour Pawandeep Kaur sharing many “tea-evenings“ with discussions, laughs and “tears”

my close friends Zoofa Shahidi, Dr. Soledad Funes as well as my neighbours Selma and Sead Granulo

my boyfriend Ensar Muratović for always finding a way to give me new energy and make me laugh

my sister Majida and my brother Senad that always find a way to encourage me

my parents Asima and Salih for the constant encouragement, the unconditional support, their conviction and belief in me

Abbreviations

aa	amino acids
Ab	antibody
AD	activation domain
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
Amp	ampicillin
AML-comp	AML with complex aberrant karyotype
AML_nk	AML with normal karyotype
ANTH domain	AP180 N-terminal homology domain
AP-2	clathrin adaptor protein complex 2
APS	ammonium persulfate
Aqua dest.	water, deionized
BD	binding domain
bp	base pairs
BSA	bovine serum albumine
cDNA	complementary DNA, synthetic DNA transcribed from a specific RNA through the action of the reverse transcriptase
cfu	colony forming unit
CME	clathrin mediated endocytosis
CML	chronic myelogenous leukemia
CoIP	co-immunoprecipitation
Conc	concentration
DAPI	4',6-diamidino-2-phenylindole
dCTP	Deoxycytosine triphosphate
ddH2O	double distilled water
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
dGTP	Deoxyguanosine triphosphate
DMF	Dimethylformamide
DNA	deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DO	drop out
DTT	dithiothreitol
dTTP	Deoxythymidine triphosphate
ECFP	Enhanced Cyan Fluorescent Protein
EDTA	ethylenediaminetetraacetic acid
e.g.	example
EGFP	Enhanced Green Fluorescent Protein
EST	expressed sequence tags
EtBr	Ethidium-bromide
EtOH	ethanol

EYFP	Enhanced yellow fluorescent protein
FAB	French-American-British classification system for acute leukemia
FBS	Fetal bovine serum
FCS	Fetal calf serum
g	gram
g	relative centrifugal acceleration
GAL	Galactose
GAL4	yeast transcription factor
GAL4-AD	GAL4 transcriptional activation domain
GAL4-DBD	GAL4 DNA binding domain
GAL4-UAS	GAL4 upstream activating sequence
Gly	Glycine
GFP	green fluorescent protein
GST	glutathione S transferase
HEPES	N-(2-Hydroxythyl) piperazine-N'-2-ethan sulphuric acid
His	Histidine
HRP	horse radish peroxidase
hr(s)	hour(s)
HSC	Hematopoietic stem cell
IPTG	isopropyl β-D thiogalactoside
KAc	Potassium acetate
kb	kilobase
KCl	Potassium chloride
kDa	kilodalton
KH	hnRNP K homology domain
KH_2PO_4	Potassium dihydrogenphosphate
l	liter
LacZ	*E. coli* gene encoding beta-galactosidase
LB	Luria Bertani bacterial medium
Leu = L	Leucine
LiAc	lithium acetate
LIM	Lin11, Isl-1 and Mec-3 domain
Lys = K	Lycine
M	Molar
m	milli (1×10^{-3})
MCS	multiple cloning site
Met = M	Methionine
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	minute(s)
ml	milliliter
MLL	Myeloid/lymphoid or mixed-lineage leukemia
mM	Milli Molar

MOPS	3-N-morpholino-propanesulfonic acid
mRNA	messenger RNA
MW	Molecular weight
n	Nano (1 x 10^{-9})
NaAC	sodium acetate
NaOH	sodium hydroxide
NaH_2PO_4	sodium dihydrogenphosphate
Na_2HPO_4	disodium hydrogenphosphate
$(NH_4)_2SO_4$	ammonium sulfate
NLS	nuclear localization signal
nt	nucleotide
O/N	overnight
ºC	degree Celsius
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEG	polyethylenglycol
Pen/Strep	Penicillin/Streptomycin
PFA	paraformaldehyde
pg	pico gram
PHD	plant homeodomain
Phe = F	Phenylalanine
PMSF	Phenylmethylsulfonyl fluoride
PtdIns(4,5)P_2	phosphatidylinositol-4,5-bisphosphate
RNA	ribonucleic acid
RNAse	ribonuclease
Rpm	revolutions Per Minute
RPMI	Roswell Park Memorial Institute culture medium
RT	room temperature
SD	synthetic defined Dropout medium
SDS	sodium dodecylsulfate
sec	second(s)
Taq	Thermus aquaticus (-Polymerase)
TBS	tris buffered saline
TE	tris-EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylendiamine
Thr = T	Threonine
Tris	trishydroxymethylaminomethane
Trp = W	Tryptophan
tot.	total
Tm	melting temperature
Tyr = Y	Tyrosine

U	unit
UTR	untranslated region
UV	ultraviolet
V	volts
Val = V	Valine
vector	plasmid or phage chromosome used to carry cloned DNA segment
vol.	volume
v/v	volume per volume ratio
w/v	weight per volume ratio
WB	Western blot
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})
µl	micro liter
µm	micro meter
µM	micro molar

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1 Introduction

1.1 Hematopoiesis

1.1.1 Hematopoietic stem cell

Our body generates billions of blood cells every day. Hematopoiesis, the formation of red and white blood cells, begins already in the embryonic yolk sac. During the development of the human fetus, hematopoietic stem cells (HSC) migrate from the yolk sac to the fetal liver and thereafter to the spleen. After the 7th month of gestation the differentiation of HSCs in the bone marrow becomes the major location of hematopoiesis. HSCs consists of two subpopulations -one with short-term repopulation ability (<10 weeks) and the second population with a long-term repopulating ability that lasts the lifetime of an organism (Morrison S. and Weissman I., 1994). There are two standards for defining a cell as a normal stem cell: it must have the ability (1) to self-renew and (2) to differentiate into the various blood cells (Till J.E. and McCullough E.A., 1961; Weissman I. L., 2000). Early in hematopoiesis the HSC differentiates either into a lymphoid progenitor cell or into a myeloid progenitor cell. Progenitor cells are cells that have lost the capacity to self-renew and are destined to a particular cell lineage. The lymphoid progenitor gives rise to B-, T- and natural killer (NK) cells, while the myeloid progenitor give rise to red blood cells (erythrocytes), white blood cells (neutrophils, eosinophils, basophils, monocytes, mast cells) and megakaryocytes. In figure 1.1 a model of the differentiation pathways of the hematopoietic system is shown.

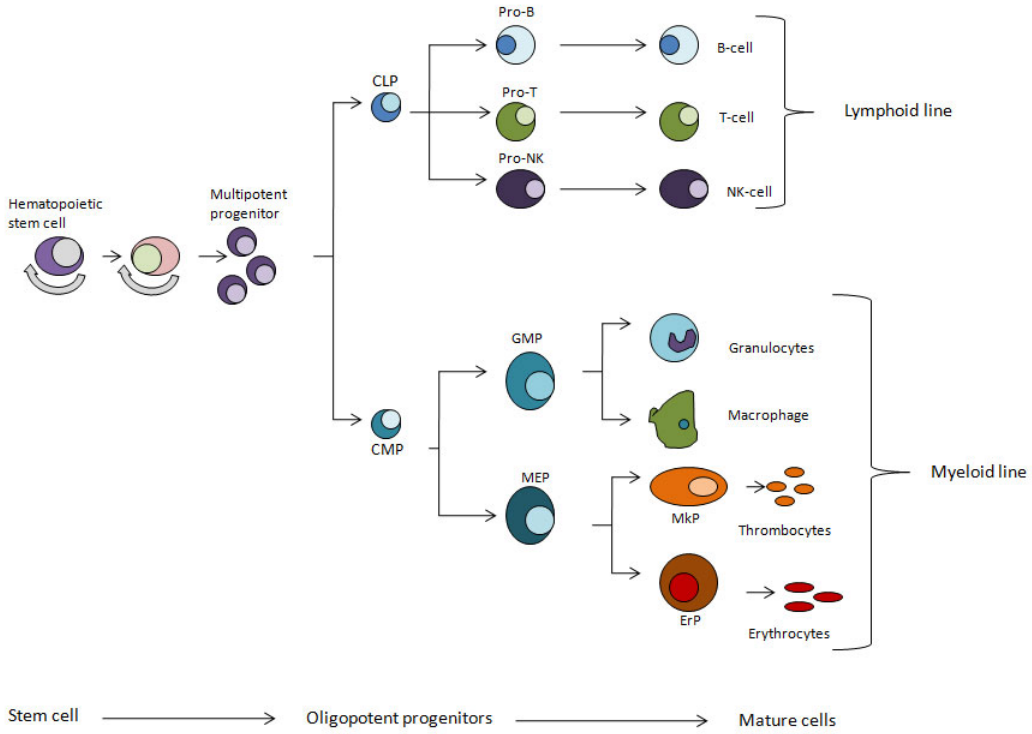


Figure 1.1 The hematopoietic system. The HSC differentiates into the common lymphoid progenitor (CLP) or into the common myeloid progenitor (CMP). The CMP differentiates in turn into red blood cells, white blood cells and megakaryocytes. The CLP differentiates into B-, T- and NK cells.

The physiological function of hematopoietic stem cells is to replace blood cells that were lost in illness or trauma, and in normal cell turnover. Under normal conditions stem cells can be in a quiescent state for a longer time period, however once they exit this state they start to self renew or differentiate to generate needed progenies (McCulloch E. and Till J.E., 2005; Moore M. et al., 2006; Weissman I. L., 2000).

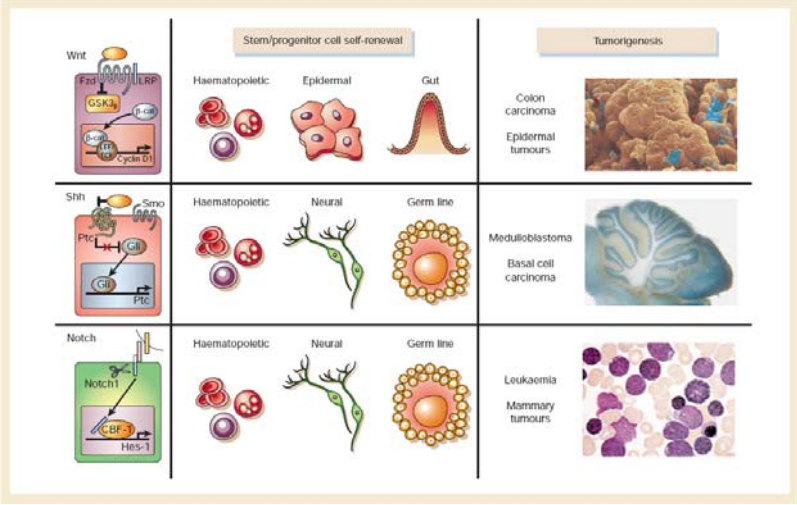
Stem cell niche

It is an evolutionarily conserved phenomenon that stem cells interact with their micro environment- the so called stem cell niche, to establish and maintain their properties (Fuchs E. et al., 2004). To be considered a stem cell niche, the environment has to enable the stem cells to reproduce or self renew (Scadden D., 2006). Recently it has been shown that osteoblasts, the bone-forming cells, are essential regulatory components of the HSC niche in the bone marrow compartment that influence HSC function through Notch

signaling (Calvi L., 2006; Stier S. et al., 2005). Once the stem cell compartment is formed in a tissue the stem cells often go into a quiescent state. This is for example the case when HSC that express the tyrosine kinase receptor Tie2 adhere to osteoblasts in the bone marrow niche. The interaction of Tie2 and its ligand angiopoietin-1 (Ang-1) results in HSCs tightly adhering to the stromal cells, which in turn leads to the maintenance of their long term repopulating activity of HSC (Suda T. et al., 2005). As the HSC progressively mature they lose contact with the stromal cells and start to proliferate. They migrate towards the central bone marrow cavity where they differentiate and proliferate and finally as mature blood cells they enter the blood vessels.

Self renewal and leukemogenesis

There is increasing evidence that the *Notch, Hedgehog* and *Wnt* pathways, that control many developmental processes, also regulate the self-renewal of hematopoietic progenitors and stem cells. These pathways have also been shown to be deregulated in cancers (Bhardwaj G. et al., 2001; Reya T. et al., 2001; Varnum-Finney B. et al., 2000) as shown in figure1.2.



Figur 1.2 Signaling pathways regulating self renewal and stem cells. *Notch, Hedgehog* and *Wnt* pathways regulate developmental processes, self-renewal of hematopoietic progenitors and stem cells, and have been shown to be involved in cancers when deregulated (Picture from Reya, 2001).

In the case of Wnt signaling, the Wnt proteins are produced by HSCs themselves but also by the surrounding microenvironment (Rattis F.M. et al., 2004), implicating that Wnt can modulate HSC indirectly by influencing the microenvironment. Since normal stem cell and cancer cell share the ability to self renew (Al-Hajj M. and Clarke MF., 2004; Bonnet D. and Dick J., 1997) it is reasonable to assume that newly arising cancer cell use the machinery for self-renewing cell division that is used by stem cells for their own ends. There are two reasons to believe that stem cells themselves are the target of transformation: (1) stem cells already have self-renewal capacity and (2) self-renewing-stem cells often persist for long periods of time, instead of dying after short periods of time like many mature cells in highly proliferative tissues. This means that stem cells have the opportunity and time to accumulate the mutations that are required for malignant transformation.

1.2 Chromosomal translocation

Nowell and Hungerford discovered in 1960s a small acrocentric chromosome that was very often observed in association with chronic myeloid leukemia (CML) (Nowell and Hungerford, 1960). It was named the Philadelphia chromosome and was one of the first chromosomal abnormalities to be associated with a human cancer. The nature of this abnormality was unclear but it was assumed that a loss of chromosomal material from one of the small G-group chromosomes had occurred and that this loss was somehow associated with the development of leukemia. Following this discovery, recurring chromosomal abnormalities and the discovery that the Philadelphia chromosome in CML was the result of a balanced chromosomal translocation (Rowley J.D., 1973) led to an enormous increase in our understanding of the genetic changes that occur in leukemic cells. At the cytogenetic level most chromosome translocations in leukemia arise in hematopoietic stem cells and are reciprocal, stable and balanced (Zhang Y. and Rowley J., 2005). In balanced chromosomal translocations, genetic material between two non-homologues chromosomes is exchanged (fig 1.3).

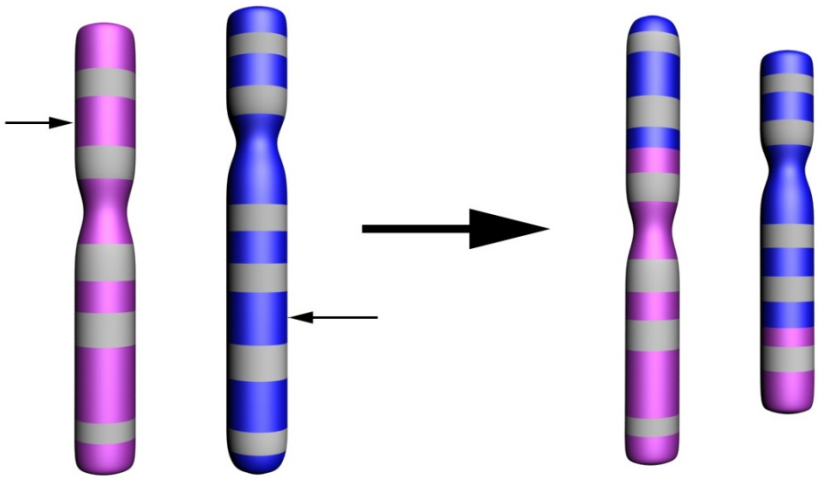


Figure 1.3 Balanced chromosomal translocation. The small arrows indicate the breakpoints.

Such balanced translocations can have either of two consequences: (1) the juxtapposition of a coding region from one gene with a promoter of another gene leading to deregulated gene expression or (2) the fusion of two coding regions which creates a new chimaeric gene that encodes for a fusion protein (Rowley J.D., 1999; Rowley J.D., 2000; Rowley J.D., 2001). A classic example of the first consequence is the first molecularly characterized translocation t(8;14) that causes Burkitt's lymphoma (Zech L. et al., 1976). The translocation juxtapposes the immunoglobulin heavy chain (IGH) promoter region to the MYC coding sequence resulting in abnormal expression of the MYC oncogene as seen in figure 1.4.

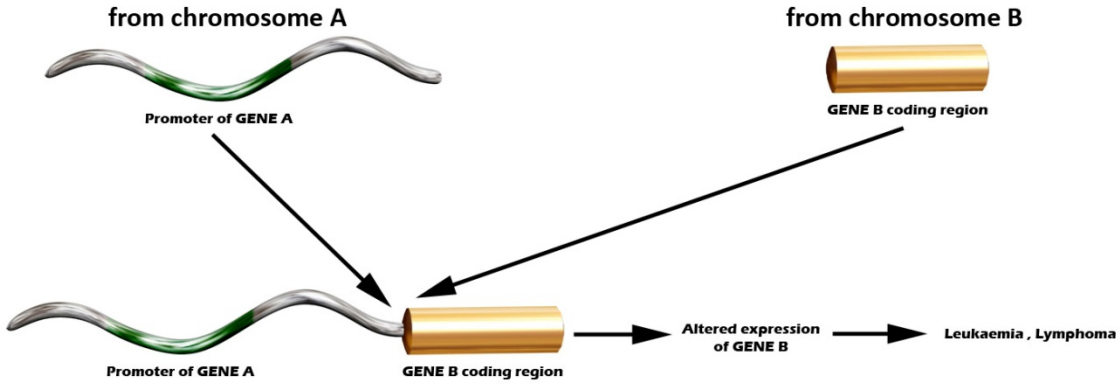


Figure 1.4 Malignant transformation-1. The juxtaposition of a coding region from one gene with a promoter of another gene inducing altered expression of Gene B. (Adapted of Rowley, 2001)

The examples for the second consequence is the t(9;22)(q34;q11) translocation which leads to a fusion between the breakpoint cluster region (BCR) gene and the Abelson tyrosine kinase (ABL) gene resulting in the production of the BCR/ABL fusion protein, figure 1.5.

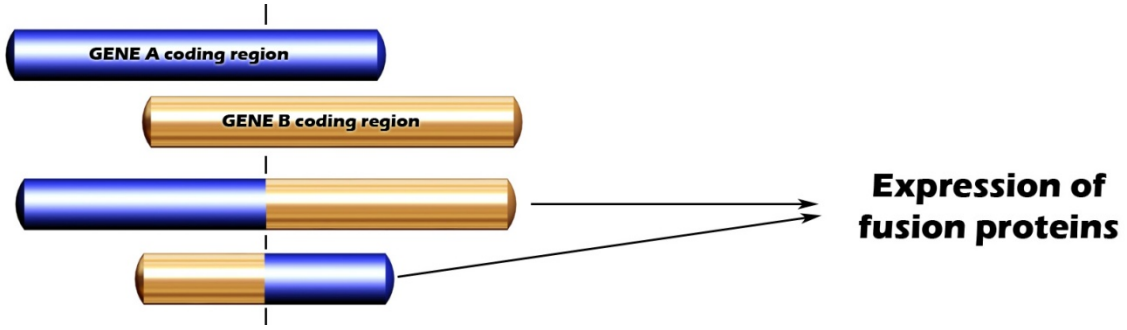


Figure 1.5 Malignant transformation-2. Fusion of two coding regions that create a new chimaeric gene that encodes a new fusion protein. (Picture adapted from Rowley, 2001)

The association of particular chromosomal translocations with specific subtypes of leukemia and lymphomas led to a strong research activity in this field and today leukemia and lymphoma are the most extensively characterized human malignant diseases. As a result of this activity a network of different fusion genes has emerged showing that some genes are involved in more than one translocation forming fusion genes with several partner genes. The most prominent genes involved in more than one translocation in this network are the *RUNX1* (AML1), *ETV6* and *MLL* genes, which have been shown to play central roles in the pathogenesis of different leukemias (Bohlander S.K. et al., 2000).

1.3 The CALM/AF10 fusion gene

1.3.1 CALM, AF10 and the CALM/AF10 fusion

The t(10;11)(p13;q14) translocation was first characterized at the molecular level in the human monocytic cell line U937 (Dreyling M. et al., 1996). This balanced translocation results in a fusion of CALM on chromosome 11, band q14, to AF10 on chromosome 10 band p13 as shown in figure 1.6.

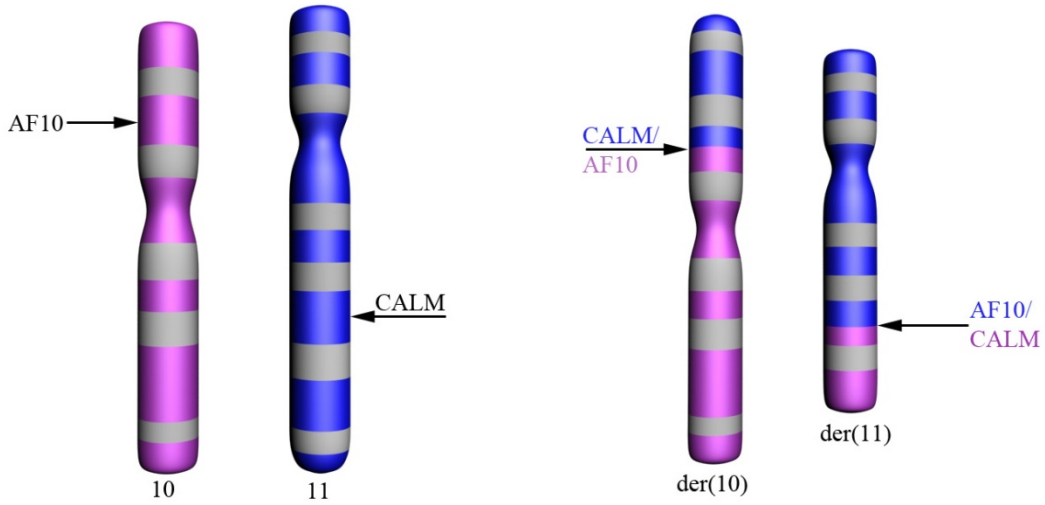


Figure 1.6 The CALM/AF10 and AF10/CALM fusion genes. The distal part of the short arm of chromosome 10 is fused to the proximal long arm of chromosome 11 resulting in the formation of an AF10/CALM fusion gene at the breakpoint. The distal part of the long arm of chromosome 11 is fused to the proximal part of the short arm chromosome 10 resulting in the formation of the CALM/AF10 fusion gene.

The breakpoint in the CALM gene is located at the C-terminus of the gene and the breakpoint in AF10 is located near the N-terminus of AF10. The CALM/AF10 fusion contains almost the complete open reading frames of both the CALM and AF10 genes (Dreyling M. et al., 1996), whereas the AF10/CALM fusion only encodes for a truncated AF10 protein consisting of 84 amino acids as seen in figure 1.7.

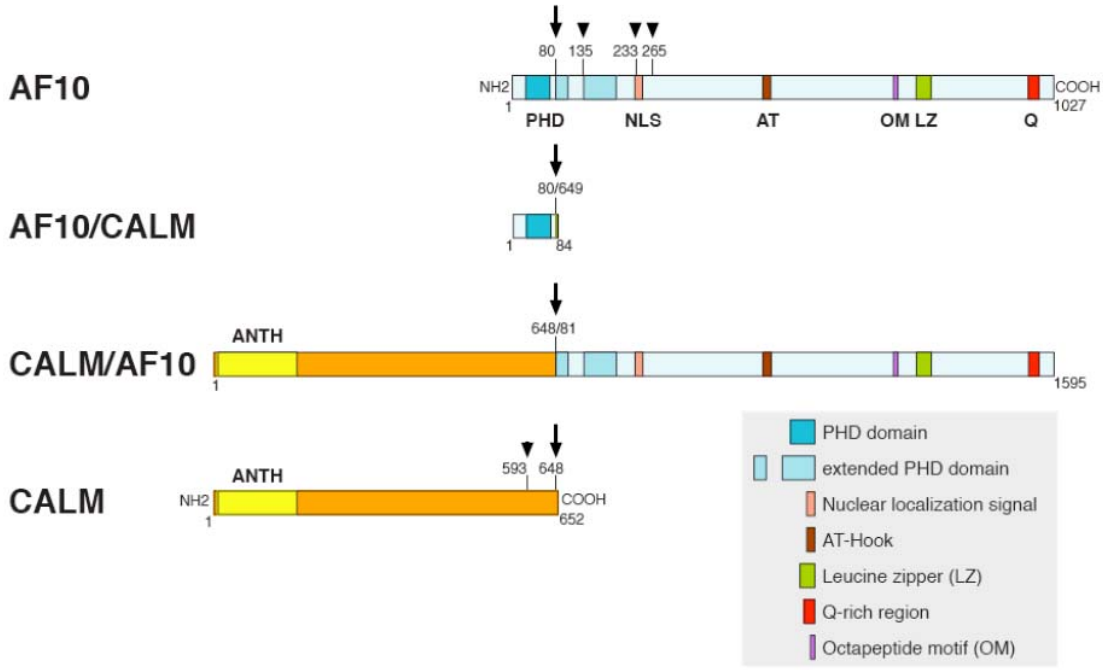


Figure 1.7 Scheme of AF10 and CALM proteins, respectively AF10/CALM and CALM/AF10 translocations. Breakpoints in the protein in U937 cell line are indicated by arrows, and breakpoints found in cells from patients are indicated by triangles. It can be seen that CALM/AF10 translocation contains almost the whole AF10 and CALM gene while AF10/CALM translocation contains the small leftover parts. 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.

Due to the location of the breakpoints in CALM and AF10 the reciprocal fusion mRNAs encode fusion proteins of unequal size. Analysis of the breakpoint region in t(10;11)(p13;q14) leukemia showed three different breakpoints in CALM and four breakpoints in the AF10 gene with no apparent correlation between the location of the breakpoints and the phenotype or outcome of disease (Bohlander S.K. et al., 2000; Kumon K. et al., 1999). The CALM/AF10 fusion transcript has mainly been identified in T-cell acute lymphoblastic leukemia (ALL), undifferentiated acute myeloid leukemia (AML) (FAB M0 or M1) and in malignant lymphoma and has a poor prognosis (Dreyling M. et al., 1998; Kumon K. et al., 1999; Narita M. et al., 1999). Later CALM/AF10 translocations were also observed in more differentiated AML subtypes (FAB M4, M5

and M7) (Abdou S.M. et al., 2002; Carlson K. et al., 2000; Jones L.K. et al., 2001; Nakamura F. et al., 2003; Salmon-Nguyen F. et al., 2000).

Most t(10;11)(p13;q14) translocations generate CALM/AF10 as well as the AF10/CALM fusion transcripts. However, AF10/CALM transcripts are not detected in all leukemia samples indicating that it is probably the CALM/AF10 fusion transcript that directs the transformation process (Carlson K. et al., 2000). Desphande *et al*, showed that the expression of the CALM/AF10 fusion protein is sufficient to cause an aggressive biphenotypic leukemia in a murine bone marrow transplantation model (Deshpande A. et al., 2006).

The *AF10* gene (ALL 1 fused gene from chromosome 10), was first identified as a fusion partner of *MLL* in 3 AML patients with a t(10;11)(p12;q23) translocation (Chaplin T. et al., 1995). In 1996, Dreyling *et al* described the second translocation involving AF10- the CALM/AF10 translocation. The AF10 gene is located on chromosome 10 band p12 and encodes a 1,027 amino acids long protein. The *AF10* gene encodes a putative transcription factor containing N-terminally two LAP/PHD (Leukemia Associated Protein/Plant Homeo Domain) zinc finger motifs, three extended LAP/PHD fingers, a nuclear localization signal (NLS), an AT-hook domain, an octapeptide motif (OM), a leucine zipper (LZ) motif and a glutamic acid rich region (Q rich region). The *Drosophila* homologue of *AF10, alhambra,* has been suggested to play a role in heterochromatin-mediated transcriptional silencing (Debernardi S. et al., 2002; Linder B. et al., 2001; Linder B. et al., 2000; Perrin L. et al., 2003; Perrin L. and Dura J.M., 2004; Saha V. et al., 1995).

CALM was first identified as the fusion partner of AF10 in the human monocytic cell line U937 that harbors the t(10;11)(p13;q14) translocation (Dreyling M. et al., 1996). The clathrin assembly lymphoid myeloid leukemia protein (CALM) is the ubiquitously expressed homologue of the neuron-specific protein AP180 (Tebar F. et al., 1999). *CALM* is located on chromosome 11 band q14 and encodes a 652 amino acid long protein. The homologous proteins of CALM: LAP (in *Drosohophila melanogaster*) and Unc11 (in *Caenorhabditis elegans*) are both implicated in clathrin-mediated endocytosis (CME). Mutations of LAP and Unc11 suggest that these proteins play a role in the regulation of

endocytic vesicle size (Kalthoff C. et al., 2002; Meyerholz A. et al., 2005; Nonet M. et al., 1999). It has also been shown that CALM plays a role in clathrin mediated endocytosis by promoting the assembly of clathrin into clathrin cages and taking part in the initial stage of coated pit formation and invagination together with clathrin, AP-2 and PtdIns(4,5)P_2 containing membranes (Ford M. et al., 2001; Kim H.L. and Kim J.A., 2000; Kim J.A. and Kim H.L., 2001; Meyerholz A. et al., 2005; Tebar F. et al., 1999). This process is probably mediated by binding of phosphoinositides through the CALM Epsin N-terminal homology (ENTH) domain and binding of clathrin through the CALM C-terminal region. The CALM- clathrin interaction has been shown, *in vitro,* to promote the assembly of clathrin triskelia into clathrin cages (Ford M. et al., 2001; Kim H.L. and Kim J.A., 2000). Both overexpression and down regulation of CALM have been shown to inhibit CME and impair the trafficking of receptors between the trans golgi network and endosomes (Meyerholz A. et al., 2005; Tebar F. et al., 1999). Point mutations in the mouse homologue *Picalm* were shown to cause abnormalities in hematopoiesis, iron metabolism and bone growth (Klebig M. et al., 2003).

However, many aspects of the function of CALM or the leukemogenic CALM/AF10 fusion protein are unknown. We chose to learn more about the function of CALM by searching for CALM interacting proteins with a yeast two hybrid screen. One of the interesting proteins discovered in our screen was the FHL2 protein (four and a half LIM domain protein 2).

2 Material and Methods

2.1 Material

2.1.1 Reagents

1-kb-DNA Ladder	Invitrogen, Karlsruhe, Germany
β-Mercaptoethanol	Sigma, Taufkirchen, Germany
Acetic acid	Merck, Darmstad, Germany
Acrylamid Rotiphorese® Gel 30 (37, 5:1)	Carl Roth, Karlsruhe, Germany
Agar	Carl Roth, Karlsruhe, Germany
Agarose	ICN Biomedicals Inc.
Amino acids (yeast two-hybrid)	Sigma, Taufkirchen, Germany
Ammonium persulfate (APS)	Sigma, Taufkirchen, Germany
Ampicillin Na-Salt	Pan Biotech, Aidenbach, Germany
BigDye™ terminator mix	PE Applied Biosystems, Foster City, CA
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
Coomassie® stain solution	BioRad, Hercules, CA
D(+)-Glucose-Monohydrate	Merck, Darmstadt, Germany
Dakocytomation Mounting Medium	DakoCytomation, Hamburg, Germany
DAPI- 4',6-diamidino-2-phenylindole	Sigma, Taufkirchen, Germany
d_2H_2O	Millipore, Eschborn, Germany
Deoxyribonuclease I, Amplification Grade	Invitrogen, 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
Dulbecco´s Modified Eagle medium (DMEM),	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
Formaldehyde 37%	Carl Roth, Karlsruhe, Germany
Formamide	Carl Roth, Karlsruhe, Germany
Glutathione-Agarose	Sigma, Taufkirchen, Germany
Glycerin 87%	neoLab, Heidelberg, Germany
Glycerol	Carl Roth, Karlsruhe, Germany
Glycine	Merck, Darmstadt, 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
Kanamycin	Pan Biotech, Aidenbach, Germany
Klenow Fragment	MBI Fermentas, St. Leon-Rot, Germany
L-Glutamine	Pan Biotech, Aidenbach, Germany
Lithium acetate	Sigma, Taufkirchen, Germany
Magnesium	Sigma, Taufkirchen, Germany
Methanol	Merck, Darmstadt, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
MOPS (3-(N-Morpholino)-propanesulfonic acid)	Carl Roth, Karlsruhe, Germany
NP40 (Nonidet P-40)	Roche, Mannheim, Germany
Oligo (dT)12-18 Primer	Invitrogen, Karlsruhe, Germany
PanScript DNA Polymerase	Pan Biotech, Aidenbach, Germany
Paraformaldehyde	Electron Microscopy Sciences, Hatfield, USA
Penicillin/streptomycin	Pan Biotech, Aidenbach, Germany
Pepstatin	Sigma, Taufkirchen, Germany
Peptone, meat pancreatic digested	Merck, Darmstadt, 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
Potassium acetate (KAc)	Calbiochem, San Diego, USA
Potassium chloride (KCl)	Calbiochem, San Diego, USA
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
Roswell Park Memorial Institute culture Medium (RPMI 1640)	Pan Biotech, Aidenbach, Germany
Roti®-Fect transfection reagent	Carl Roth, Karlsruhe, Germany
SDS- Sodium dodecyl sulfate	Carl Roth, Karlsruhe, Germany
SeeBlue® Plus2 pre-stained standard	Invitrogen, Karlsruhe, Germany
Shrimp Alkaline Phosphatase (SAP)l	MBI Fermentas, St. Leon-Rot, Germany
Sodium acetate, Anhydrous (NaAC)	Calbiochem, San Diego, USA
Sodium chloride (NaCl)	Merck, Darmstadt, 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 (Na_2HPO_4)	Calbiochem, San Diego, USA
Sucrose	Sigma, Taufkirchen, Germany
Sybr Green	Roche, Mannheim, Germany

SuperScript™ II RNase H- Reverse Transcriptase	Invitrogen, Karlsruhe, Germany
T4 DNA Ligase	NewEngland Biolabs, Schwalbach, Germany
Trichloroacetic acid (TCA)	Carl Roth, Karlsruhe, Germany
Tris- trishydroxymethylaminomethane	Carl Roth, Karlsruhe, Germany
Triton X-100	Carl Roth, Karlsruhe, 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 amino acids	Difco, Detroit MI, USA

2.1.2 Material and Kits

Blotting paper GB003 and GB002	Schleicher & Schuell, Dassel, 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
Filter 0.22µm	Millipore, Belford, USA
Genomed Plasmid Mega Kit	Genomed, St. Louis, USA
Glass beads	Sigma, Taufkirchen, Germany
Glassware	Schott, Jena, Germany
Hybond-N nylon membranes	Amersham, Freiburg, Germany
Hybond™ ECL™ nitrocellulose membrane	Amersham, Freiburg, Germany
Hypercassete™ 18x24 cm	Amersham, Freiburg, Germany
Hyperfilm ECL high performance	Amersham, Freiburg, Germany
JETSTAR MEGA Cartridge	Genomed, Bad Oeynhausen, Germany
Luminometer tube (Disposable Cuvettes 12 mm x 50 mm)	Promega, Mannheim, Germany
Microcentrifuge tubes (1.5 ml)	Eppendorf, Hamburg, Germany
Microscope slides	Menzel-Gläser®, Braunschweig, 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
Qiagen Gelextraction Kit	Qiagen, Hilden, Germany
Qiagen RNEasy Mini Kit	Qiagen, Hilden, Germany
Qiagen Midi Kit	Qiagen, Hilden, Germany
Qiagen EndoFree Maxi Kit	Qiagen, Hilden, Germany
QIAquick columns	Qiagen, Hilden, Germany
Qiashredder™ columns	Qiagen, Hilden, Germany
RNeasy™ mini kit	Qiagen, Hilden, Germany
Surgical blades	Feather Safety Razor Co. Med. Div., Japan

Syringe	Braun, Melsungen, Germany

2.1.3 Laboratory 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
Cryotome CM 1850	Leica Microsystems, Wetzlar, Germany
Electroporator (Easyject Prima)	EquiBio Peqlab, Erlangen, Germany
Gel Air™ Drying System	BioRad Laboratories, Hercules, CA
Genetic Analyzer automated sequencer ABI PRISM 310	PE Applied Biosystems, Foster City, CA
GS Gene Linker™ UV Chamber	BioRad Laboratories, Hercules, CA
Cell Culture Incubators	Heraeus Instruments, Langenselbold, Germany
Innova™ 4400 Incubator Shaker	New Brunswick Scientific, Nürtingen, Germany
Orion Microplate Luminometer	Berthold Detection System
Thermomixer 5436	Eppendorf, Hamburg, Germany
Trans-Blot® SD Semi -Dry Transfer Cell	BioRad Laboratories, Hercules, CA
X-Ray Automatic Processor Curix 60	Agfa, Köln Germany

2.1.4 Software

Microsoft Office 2003 (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 convert documents into PDF format. Pictures were processed with Adobe Photoshop CS3 (Adobe Systems, Mountain View, U.S.A.). The Vector™ NTI Suite 9 (Oxford Molecular Group) was used for sequence analysis and primers design. The Leica Software was used for confocal imaging (Leica Microsystems, Germany).

2.1.4.1 R program

R (Bell Laboratories) is an object oriented program for statistical computing and graphics, written initially by Robert Gentleman and Ross Ihaka at Statistics Department of the University of Auckland, New Zealand. R can be used to analyze probe sets from micro array data. R is a free open source environment. Its strength is the graphical facilitiy that produce publication-quality graphs which can include mathematical symbols.

2.1.5 Buffers and Solutions

All buffers and solution were prepared with d_2H_2O, unless they had to be RNase-free, in which case they were prepared with DEPC-treated-H_2O.

Ampicillin stock	100 mg/ml	Ampicillin Aqua bidest. Sterilized by filtration. Aliquots 1 ml; stored -20°C
Ammonium chloride solution	0.8% 0.1 mM	NH_4Cl in sterile water EDTA
Blue Juice loading buffer (DNA)	65% 10 mM 10 mM 0.3%	Sucrose Tris/HCl pH 7.5 EDTA Bromophenol blue Storage at room temperature (RT)
Blocking milk solution	1 x 5% 0.05%	TBS milk powder Tween 20
DEPC-H_2O	0.01%	Diethyl pyrocarbonate (DEPC) in d_2H_2O Mix well, incubate O/N and autoclave
Electrophoresis running buffer	250 mM 2 M 1%	Tris HCl, pH 8,5 glycin SDS
P1 and E1 resuspension buffer	50 mM 10 mM 100 µg/mL	Tris/HCl pH 8.0 EDTA RNAse A Storage at 4°C
P2 and E2 lyses buffer	200 mM 1%	NaOH SDS Autoclave, storage at RT
P3 and E3 neutralization buffer	3 M	Potassium acetate pH5.5 (with acetic acid) Autoclave, storage at RT
E4	100 mM 600 mM 0.15%	Potassium acetate (pH 5.0 with acetic acid) NaCl Triton X-100
E5	100 mM 800 mM	Sodium acetate(pH 5 with acetic acid) NaCl
E6	100 mM 1500mM	Sodium acetate NaCl

Gel fixation solution	20% 10%	methanol (v/v) acetic acid (v/v)
40% Glucose	40% w/v (400g)	D-(+)-Glucose Fill up to 1l of d_2H_2O Autoclave
GST buffer	100 mM 25 mM 0.1%	NaCl Tris pH 7.5 NP-40
IP buffer	0.1 %	NP-40 PBS
Kanamycin stock	50mg/ml	Kanamycin Aqua bidest. Sterilized by filtration. Aliquots 1 ml; stored at -20°C
Lyses solution for yeast cells	10 mM 1 mM 100 mM 2% 1%	Tris/HCl pH 8.0 EDTA NaCl Triton X-100 SDS
2x Lämmli sample buffer	10% 6% 20% 0.2 mg/ml 0.025X	β-mercaptoethanol SDS Glycerol Bromophenol blue Lämmlie stacking buffer (optional) Store up to 2 month at RT
2.5x Lämmli stacking buffer	0.3 M 0.25%	Tris/HCl pH 6.8 SDS Store up to 1 month at 4°C
NETN buffer	150 mM 20 mM 1 mM 0.5%	NaCl Tris/HCl pH 8.0 EDTA pH 8.0 Triton X-100

Buffer A for cytoplasmic extract	20 mM	HEPES
	10 mM	NaCl
	3 mM	$MgCl_2$
	1%	NP 40
	10%	Glycerol
	0.2 mM	EDTA
PBS-DEPC	30 ml	5 M NaCl
	15 ml	1 M Sodium phosphate buffer pH 7.3
	955 ml	DEPC-H_2O
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
2% PFA	1 ml	PFA (Paraformaldehyde)
	4 ml	PBS
Phosphate buffer saline (PBS)	140 mM	NaCl
	5.4 mM	KCl
	9.7 mM	Na_2HPO_4 x $2H_2O$
	2 mM	KH_2PO_4
	800 ml	d_2H_2O adjust pH 7.4 with HCl, complete to 1 l vol. and autoclave
RIPA buffer	1X	10XPBS
	1%	Triton X-100
	0.5%	Sodium deoxycholate
	0.1%	SDS
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)
RNase solution (TNT)	10 mM	Tris/HCl pH 7.5
	0.5 M	NaCl
	0.1%	Tween® 20 in d_2H_2O

Rödel Mix	1.85M 7.4% 20 mM 641 µl	NaOH β-mercaptoethanol 200mM PMSF d_2H_2O
10x MOPS RNA running buffer	0.2M 20 mM 10mM	MOPS, NaAC EDTA Add DEPC-H_2O to complete 1 l vol., adjust to pH 7.0. Filter sterile and store at RT protected from light
SDS 10%	10% (w/v)	Sodium dodecyl sulfate Aqua bidest. Autoclave
Sodium phosphate buffer	684 ml 316 ml	1 M Na_2HPO_4 1 M NaH_2PO_4 pH 7.2
Stripping solution for WB	62.5 mM 0.1 M 2%	Tris/HCl pH 6.8 β-mercaptoethanol SDS
5 x TBE	445 mM 445 mM 10 mM	Tris/HCl pH 8.0 Boric acid EDTA
TE buffer	10 mM 1 mM	Tris/HCl (stock 1M pH 8.0) EDTA Aqua bidest.
10x TE buffer	100 mM 10 mM	Tris/HCl (stock 1M pH 8.0) EDTA pH 8.0 or 7.5 (for Y2H experiments)
1x TBS	10 mM 150 mM	Tris/HCl pH 8.0 NaCl
1x TBST washing buffer	1X 0.1%	TBS Tween®20
Transfer buffer(1 l)	25 mM 1.44%(w/v) 20%	Tris Base Glycin Methanol Aqua bidest.
72% Trichloroacetic acid (TCA)	72 g 100 ml	Trichloroacetic acid (TCA) Aqua bidest

10x Tris-Glycine electrophoresis buffer	250 mM 2.5 M 1%	Tris Glycine SDS
10x TE buffer	100 mM 10 mM	Tris, bring to pH 7.5 with HCl EDTA d_2H_20
1x TE /1x LiAc	0.2 ml 0.2 ml 1.6 ml	10X TE LiAc solution d_2H_2O
Tfb 1	30 mM 50 mM 100 mM 10 mM 15%	KAc $MgCl_2$ KCl $CaCl_2$ Glycerol adjust to pH 5.8 (with 0.2 M NaOH), filter sterilize, aliquot and store at -20°C
Tfb 2	10 mM 75 mM 10 mM 15%	MOPS $CaCl_2$ KCl Glycerol adjust to pH 6.8 (with acetic acid), filter sterile, aliquot and store at -20°C
1x Thermal buffer (PCR)	20 mM 10 mM 10 mM 2 mM 0.1% (pH 8.8)	Tris-HCl $(NH_4)_2SO_4$ KCl $MgSO_4$ Triton X-100
Trypan blue solution	0.9% 0.5%	NaCl Trypan blue dye Aqua bidest.

2.1.6 Culture media

2.1.6.1 Culture medium for bacteria

The components for the Luria Bertani (LB) medium were dissolved in d_2H_2O and thereafter the medium was autoclaved

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

To select transformed cells, ampicillin or kanamycin was added to the medium to a final concentration of 50 µg/ml or 25 µg/ml, respectively. LB-agar medium with antibiotics was poured into Petri dishes and stored at 4˚C.

2.1.6.2 Culture medium for yeast

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

YPD medium	20 g/l	peptone
	10 g/l	yeast extract
	20 g/l	agar (for YPD agar plates only)
	950 ml	d_2H_2O, autoclave

The pH was adjusted to 6.5 (with NaOH) and the medium was autoclaved. When medium cooled to approximately 55°C, 50 ml of 40% glucose stock solution was added to 950 ml of medium. The YPD-agar medium was poured into Petri dishes and stored at 4°C.

To make minimal yeast medium lacking one or more specific nutrients, a combination of minimal SD base and a dropout (DO) solution was used. For each selective SD/DO medium, nutrients were added separately according to the desired selection. 10x stock dropout solution was prepared lacking histidine, adenine, leucine, or tryptophan (-H/-A/-L/-W).

10x DO -His, -Ade, -Leu, -Trp	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	d_2H_2O, autoclave
SD -His, -Ade, -Leu, -Trp	6.7 g	yeast nitrogen base without amino acids
	20 g	agar (for SD plates only)
	850 ml	d_2H_2O
	100 ml	10x dropout solution (-His, -Ade, -Leu, -Trp)
For SD –His, -Leu, -Trp	200 mg	Adenine was added
For SD –Leu, -Trp	200 mg	Adenine, and Histidine were added

The pH was adjusted to 5.8, and the medium was autoclaved. When the medium had cooled to approximately 55°C, 50 ml of 40% glucose stock solution was added to 950 ml of medium. For SD -His, -Ade, -Leu, -Trp plates supplemented with X-α-gal, 1:1000 volume of X-α-gal (20 mg/ml in DMF- dimethylformamide) was added to the medium. SD-agar medium was poured into Petri dishes and subsequently stored at 4˚C.

2.1.6.3 Culture medium for cells

Cell culture media and solutions were handled in sterile conditions and were stored at 4°C. All cell lines used were cultivated in an incubator set at 37˚C with 95% relative air humidity in the presence of 5% CO_2. The cell culture media RPMI-1640 and DMEM were supplemented with 20% and 10% FBS, respectively. 5 U/ml each of penicillin and streptomycin were added.

2.1.7 Bacterial strain (Escherichia coli)

Strain	Usage
XL1’ Blue	host for plasmid amplification
XL 10	host of choice for GST fusion protein expression and plasmid amplification
DH5α	host for plasmid amplification

2.1.8 Yeast strain

Yeast reporter strain was purchased from DB Biosciences Clontech, Heidelberg, Germany

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

2.1.9 Mammalian cell lines

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

2.1.9.1 Murine and rat cell lines

Name	Cell type/ Origin
NIH3T3	swiss mouse embryo fibroblast
TGR	rat fibroblast
BA/F3	mouse pro B-cells, IL-3 dependent line established from peripheral blood

2.1.9.2 Human cell lines

Normal cell lines

Name	Cell type/ Origin
293T	human embryonal kidney
LCL 3.1*	B-cell EBV mortalized

*courtesy of Dr. Martin Dreyling, CCG-Leukemia, Grosshadern, 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

B-cell leukemia and B-cell lymphoma cell lines

Name	Cell type/ Origin
DG-75	Cell line established from the pleural effusion of a 10-year-old boy with Burkitt lymphoma in 1975
Granta 519*	Cell line established from the peripheral blood from a 58-year-old Caucasian woman with previous history of cervical carcinoma at relapse of a high-grade B-NHL (leukemic transformation of mantle cell lymphoma, stage IV)
HBL-2*	human mature B-cell line derived from B-NHL (diffuse large cell), carries the t(11;14)(q13;q32) which juxtaposes the IGH promoter to the BCL1 gene
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 the t(11;14)(q13;q32) which juxtaposes the IGH promoter to the BCL1 gene
Rec*	Human B-cell lymphoma, established from the lymph node or peripheral blood from a 61-year-old man with B-cell Non-Hodgkin lymphoma.

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

T-cell leukemia

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
HUT78	human mature T-cell line derived from the peripheral blood of a 50-year-old male patient with Sézary syndrome (cutaneous T-cell lymphoma).
MOLT-4	human immature T cell line established from the peripheral blood of a 19-year-old man with ALL

* courtesy of Dr. Martin Dreyling, CCG-Leukemia, Grosshadern, 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 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
K-562 ♣	Human chronic myeloid leukemia in blast crisis, established from the pleural effusion of a 53-year old woman with chronic myeloid leukemia (CML) Expresses BCR-ABL fusion gene
MOLM-13 ♣	Human acute myeloid leukemia, established from the peripheral blood of a 20-year-old man with AML (FAB M5a)
U937	human histiocytic lymphoma. Cells express markers and properties of monocytes and carry the t(10;11) resulting in the CALM-AF10 fusion gene

* courtesy of Dr. Martin Dreyling, CCG-Leukemia, Grosshadern, Munich
♣courtesy of Dr. Tobias Kohl, CCG-Leukemia, Grosshadern, Munich

2.1.10 Plasmids

pACT2	Shuttle vector for expression of GAL4-AD (amino acids 768-881) fusion proteins in yeast, DB Biosciences Clontech, Heidelberg, Germany
pcDNA6/V5	Contains V5 epitope, Invitrogen, Karlsruhe, Germany
pGEM®-T	Cloning vector with 3'-T overhangs at insertion site for direct cloning of PCR products, Promega, Madison, USA
pGEM®-T Easy	Cloning vector with 3'-T overhangs at insertion site for direct cloning of PCR products, Promega, Madison, USA
pGBKT7	Shuttle vector for expression of GAL4-DBD (amino acids 1-147) fusion proteins in yeast, DB Biosciences Clontech, Heidelberg, Germany
pGADGH; pGADT7	Shuttle vector for expression of GAL4-AD (amino acids 768-881) fusion proteins in yeast, DB Biosciences Clontech, Heidelberg, Germany
pEYFP-C1	Mammalian expression vector for yellow (YFP) fluorescent fusion proteins, DB Biosciences Clontech, Heidelberg, Germany
pGEX-4T1	Expression of GST fusion proteins in bacterial cells. Vector contains a tac promoter for protein expression after IPTG induction, Amersham Pharmacia Biotech
pM1	Mammalian expression vector for GAL4-DBD (1-147) fusion proteins, DB Biosciences Clontech, Heidelberg, Germany
pGAL4-LUC	Reporter plasmid encoding the luciferase gene under the transcriptional control of 5 GAL4-binding sites and the min SV40 promoter
$pGAL4_5tkLUC$	Reporter plasmid encoding the luciferase gene under the transcriptional control of the Herpes simplex virus thymidine kinase promoter and 5 GAL4-binding sites, (Netzer C. et al., 2001)
pRL-null	Co-reporter vector encoding the Renilla luciferase protein, Promega, Madison, USA

2.1.11 Constructs

pcDNA3-FLAG-FHL2 (aa 1-279)	Dr. Paul Riley, Molecular Medicine Unit Institute of Child Health, London, UK
pCMX-GAL4DBD-Flirt1 (aa 1-279)	Dr. Judith Müller, Universität-Frauenklinik, Freiburg, Germany
pGBKT7-CALMΔ10 (aa 1-408)	Britta Kaltwasser, CCG Leukemia, GSF, Munich, Germany
pGBKT7-LIM ½ (aa 1-75)	Zlatana Pasalic, CCG Leukemia,GSF, Munich, Germany
pGBKT7-LIM ½-1 (aa 1-143)	Zlatana Pasalic, CCG Leukemia,GSF, Munich, Germany
pGBKT7-LIM ½-2 (aa 1-202)	Zlatana Pasalic, CCG Leukemia,GSF, Munich, Germany
pGBKT7-LIM ½-3 (aa 1-262)	Zlatana Pasalic, CCG Leukemia,GSF, Munich, Germany
pGEX-4T-1-FHL2 (aa 1-279)	Dr. Judith Müller, Universität-Frauenklinik, Freiburg, Germany
GAL4-CALMΔ3 (aa 1-652)	Sigrun Bartels, Institute für Humangenetik, Göttingen, Germany
pAS2-CALMΔ1 (aa 1-294)	Sigrun Bartels, Institute für Humangenetik, Göttingen, Germany
pAS2-CALMΔ4 (aa 1-335)	Sigrun Bartels, Institute für Humangenetik, Göttingen, Germany
pAS2-CALMΔ7 (aa 1-221)	Sigrun Bartels, Institute für Humangenetik, Göttingen, Germany
pAS2-CALMΔ8 (aa 1-105)	Sigrun Bartels, Institute für Humangenetik, Göttingen, Germany
pEYFP-FHL2 (aa 1-279)	Zlatana Pasalic, CCG Leukemia,GSF, Munich, Germany
YFP-CALM/AF10	Dr. Alexandre Krause, CCG Leukemia, GSF, Munich, Germany

2.1.12 Oligonucleotides

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

FHL2 specific primers

Oligonucleotide	Sequence	Position at FHL2 sequence*	Tm °C
DRAL66T	5'-GCTGAGAACTGTGTCTTCCTGGAG-3'	66	61
DRAL450B	5'-GCATTCCTGGCACTTGGATGAG-3'	450	61
FHL2-T-143-BamHI	5'-CATTGGATCCACCATGACTGAGCG CTTTGA C-3'	143	60
FHL2-B-961EcoRI	5'-TGTGTTGAATTCGATGTCTTTCCC ACAGTC-3'	961	60
FHL2T-XhoI	5'-CCTCTCGAGAAATGACTGAGCGCTTTG-3'	140	57
FHL2B-EcoRI	5'-GTGTTGAATTCAGATGTCTTTCCC-3'	980	57
FHL2-T94	5'-ATTACCATGGATGGCTGGCATTTT GACTTTGGG-3'	94	63
FHL2-LIM1/2	5'-CGGGATCCCTGACGAACAGGGTCT CAAAGCACAC-3'	226	63
FHL2-LIM1/2-1	5'-CGGGATCCCGCATTCCTGGCACTT GGATGAG-3'	429	63
FHL2-LIM1/2-2	5'-CGGGATCCCTGACGCACTGCATGG CATGTTG-3'	607	63
FHL2-LIM1/2-3	5'-CGGGATCCCTGACCCAGCACACTT CTTGGCATAC-3'	786	63

*GenBank acc. Nr L42176 (HUMDRAL)

Vector primers

Oligonucleotide	Sequence	Position	Tm °C
GAPDHforward	5'-GCACCACCAACTGCTTAGCACC-3'	Exon 7 (530-551)	60
GAPDHreverse	5'-GTCTGAGTGTGGCAGGGACTC-3'	Exon 9 (1166-1146)	60
NG2T7/T1096	5'-TGGCAAGCACTGGTCTAT-3;	1073-1091	
Y2H2	5'-TCATCGGAAGAGAGTAG-3'	(1155-1171 pGBKT7)	55
Y2H3'AD	5'-AGATGGTGCACGATGCACAG -3'	(2102-2083 pGADT7)	55
pGEX5'	5'-GGGCTGGCAAGCCACGTTT GGTG-3'	(869-891)	55
pEGFPC-1240-1258	5'-AAAGACCCCAACGAGAAGC-3'	(1240-1258)	55

*GenBank acc. Nr.: U55763 (pEGFP-C1), U13854 (pGEX-4T2), NM_002046 (human GAPDH)

2.1.13 Antibodies

Name	Company	dilution
Alexa Fluor® 555 goat anti-mouse	Invitrogen	1:500
Anti-β-Actin clone AC-15	Sigma®	1:2000
Anti-FLAG	Sigma®	1:6000
Anti-FHL2 (F4B2-B11)	Cell Sciences , Inc	1:250
Anti-GFP rabbit IgG fraction	Molecular Probes, Inc.	1:6000
Anti-V5	Invitrogen	1:6000
CALM (S-19)	Santa Cruz Biotechnology, Inc.	1:1000
CALM (G-17)	Santa Cruz Biotechnology, Inc.	1:1000
CALM (C-18)	Santa Cruz Biotechnology, Inc.	1:1000
GAL4 (DBD) (RK5C1)	Santa Cruz Biotechnology, Inc.	1:1000
Donkey anti-rabbit IgG-HRP conjugated	Santa Cruz Biotechnology, Inc.	1:2000
Donkey anti-goat IgG-HRP conjugated	Santa Cruz Biotechnology, Inc.	1:2000
goat anti-mouse IgG-HRP conjugated	Chemicon International	1:1000-3000
Goat anti-mouse IgG + IgM-Cy™3 conjugated	Jackson ImmunoResearch Laboratories, Inc.	1:500

2.2 Methods

The molecular biology methods in this work were performed according to the "Molecular Cloning" (Sambrook and Russell, 2001) with minor or no modifications. All buffers used in this work are described in chapter 2.1.5 with exception for some Qiagen and Promega buffers.

2.2.1 Isolation of DNA

Plasmids were prepared from E.coli XL-1' blue, DH5α and XL10 bacterial cultures grown in the presence of selective antibiotics. All Mini, Midi, Maxi and Mega preparations are procedures that isolate plasmid DNA from bacteria, using the alkaline method followed by isopropanol precipitation.

2.2.1.1 Mini preparation (Mini Prep)

Miniprep were performed during the cloning procedure to isolate small amounts of DNA from a large number of clones. Each single colony was inoculated in 4 ml of LB medium (plus 100 µg/ml Amp or 50 µg/ml Kan) and grown O/N at 37°C with vigorous shaking (260 rpm). 1.5 ml of the overnight culture was transferred to Eppendorf tubes and bacterial cells were harvested by centrifugation at 14000 rpm for 2 min. The pellet was resuspended in 300 µl of cold buffer P1 containing RNase (0.1 mg/ml). 300 µl of buffer P2 was added, mixed gently, and the mixture was incubated at RT for 5 min. The lysed pellet was neutralized by gently mixing with 300 µl of Buffer P3, which was immediately thereafter centrifuged at 14'000 rpm for 15 min. 800 µl of the supernatant was transferred to a new sterile Eppendorf tube and 0.7 volume of isopropanol was added. The precipitate was vortexed and incubated at -20°C for 10-20 min and then centrifuged at 14'000 rpm for 20 min. The pellet was washed with 500 µl of 70% ethanol and centrifuged 10 min at 14'000 rpm. Then the pellet was dried for 10-20 min and redissolved in 20 µl of sterile water. The DNA was stored at -20°C.

2.2.1.2 Midi preparation (Midiprep)

To isolate larger amount of plasmid DNA for cloning, Midi preparation was done. A bacterial colony or 20 µl of a glycerol stock was inoculated in 25 ml of LB medium containing appropriate selective antibiotics and grown overnight at 37°C with vigorous shaking (270 rpm). Cells were harvested by centrifugation at 6000 x *g* for 15 min at 4°C. 4 ml of buffer P1 was used to resuspend the pellet. For cell lysis 4 ml of Buffer P2 was added and mixed by inversion, and the mixture was incubated at RT for 5 min. 4 ml of pre-chilled Buffer P3 was added, mixed and incubated on ice for 15 min. After incubation the mixture was centrifuged at ≥20'000 x *g* for 30 min at 4°C. The supernatant was transferred to fresh 50 ml Falcon tube and once more centrifuged at ≥20'000 x *g* for 15 min at 4°C. During centrifugation the Qiagen tip 100 was equilibrated with 4 ml of Buffer QBT. The supernatant was then applied to the column, whereafter the flow-through was discarded and the column-bound DNA was washed twice with Buffer QC. The DNA was eluted with 5 ml of Buffer QF and precipitated with 0.7 volumes RT isopropanol (in order to minimize the salt precipitation). The mixture was mixed and centrifuged at ≥15'000 x *g* for 30 min at 4°C. The supernatant was carefully discarded and the pellet was washed with 2 ml of RT 70% ethanol by centrifugation at ≥15'000 x *g* for 10 min at 4°C. Ethanol removes the precipitated salt and replaces isopropanol with the more volatile ethanol. The pellet was air dried for 5-10 min and redissolved in 100 µl or appropriate volume of 1x TE buffer or sterile d_2H_2O. To ensure that the DNA plasmid obtained was correct a digestion with appropriate restriction enzymes was performed.

2.2.1.3 MaxiEndofree preparation (Maxi Prep)

For transfection experiments the DNA was prepared using Qiagen Maxi Endofree kit. A bacterial colony or 20 µl of a glycerol stock were inoculated in 100 ml of LB medium in the presence of ampicillin (50 µg/ml) or kanamycin (25 µg/ml), and grown overnight at 37°C with vigorous shaking (230 rpm). The cells were harvested by centrifugation at 6000 x *g* for 15 min at 4°C. 10 ml of buffer P1 was used to resuspend the pellet. For cell lysis 10 ml of Buffer P2 was added and mixed by inversion, and the mixture was incubated at RT for 5 min. The solution was neutralized by adding 10 ml of ice-cold

buffer P3 and gently inversion. The suspension was applied into the Qiafilter Cartridge and incubated at RT for 10 min. Thereafter the suspension was filtered through the Qiafilter Cartridge. 2.5 ml of ER buffer was added to the lysate and the solution was incubated at ice for 30 min. During the incubation a Qiagen column was equilibrated with 10 ml Buffer QBT. The supernatant from the cell lysate was subsequently poured into the column to bind the DNA to the column. The DNA was eluted with 15 ml of Buffer QN and precipitated with 0.7 volumes of isopropanol by centrifuging ≥ 15'000 x *g* for 30 min at 4°C. To neutralize the isopropanol the DNA pellet was washed with 15 ml of 70% ethanol by centrifuging ≥ 15'000 x *g* for 15 min at 4°C. The supernatant was discarded and the pellet was dried for 10-20 min and dissolved in 200-500 µl, depending of the size of the pellet, of endotoxin Free buffer (EB). After determining the concentration the DNA was stored at -20°C. To ensure that the DNA plasmid obtained is correct a restriction enzyme digestion analyses is done.

2.2.1.4 Mega Preparation (Mega Prep)

After amplifying the yeast two hybrid thymus cDNA library as single colonies on 150 mm LB plates a plasmid mega preparation was performed. The colonies from the plates (50 plates) were scraped into 500 ml LB medium with selective antibiotics, and grown at 37˚C O/N with vigorous shaking (300 rpm). The overnight culture was harvested by centrifuging at 6000 x *g* for 15 min at 4˚C. The pellet was resuspended in 50 ml buffer E1. For cell lysis 50 ml E2 was added and mixed until a homogenous lysate was obtained. Neutralization of lysis was performed by mixing 50 ml E3. The mixture was centrifuged for 20 min at 12'000 x *g* at RT, meanwhile the MEGA Cartridge was equilibrated with 100 ml buffer E4. The suspension was filtered through the MEGA-cartridge and the DNA absorbed to the column. DNA was eluted with 50 ml of elution Buffer E6 and precipitated with 0.7 volumes of isopropanol by centrifuging ≥ 13'000 x *g* for at least 30 min at 4°C. The precipitated DNA was washed with 10 ml of 70-80% ethanol by centrifugation for 5 min at 13'000 x *g* at 4°C. Pellet was air dried for 10 min at RT and then redissolved in appropriate volume of 1xTE. The DNA was stored at -20°.

2.2.2 Determination of nucleic acid concentration

To determine the quality and yield, the DNA was measured with an UV spectrophotometer (BioPhotometer 6131, Eppendorf) at a wavelength of 260 and 280 nm. The dilution used for measurements was 1:100. DNA preparations with a concentration of less than 0.2 µg/ml and a ratio below 1.5 were discarded. The DNA concentration is calculated by the spectrophotometric conversion in which $1A_{260}$ unit corresponds to 50 µg/ml of double-stranded DNA or 40 µg/ml of RNA (Sambrook, 1989).

2.2.3 Digestion of DNA

Type II restriction endonucleases are essential for specific digestion of DNA. These enzymes recognize, bind and cleave short defined mostly palindromic base sequences in the DNA. Different buffers are used for different enzymes, but for multiple enzyme digestion the Five Buffer Plus System such as Y+/Tango™ (MBI Fermentas), was required. The digest with a single restriction enzyme was performed in an Eppendorf tube as follows:

1 µg	DNA
5U	restriction enzyme
1-2X	appropriate restriction enzyme buffer
x µl,	up to 20 µl, of sterile water

The reaction was incubated from 2-3 h to O/N at 37°C (or at the recommended temperature) depending on enzymes used.

2.2.4 Ethanol precipitation of DNA

Ethanol precipitation was used to purify DNA from buffers in previous reactions (e.g. digestion and sequencing). 1 volume of DNA sample was mixed with 2.5 volumes of 100% - ethanol and 1:10 volumes of 3M NaAc (pH 5.2). The mixture was incubated at -20°C for 10 min and centrifuged at 14 000 rpm in a table top centrifuge for 30 min at 4˚C. The supernatant was discarded and the pellet was washed with 500 µl of 70% ethanol by centrifuging at top speed for 10 min at 4°C. The pellet was dried at RT or for 10 min at 56˚C in a thermo block. The pellet was resuspended in 20µl 1x TE or sterile d_2H_2O. The purified DNA was stored at -20˚C.

2.2.5 Ligation

Both the vector and the insert (the restriction fragment) were restricted in such a way that they contained compatible ends. A 1:4 (vector: insert) ratio was used and mixed in a 1.5 ml eppendorf tube as described below:

X ng	restricted vector DNA (2.2.3) and
X ng	insert purified (2.2.4)
4 µl	5X T4 DNA ligase buffer
1 µl	T4 DNA ligase (5 U/µl)
to 20µl	d_2H_2O

The ligation reaction was incubated O/N at 4 ˚C.

2.2.6 DNA transformation into bacterial cell

For bacterial transformation two different protocols were used, the heat shock and the electroporation transformation. For chemical transformation the XL10 cells were used while the electrocompetent *Epicurian coli XL1-blue* cells were used for the electroporation. The buffers used are described in 2.1.5.

2.2.6.1 Preparation of chemically competent cells

An aliquot (200 µl) of frozen cells (E. coli strain XL10) was inoculated into 10 ml LB medium and incubated O/N with vigorous shaking at 37°C. The O/N culture was diluted in 300 ml LB medium and incubated for an additional 2-3 hrs until the culture reached an OD_{600} of 0.5-0.7. The bacterial culture was cooled on ice and cells were harvested by centrifugation at 4000 rpm at 4°C. The cell pellet was resuspended in 100 ml Tfb1 and incubated on ice for 10-15 min. The suspension was centrifuged at 4 000 rpm at 4°C for 10 min, whereafter the supernatant was discarded and the cells were resuspended in 4 ml of Tfb2. The competent cells were aliquoted (200 µl), frozen in liquid nitrogen and stored at -80°C.

2.2.6.2 Transformation

100 µl of the chemically competent cells were mixed with 1 to 5 µl of 50-100 ng/µl plasmid DNA or 5µl ligation reaction (2.2.5). The reaction was first incubated on ice for 30 min then heat shocked for 50 sec at 42°C and then again cooled on ice for one more minute. 900 µl LB medium was added to the reaction, which was incubated for 1h

shaking at 37°C. After incubation, cells were streaked on LB agar plate with appropriate selective antibiotics. The plates were then incubated O/N at 37°C.

2.2.6.3 Preparation of XL1-Blue electrocompetent cells

An aliquot (50µl) of frozen cells (E. coli strain XL1-blue MRF') was inoculated into 10 ml LB medium and incubated O/N with vigorous shaking at 37°C. The overnight culture was diluted in 400 ml LB medium and incubated for 2-3 hrs until the culture reached an OD_{600} of 0.5-0.6, and was then cooled on ice. The cells were harvested by centrifugation and the pellet was washed twice with 40 ml ice cold sterile d_2H_2O, twice with 20 ml ice cold 10% glycerol (in d_2H_2O) and then resuspended in 800 µl 10% glycerol. The electrocompetent cells were aliquoted in 50 µl and stored at -80°C. The centrifugation steps were all carried out at 4 500 rpm for 5 min at 4°C.

2.2.6.4 Electroporation

Electrocompetent cells (50 µl aliquot) (2.2.6.3) were mixed with 1 µl plasmid DNA (50-100 ng) or 1 µl ligation reaction (2.2.5). They were thereafter incubated for 1 min on ice and transferred to a pre-cooled electroporation cuvette. The cuvette was placed in an electroporator and cells were electroporated at 2.5 kV in 3-4 msec pulse (Easyject-Equibio, 6.125 kV/cm maximum field strength). 1 ml of pre-warmed LB medium (37°C) was immediately added to the cells and the suspension was transferred to a fresh Eppendorf tube. The cells were incubated for 1 h vigorously shaking at 37°C, then plated on LB agar plates containing selective antibiotics, and incubated O/N at 37°C.

2.2.7 Agarose-gel electrophoresis for DNA

To separate DNA fragments both for analysis and isolation, non denaturing agarose gel electrophoresis was used. According to the size of the linear DNA fragments to be separated, gels of different concentration were used.

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

Agarose was mixed and boiled in 50 ml 0.5x TBE. 3 µl Ethidium bromide was added to the solution and the gel was poured into a gel chamber. The DNA samples were mixed with 1x Blue Juice loading buffer (2.1.5) and then loaded in the wells. The negatively charged nucleic acids were run under electric current (60-110 V) moving from the negative to the positive pole.

2.2.8 Isolation of DNA fragments

After the gel electrophoresis the DNA fragments were cut out of the gel under the UV light (312 nm) and transferred to an Eppendorf tube. The Qiagen Gel Extraction Kit (using microcentrifuge) was used to extract the DNA from the agarose gels. For each 100 mg gel slice 300 µl buffer QG was added and the reaction was incubated at 50°C for 10 min. Every 2-3 min the tubes were vortexed. When the gel slice had dissolved completely, 1 gel volume of the isopropanol was added and mixed. To bind the DNA, 800 µl of the reaction was applied to a QIAquick column and centrifuged for one minute. Flow-through was discarded and the column was soaked with 750 µl wash Buffer PE for 3 min and thereafter the bound DNA was washed by centrifugation for one minute. The flow-through was discarded and the QIAquick column was centrifuged for additional one minute to remove the residual ethanol from the buffer. The QIAquick column was transferred to a fresh sterile microcentrifuge tube and the DNA was eluted with 30 µl elution buffer EB or sterile d_2H_2O by centrifugation for one minute. The DNA was stored in -20°C. The purified DNA fragments were used for reactions such as ligations (2.2.5).

2.2.9 RNA extraction

2.2.9.1 RNeasy® Mini Kit

Total RNA was prepared from cultured cell lines (2.2.13) with the RNeasy® Mini Kit (Qiagen). For RNA extraction, approximately 5.5 x 10^6 cells were lysed by the addition of 600 µl RLT buffer. For homogenization, samples were applied to a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2 min at 14'000 rpm at 20-25˚C. 1 volume (600 µl) of 70% ethanol was added to the homogenized lysate and mixed thoroughly by pipetting. 700 µl of 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 RNA bound to the column was washed once with 700 µl RW1 buffer (15 sec at 8 000 x *g*) and twice with 500 µl RPE buffer (2 min at 8 000 x *g*). The flow-through was discarded after each washing step. RNeasy column was placed in a fresh 1.5 ml tube. To elute the RNA that was bound to the column 50 µl RNase-free H_2O was applied to the column and incubated for 1 min at RT. The RNA was collected By centrifuging for one minute at 8000 x *g*. The RNA concentration was measured as described in (2.2.2), and the quality was determined by loading 2 µl of the prepared RNA on a 1% agarose gel (2.2.10). The RNA was stored at -80°C.

2.2.9.2 Trizol RNA extraction

For total RNA extraction approximately 5.5 x 10^6 cells were harvested by centrifugation at 1000 x *g* for 5 min at 4˚C. The cells were lysed in 1 ml TRIzol Reagent by repetitive pipetting and then incubated at RT for 5 min. 0.2 ml chloroform per sample was added and shaked vigorously by hand for approximately 15 sec and then incubated at RT for 2-3 min. The phase separation was performed by centrifugation at 12'000 x *g* for 15 min at 4˚C. The mixture separated in lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase, which contains the RNA. The aqueous phase was transferred to a fresh RNAse free tube and 0.5 ml of isopropanol was added. The samples were incubated for 10 min at RT and centrifuged at 12 000 x *g* for 10 min at 4˚C and RNA precipitate formed a gel like pellet. The supernatant was discarded and 1 ml of 75% ethanol was added to the RNA pellet, vortexed and centrifuged at 7'500 x *g* for 5 min at 4˚C. RNA pellet was air dried for 10 min at RT or 1 min at 58°C and dissolved in an appropriate volume, 50-75 µl, of DEPC-H_2O. The samples were incubated at 58°C for 10 min. RNA aliquots were stored in -80°C.

2.2.10 Agarose-gel electrophoresis for RNA

To analyze the quality of the RNA, a denaturing 1%- agarose gel was prepared by boiling 1g of Agarose in 74 ml DEPC-H_2O and 10 ml 10x MOPS RNA running buffer. When the gel was cooled down 16.6 ml of 37% formaldehyde was added. Gel solution was poured into an RNase free gel chamber and the RNA samples were prepared on ice to avoid degradation. 1 µl of the RNA sample was mixed with 5µl DEPC-H_2O, 5 µl 1.5x RNA

sample buffer and 1 µl EtBr (1 µg/ µl). The RNA samples were then incubated at 65°C for 10 min and then cooled on ice for one minute. 3 µl of RNA loading buffer was added to the mixture and the samples were run on the gel. The electrophoresis was carried out in 1x MOPS running buffer (diluted in DEPC- H_2O) at RT for 1,5 hr at 60V.

2.2.11 cDNA synthesis – reverse transcriptase reaction

cDNA is DNA synthesized *in vitro* on a RNA template by the reverse transcriptase enzyme. The most important advantage of cDNA is that it is more stable than mRNA and can be used as template in PCR reactions. A complementary DNA strand -cDNA, of mRNA is synthesized with the extension of oligo(dT) primers by the reverse transcriptase enzyme. cDNA is used as a template for subsequent PCR reactions as described in 2.2.12. To avoid genomic DNA contamination the RNA (2.2.9) was pretreated with DNAse I as follows: 1 µg of RNA was mixed with 1 µl 10x DNase I reaction buffer, 1 µl DNase I (1 U/µl) and DEPC- H_2O to a final volume of 10 µl and then the mixture was incubated for 15 min at RT. The reaction was inactivated by adding 1 µl of 25 mM EDTA and heating it at 65ºC for 10 min. For the cDNA synthesis 2 µg DNAse-free RNA was mixed with 5 µl dNTP mix (2 mM), 1 µl oligo(dT) primer (500 µg/ml) and DEPC- H_2O to a final volume of 12 µl. The mixture was denatured at 65ºC for 5 min and then chilled on ice for 1 min. The contents of the tube was collected by brief (5-10 sec) centrifugation and 4 µl of 5x First-Strand Buffer, 2 µl of 0.1M DTT and 1 µl RNaseOut recombinant ribonuclease inhibitor (40 U/µl) were added to the reaction. The mixture was gently mixed and incubated at 42ºC for 2 min. 1 µl of SuperScript II enzyme was added to the reaction and the mixture was incubated at 42°C for 50 min. The RT enzymes was nactivated incubating the reaction at 70ºC for 15 min. For the PCR reactions (2.2.11) 1 µl of the synthesized cDNA (20 µl total vol.) was used.

2.2.12 Polymerase chain reaction- PCR

The PCR technique is employed to amplify specific regions of DNA *in vitro*. This is achieved by using two primers (synthetic DNA oligonucleotides) flanking the sequence to be amplified. The principle of the PCR is that the reaction needs a brief heat treatment to separate the two strands of the DNA double helix. Subsequent cooling of the reaction

and an excess of the two primers DNA oligonucleotides allows the primers to hybridize to the target sequence. The annealed mixture is then incubated with DNA polymerase and dNTPs so that the region downstream of the primers is synthesized. When the procedure is repeated the newly synthesized fragments serve as template, and already after few cycles there is an excess of the desired DNA fragment because it is amplified in an exponential fashion. As the template 1 µl cDNA (2.2.11), or 0.5 ng plasmid DNA was used. The PCR reaction was performed by mixing template, 2 µl 10x PCR Buffer, 1.5-5mM total concentration of $MgCl_2$ depending on the primer pairs, 2 µl dNTP (2 mM), 0.2 µM- 0.5 µM forward primer (tot. conc.), 0.2 µM- 0.5 µM reverse primer (tot. conc.), 0.16 µl Taq polymerase (5 U/µl) and d_2H_2O to a final volume of 20 µl. Sometimes reactions were scaled up to a volume of 50 µl.

Reaction was run in a thermocycler as follows:

95°C : 2 min	(denaturation)
94°C : 30- 45 sec	(denaturation),
56°C-62°C: 30 sec to 1 min	(annealing),
72°C : 40-45 sec	(extension) (25-35 cycles)
72°C : 5 min	(final extension)

Depending on the primer pair, the annealing temperature varied. The extension time varied according to the expected size of the PCR product. As a general rule of thumb 1 min per kb was used.

2.2.12.1 DNA Sequencing

DNA Sequencing was performed to identify a clone or to examine the fidelity of plasmid DNA after a cloning step. The sequencing reactions were performed according to the Sanger chain-termination method. Cycle-sequencing, like ordinary PCR, consists of cycles of denaturation, annealing and extension in presence of polymerase and dNTP's, with the exception that by sequencing additional dye labeled ddNTP's are also added.

Reaction was prepared as follows:

4 µl BigDye terminator
1 µl primer (1 µM tot.conc)
1 µg DNA was used of plasmid DNA,
4 µl d_2H_2O
To 10 µl total volume of per reaction.

Reaction was run in a thermocycler as follows:

96°C: 2 min (denaturation),
96°C: 30 sec (denaturation),
50°C: 15 sec (annealing),
60°C: 4 min (extension)
(25 cycles)

After cycling had been completed, 10 µl d_2H_2O was added to the reaction followed by ethanol precipitation (2.2.4). The precipitated DNA was diluted in 20 µl sterile d_2H_2O whereafter 10µl was used to run the samples into the Genetic Analyzer automated sequencer ABI PRISM 310 (PE Applied Biosystems).

2.2.13 Cloning of constructs

For mapping the CALM interaction domain of FHL2 in the yeast two hybrid system, various portions of the *FHL2* cDNA were inserted into the pGBKT7 vector to produce a series of GAL4 DBD-FHL2 deletion mutants. The appropriate portions of the *FHL2* cDNA were amplified with the primers FHL2-T, FHL2-LIM1/2, FHL2-LIM1/2-1, FHL2-LIM1/2-2 and FHL2-LIM1/2-3 (2.1.12) and inserted into the *NcoI* and *BamHI* restriction sites of the pGBKT7 vector (fig 3.4 A).

For immunofluoroscence, the pEYFP-FHL2 construct was cloned. The full length *FHL2* DNA fragment was generated by PCR amplification using pACT2-FHL2 with primers containing sites for the restriction enzymes *XhoI* and *EcoRI* (FHL2T-XhoI and FHL2B-EcoRI). The 840 bp PCR product corresponding to FHL2 isoform 2 was digested with these restriction enzymes and cloned in frame with the green fluorescent proteins into the pEYFP-C1 vector (2.1.10).

For immunoprecipitation, the pcDNA6/V5-FHL2 construct was cloned. The full length *FHL2* DNA fragment was generated by PCR amplification using pACT2-FHL2 with primers containing sites for the restriction enzymes *BamHI* and *EcoRI* (FHL2-T-143-BamHI and FHL2-B-961EcoRI. The 818 bp PCR product corresponding to FHL2 isoform 2 was digested with these restriction enzymes and cloned in frame with the V5 proteins into the pcDNA6/V5 vector (2.1.10).

2.2.14 Culture of mammalian cells

Adherent cells were grown in DMEM supplemented with 10% heat inactivated FBS and 1% penicillin/streptomycin. Suspension cells were grown in RPMI medium supplemented with 20% heat inactivated FBS. Cells were cultured in a humidified incubator with 5% carbon dioxide at 37°C. Splitting of confluent cultures of adherent cells was done every 2-4 days in a 1:4- 1:6 ratios. The cells were first washed with DPBS and then trypsinized with Trypsin/EDTA for approximately 5 min at 37°C. Fresh medium was added to the trypsinized cells, whereafter the cells were replated in new Petri dishes. Suspension cells were split by aliquoting part of the growing culture in a new flask and then adding fresh medium to these cells. For long-term storage, the cells were harvested by centrifuging for 5 min at 1 000 rpm at 4°C. The pellet was then washed with DPBS and resuspended in 90% FBS and 10% DMSO. The cells were aliquoted in Cryotube™ vials and were put in pre cooled ice box that was then placed in a -80°C freezer.

2.2.15 Transient transfection of adherent cells

For protein co-localization and protein expression, transient transfections were performed, using Roti®-Fect and polyethylenimine (PEI) transfection reagents. For co localization, one day before transfection, approximately $4x10^5$, cells were seeded and grown in 6-well plates, with 18x24 mm cover slips placed in the wells. 2 ml of growth medium was added and the cells were incubated O/N (or until the cells were 30-40% confluent).

For reporter gene assay, $5x10^4$, HEK 293T cells were seeded the day before transfection and grown in 24 well plates with 0.5 ml of growth medium containing serum. The cells were incubated O/N. The transfection was performed on the next day. To examine the functional role of the CALM on GAL4 DBD-FHL2 the total amount of 1020 ng DNA was transfected into, $5x10^4$cells; 400 ng of plasmid encoding GAL4 SV40 minimal promoter, 20 ng of p-RL co reporter vector (Promega), 200 ng GAL4 DBD-FHL2 and 100, 200 respectively 400 ng YFP-CALM.

To investigate the role of CALM/AF10 on GAL4 DBD-FHL2, the total amount of 1020 ng DNA was transfected; 400 ng of plasmid encoding SV40 minimal promoter, 20 ng of

p-RL co reporter vector (Promega), 200 ng GAL4 DBD-FHL2 plasmid and 100, 200 or 400 ng YFP-CALM/AF10 plasmid.

To study the role of FHL2 on GAL4 DBD-CALM/AF10, cells were co transfected with 100 ng plasmid encoding GAL4 SV40 minimal promoter, 10 ng of p-RL co reporter vector (Promega), 100 ng GAL4 DBD alone or GAL4 DBD-CALM/AF10 expression plasmids and 100 ng FLAG-FHL2. The total amount of the transfected DNA was kept constant by adding pcDNA6/V5 in all experiments.

For the protein extractions, $2x10^6$ cells were seeded in a 100 mm Petri dish the day before the transfection. On the next day, the cells were washed with DPBS and 8 ml fresh medium was added.

For reporter gene assay-the different DNA concentrations described, for co localization-2 µg plasmid DNA and for the protein extraction 8 µg plasmid DNA was mixed with 30, 60 or 200 µl pure medium (free from serum and antibiotics) in reaction A. In reaction B - 30, 60 or 200 µl medium and 10-50 µl Roti®-Fect or 1.5 volumes of PEI was mixed. In order to form DNA lipid complexes reaction A and B were mixed and incubated for 30 min. The cells were meanwhile washed with DPBS and growth medium with serum was added to the cells. The mixture of the DNA lipid complex was added to the cells and incubated for 24-48 hrs. After incubation, the cells were either harvested for reporter gene assay (2.2.23) and protein extraction (2.2.17) or fixed for microscopy (2.2.15).

2.2.16 Fixation of transfected cells and microscopy

After the transfection (2.2.14), cells were once washed with ice cold PBS and fixed for 10 min in RT with freshly prepared 2% paraformaldehyde (in PBS). After fixation, the cells were washed with ice cold PBS and stained with 2 ml DAPI solution (5 mg/ml stock diluted 1:10'000 in PBS) for 2 min. The cells were washed one more time with PBS whereafter the coverslips were mounted on glass slides with Dakocytomation Mounting Medium and sealed with nail polisher. The Axiovert 200M microscope (Carl Zeiss, Jena, Germany), with filter sets for visualization of DAPI, cyan (CFP) and yellow (YFP) and Texas Red was used to observe the cells. Openlab 3.08 software (Improvision, Coventry, UK) was used to record the images of the cells.

2.2.17 Yeast two hybrid

The yeast two hybrid assay is a sensitive method for detecting weak and transient protein interactions in living yeast cells. Even though some interactions cannot be biochemically detectable they may be critical for proper functioning of complex biological systems, (Van Criekinge W. and Beyaert R., 1999). The yeast two hybrid assay is a GAL4-based assay, based on the coexpression of the bait protein (here CALM fused in frame to the GAL4- DBD) and a prey protein (a library of random cDNA's expressing protein X fused in frame to the GAL4-AD). The plasmid cDNA library used for the screen was a commercially available HeLa cDNA-library that was cloned into the pGAD-GH vector and a human thymus cDNA library cloned into pACT2. When an interaction between the bait and prey proteins occurs, the GAL4-DBD and GAL4-AD are brought into proximity and a transcriptional activator complex is reconstituted that activates the expression of reporter genes in the yeast (Figure 2.1). The yeast strain used is AH109 that contains the reporter constructs in which the ADE2, HIS3 and lacZ/MEL1 genes are under control of different GAL UAS and TATA boxes. The single yeast clones can be obtained by plating them on selective plates. Expression of HIS3 and ADE2 enables the yeast cells to grow on plates lacking nutrients (2.1.6.2). MEL1 provides the catalytic color reaction on the plates supplemented with X-α-GAL. The product of MEL1 gene converts X-α-GAL into a blue color.

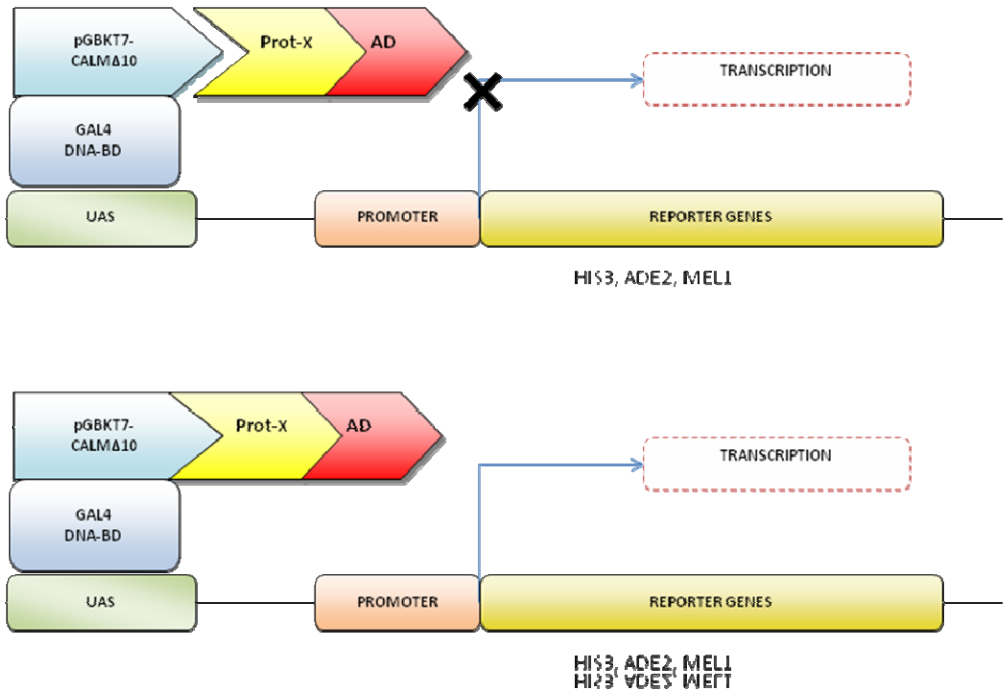


Figure 2.1The CALMΔ10 fused to GAL4 DBD binds to GAL4-UAS upstream of the reporter genes. If no interaction occurs with a protein fused to AD the transcription of the reporter genes is not activated. If the interaction appears the transcription of the reporter genes will be activated.

The LiAc- transformation method is a method of introducing foreign DNA into the yeast. Typically it has a transformation efficiency of 10^5 transformants per µg of DNA when a single type of plasmid is used. If the yeast is simultaneously co-transformed with two plasmids, the efficiency is usually an order of magnitude lower due to the lower probability that a yeast cell will take up both plasmids. The lithium acetate method is based on the fact that the competence of DNA uptake level of the cell wall is induced by treatment with lithium ions and PEG.

The day before the experiment a pre-culture was prepared with 50 ml of YPD with 2% glucose inoculated with a fresh (< 1 month old) AH109 yeast colony. Thereafter it was vortexed vigorously for 5 min to disperse any clumps. The pre-culture was incubated at 30˚C overnight shaking at 250 rpm. The OD_{600} was measured until it had reached 1.5. 30 ml of the overnight culture was diluted into 300 ml of YPD or to an OD_{600} of 0.2-0.3. The incubation of the diluted culture is continued for 3 hrs and the OD_{600} is again measured. It should have a value of 0.4-0.6 when the yeast is in its log-phase growth. Cells are harvested at that moment by centrifuging them at 1000 x *g* for 5 min at RT. The cell

pellet is resuspended in 25 ml of sterile water and recentrifuged at 1000 x *g* for 5 min at RT. Then the cells are resuspended in sterile 1xTE/ 1xLiAc. For each transformation reaction, 100 µl of competent yeast cells, 0.1 mg herring testes carrier DNA and 0.1 µg plasmid DNA were mixed in a sterile 1.5 ml eppendorf tube, and 600 µl of 1x PEG/LiAc solution was added. The transformation reaction was incubated at 30˚C, vigorously shaking at 200 rpm for 30 min. Then 70 µl of DMSO was added and the culture was mixed gently by inversion, followed by a heat shock at 42˚C for 15 min. The cells were cooled on ice for 1-2 min and then centrifuged in RT at top speed for 5 sec. The pellet was resuspended in 1xTE buffer. 100 µl of the cells were streaked on selective plates and incubated at 30˚C until the colonies appeared (3-7 days).

2.2.17.1 Test of the bait protein

Using the lithium acetate transformation protocol, pGBKT7-CALMΔ10 and pGBKT7 (used as the control) were transformed separately into the yeast strain AH109. The cells were streaked on SD –Trp plates. The transformed yeast cells were then replated on plates lacking Trp and His. If growth occurred on this selection plate, it would indicate an activation of the HIS3 reporter gene through the bait protein alone. This would mean that the construct would have to be modified to exclude any domain that acts as an activation domain.

2.2.17.2 Large scale cDNA library transformation

To obtain high transformation efficiency e.g. high number of transformants expressing individual library plasmids, a large scale transformation was done with the Hela S3 and the human thymus cDNA libraries. By sequential transformation the pGBKT7-CALMΔ10 bait plasmid was introduced into the yeast cells as in small scale transformation described in 2.2.16.1. The pGBKT7-CALMΔ10 positive colonies were grown on SD –Trp plates whereafter they were made competent as described in 2.2.16.1. 16 µl (8µg) of Thymus cDNA and 2 µl (3µg) of HeLa cDNA was used to obtain a maximal amount of transformation efficiency (this was calculated in pilot experiments), that in this case was $4x10^4$ transformants/µg DNA. After the transformation cells were incubated in 12.5 ml (250 µl/plate) of YPD medium for 1hr in 30°C. After centrifuging,

the pellet was washed and resuspended in 12.5 ml 1xTE buffer. 250 µl of the transformed yeast cells were plated on 50 medium stringency (SD –Trp, -Leu, -His) selection plates, which were incubated at 30°C for 3 to 7 days or until the colonies appeared. To estimate the transformation efficiency, 2 µl of the transformation mixture was streaked on SD –Leu and SD –Trp, -Leu.

2.2.17.3 Isolation of plasmid DNA from yeast

The positive clones were inoculated into 2 ml of SD-medium in a sterile 15 ml Falcon tube, and grown O/N at 30°C with vigorous shaking (230rpm). 1.5 ml of each overnight culture was transferred to an Eppendorf tube and was spun for 5 sec at top speed. The pellet was vortexed and thereafter resuspended in 200 µl of breaking buffer, 0.3 g of glass beads (0.45-0.52 mm acid-washed) and 200 µl of phenol/chloroform/isoamyl (25:24:1). This mixture was vortexed for 2 min at high speed, and thereafter centrifuged for 5 min at top speed at RT. 250 µl of the supernatant was then transferred to a new Eppendorf tube, and the DNA was ethanol precipitated as described in 2.2.4. The DNA was resuspended in 20 µl sterile water and 5 µl were used for transformation into chemically competent bacterial cells 2.2.6.1.

2.2.18 Protein extraction

2.2.18.1 Protein extraction from yeast cells

A fresh (< 1 month old) yeast colony was inoculated in 5 ml YPD medium, mixed by vortexing and incubated O/N at 30°C with vigorous shaking (230 rpm). The overnight culture was measured with UV-spectrometer as described in chapter 2.2.2 and a total volume of 2 OD's were transferred to fresh Eppendorf tubes. The culture was centrifuged for 15 min at 14'000 rpm at RT. The pellet was resuspended in 250 µl of 50 mM Tris (pH 8). 50 µl of Rödel mix (2.1.5) was added to the mixture and incubated for 10 min on ice. 220 µl of 72% Trichloroacetic acid (TCA) was added and the mixture was mixed by vortexing. Another incubation was done on ice for 15 min, whereafter the mixture was centrifuged for 40 min at 14 000 rpm in 4ºC. The pellet was washed with 1ml pre cooled acetone by centrifuging 10 min at 14 000 rpm at 4ºC. Pellet was air dried for 10 min at RT, and resuspended in 200 µl of 2x Lämmli buffer. Samples were subsequently boiled for 5 min at 95ºC, and 30 µl of the samples were loaded on a 10% SDS PAGE.

2.2.18.2 Protein extraction from mammalian cells

Extraction of proteins from transfected and non-transfected cells was performed by washing the cells with ice-cold PBS (2000 rpm for 5 min at 4˚C). The cells were lysed in RIPA buffer (for Western blot), with freshly added protease inhibitors (cocktail diluted 1:100). The cells were homogenized by rotating them (15 rpm) at 4ºC for 30 min and thereafter the cell extract was cleared by centrifugation at top speed for 15 min at 4ºC. The supernatant was transferred to a fresh sterile microcentrifuge tube and stored at -80ºC.

2.2.18.3 Cytoplasmic protein extraction for immunoprecipitation (IP)

$1x10^7$ 293T cells were harvested by centrifuging at 1 000 rpm for 5 min. The cells were washed three times with 5 ml ice cold PBS by centrifuging at 2 000 rpm for 5 min. The pellet was resuspended in buffer A (2.1.5) and incubated on ice for 10-15 min with occasional tapping. Nuclei were pelleted by centrifugation at 2 000 rpm for 5 min at 4°C.

The cytoplasmic fraction (the supernatant) was transferred to a fresh sterile microcentrifuge tube and was further used for IP (2.2.21).

2.2.19 Determination of protein concentration

The Bradford method was used to determine of protein concentration. A standard Bovine Serum Albumine- BSA curve, against which the protein concentration was measured, was calibrated with diluted stock solution (1 µg/µl BSA) in the volume of d_2H_2O shown in the table below.

BSA	0	1	5	10	15	20	25
d_2H_2O	800	799	795	790	785	780	775
Bradford reagent	200	200	200	200	200	200	200

For measuring the protein, 1 µl of the protein extract was diluted in 799 µl d_2H_2O and 200 µl of Bradford reagent was added to each sample. Measurements were performed with an Eppendorf BioPhotometer 6131.

2.2.20 SDS-PAGE gel electrophoresis

The protein samples prepared for the analyses were all denatured and coated with detergents by heating in the presence of SDS and a reducing agent. This results in a net negative charge which is proportional to the size of the protein. The proteins are then separated on the basis of their molecular mass. To separate the proteins, SDS-Polyacrylamide (SDS-PAGE) gel electrophoresis with denaturing conditions was used. Depending on the size of the proteins, the concentration of the gels was determined according to the table below:

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

The components for the resolving gel were mixed as follows:

Components (5 ml)	8%	10%	12%
d_2H_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	0.050 ml	0.050 ml	0.050 ml
10% APS	0.050 ml	0.050 ml	0.050 ml
TEMED	0.003 ml	0.002 ml	0.002 ml

The stacking gel was prepared after the resolving gel was polymerized as follows:

Components (2 ml)	Stacking gel 5%
d_2H_2O	1.4 ml
30% Acrylamide mix	0.33 ml
1 M Tris/HCl (pH 6.8)	0.25 ml
10% SDS	0.02 ml
10% APS	0.02 ml
TEMED	0.002 ml

As the resolving gel already was polymerized the stacking gel was poured directly on it.

2.2.20.1 Coomassie staining

To detect the proteins in the gel after the electrophoresis, the PAGE gel was Coomassie stained by incubating the gel for 1 h in Coomassie® solution (BioRad) at RT with gentle shaking. The proteins were fixed and the unspecific binding of the Coomassie® solution was washed away with 10% acetic acid and 40% methanol diluted in d_2H_2O. Destaining was performed at RT O/N, or until the protein bands were visible. The stained gel was fixed by incubation in 1% glycerol diluted in d_2H_2O for 30 min to 1 hour with gentle shaking at RT. Finally, the gel was dried for 2 hrs at 80˚C in a Gel Air™ Drying System (BioRad).

2.2.20.2 Western blot analysis

After separation on a SDS PAGE gel, the proteins were transferred to membranes for further analysis. The transfer from the SDS PAGE gel to a nitrocellulose membrane was performed with a semi-dry system. The blotting papers (GB003) and the nitrocellulose membrane were pre-wetted in transfer buffer. 3 sheets of blotting paper, the nitrocellulose membrane, the gel and finally again the 3 blotting papers were laid on top of each other on a Trans-Blot® SD Semi–Dry Transfer Cell (BioRad). The negatively charged proteins were transferred to the membrane under electric current from negative to the positive

pole at 200 mA for 1 hr. After transfer, the non specific binding sites on the membrane were blocked with 5% nonfat dried milk for one hour at RT. The specific antibodies were diluted in 5% nonfat dried milk to their optimal concentration. Immunoblotting was carried out by incubating the primary antibodies O/N at 4˚C. Then the membranes were washed 3x10 min with TBST, to remove all the unbound antibodies. To detect the primary antibody, a secondary antibody[1] was applied to the membrane and incubated for 1 hr at RT whereafter the membrane was washed (3x10 min) with TBST. Finally, enhanced chemiluminescent reagent was applied for 5 min at RT (ECL, Amersham Pharmacia biotech). The membrane is then wrapped in Saran wrap and placed in an X-ray cassette. The films were exposed between 15 sec and 15 min depending on the strength of the signals.

Re use of the membranes was possible by stripping, which was performed by incubating membranes in the stripping solution (2.1.5) at 56˚C for 1hr. The stripped membrane was washed twice with TBST for 10 min and the blocking procedure with milk was repeated as described above.

2.2.21 Expression of GST fusion proteins

To express the GST-FHL2 fusion protein the bacterial strain XL1'blue (2.1.7) was transformed (2.2.6.3), with the pGEX4T1-FHL2 construct (2.1.11). The transformed colonies were inoculated into 10 ml LB medium with Ampicillin and inoculated O/N at 37°C with vigorous shaking. The O/N culture was diluted in 100 ml LB medium with Ampicillin and grown to an OD_{600} of 0.6-0.8 at 37°C. The expression of the GST fusion protein was induced by incubating the cells for 2 hrs in the presence of 1 mM IPTG. The cells were harvested by centrifugation at 4 500 rpm for 15 min at 4°C and the pellet was resuspended in 5 ml ice-cold NETN buffer plus protease inhibitors (1:100 protease inhibitor cocktail and 1 mM PMSF). Sonification of the cells was performed for 30 sec on ice, whereafter the cell lysates were transferred to Eppendorf tubes and centrifuged at 14 000 rpm for 5 min at 4°C. The protein extracts were stored at -80°C. To examine if the

[1] The secondary antibody, conjugated to horseradish peroxidase (HRP) enzyme catalyzes a colorimetric reaction when appropriate reagent is added, here ECL (Amersham Pharmacia biotech), resulting in a deposition of the substrate on the membrane at the reaction site.

induction of proteins had occurred, 20 µl of the lysates were run on a SDS PAGE gel whereafter the gel was Coomassie stained (2.1.5).

2.2.21.1 Purification of GST fusion proteins

The GST fusion proteins were purified by incubating them with 100 µl Glutathione-agarose beads (pre washed 3x in NETN buffer) O/N at 4˚C with 15 rpm rotation. The beads were pelleted by centrifugation at 1000 x g for 5 min at 4˚C, and subsequently washed 3 times with ice cold NETN buffer. 10 µl of the slurry beads in buffer were ran on SDS PAGE gel and stained with Comassie solution (2.2.19.1).

2.2.21.2 In-vitro-expression of the radiolabeled protein

The *in vitro* expression system, TNT® -reticulocyte-lysate system from Promega was used to label proteins with radioactive S^{35}-methionine. The proteins to be expressed were cloned in-frame into the pGBKT7 vector (2.1.10). This vector contains a T7 promoter sequence at the 5' end of the multiple cloning sites. T7 RNA polymerase is used to transcribe the insert at high copy number in the reticulocyte lysate and the resulting RNA is then translated into proteins. The Master Mix contains the reticulocyte lysate, RNA polymerase, amino acids excluding the methionine, RNasin ribonuclease inhibitors and buffer.

The procedure was performed as follows:
40 µl of TNT Master Mix
2 µl of [S^{35}] Methionin (>1.000Ci/mmol at 10mCi/ml, Amersham)
1 µl of DNA Template(s) (1 µg/µl)
7 µl Nuclease-Free Water

The reaction was incubated at 30°C for 90 min.

2.2.21.3 In vitro GST pulldown assay

The purified GST fusion protein (2.2.20.2) was used for the GST pulldown assay. The reaction for the pulldown was mixed as follows:

20 µl of in vitro translated (S^{35}-methionine-labeled) pGBKT7-CALMΔ10
10 µl of purifed GST-FHL2
100 µl GST-buffer

The reaction was incubated ON at 4˚C on a rotating platform. After incubation the reaction was centrifuged for 5 min at 2000 rpm at 4˚C. The beads were washed 4 times with 500 µl GST buffer (2.1.5) and finally resuspended in 20 µl of 2x Lämmli loading buffer (2.1.5). The reaction was boiled at 95°C for 5 min and run on 10% SDS PAGE gel. The gel was stained with Comassie blue for 1h and thereafter destained with a solution consisting of 10% acetic acid and 90% d_2H_2O. To avoid damage to the gel during drying, the gel was incubated in 1% glycerol water for 30 min. The gel was placed onto pre-wetted 3 MM Whatman paper, covered with Saran wrap and dried at 80°C under constant vacuum for 2 hrs. The Saran wrap was then removed, and the gel was exposed to an X-ray film (Kodak) O/N at RT.

2.2.22 Immunoprecipitation

Plasmids expressing the desired proteins were co transfected into HEK293T cells by the lipofectamin method (2.2.14) Cells were lysed as described in 2.2.17.3 24 h after the transfection. Protein G-agarose beads (Roche) were washed with DPBS. 200 µl lysate was incubated with 3µg anti-CALM antibodies (2.1.13) for 1 hr. Thereafter the protein G-agarose beads were added to the total lysate and the mixture was incubated for another 3 hrs rotating in 4˚C and then put on ice for 30 min. The mixture was centrifuged for 5 min at 2000 rpm at 4˚C. The supernatant was discarded and the beads were washed with 1 ml NETN buffer (2.1.5) 4 times. The beads were resuspended in 5 µl of Lämmli buffer and boiled at 95 ˚C for 5 min. The immunoprecipitated proteins were analyzed by 10% SDS-PAGE (2.2.19), blotted, and detected with, respectively, monoclonal mouse anti-V5 and a mixture of polyclonal goat anti-CALM antibodies. Secondary antibodies used were the horseradish peroxidase-coupled goat anti-mouse and the donkey anti-goat IgG. Detection was achieved with the ECL detection system (2.1.1).

For the second immunoprecipitation experiment performed with GFP binder beads (Rothbauer U. et al., 2008)200 µl lysate as described in 2.2.17.3, 15µl slurry pre washed GFP binder beads, and 200 µl IP buffer (2.1.5) were incubated for 30 min rotating at 4ºC. The beads were then washed 3x with the IP buffer whereafter they were resuspended in 5 µl of Lämmli buffer and boiled at 95 ˚C for 5 min.

2.2.23 Immunofluoroscence

2x 10^5 U2OS cells were seeded and grown on 18 x 24 mm coverslips placed in 6 well plates. Cells were incubated for 24 hrs at 37°C in a CO_2 incubator. Thereafter they were washed with PBS and fixed for 10 min in 2% PFA (2.1.5). After fixation, the cells were washed 3x with PBS and incubated in the dark for 15 min in PBS/NP-40 (0.1%) whereafter the cells were blocked with PBS/FBS (10%) for 1.5 hour in RT. For the immunofluoroscence reaction the FLAG antibodies were diluted 1:500 in 1.5 ml PBS/FBS (1.5%), added to the fixed cells and incubated O/N at 4°C in a humidified chamber. Next step was to wash the cells 3x6 min with PBS/NP-40 (0.1%) at RT. The secondary antibody Alexa 555 diluted 1:500 in PBS/FBS (1.5%) and the cells were incubated for 45 min at RT. The cells were subsequently washed 3x15 min with PBS/NP-40 (0.1%). Finally a DAPI counter-stain (5 mg/ml stock solution, diluted 1:10'000 in PBS) was applied for 2 min and the cells were washed once with PBS. Coverslips were mounted on slides with Dakocytomation Mounting Medium and sealed with nail polish. Cells were visualized with an automated Axiovert 200M microscope (Carl Zeiss, Jena, Germany) equipped with single-band pass filter sets for visualization of DAPI, yellow (YFP) and red (Cy3) fluorescence.

2.2.24 GAL4-based reporter gene assays

Reporter gene assays are used to study eukaryotic gene expression. They are most frequently used as indicators for the transcriptional activity in the cells. The reporter system used is based on the expression of a protein of interest fused to GAL4-DNA binding domain (DBD) in the mammalian system. The protein is targeted to the cell nucleus and bound to the $GAL4_{UAS}$ that is located in the promoter region of the reporter gene. If the protein of interest has transcriptional properties it will either repress or activate the expression of the luciferase reporter genes as seen in fig 2.2.

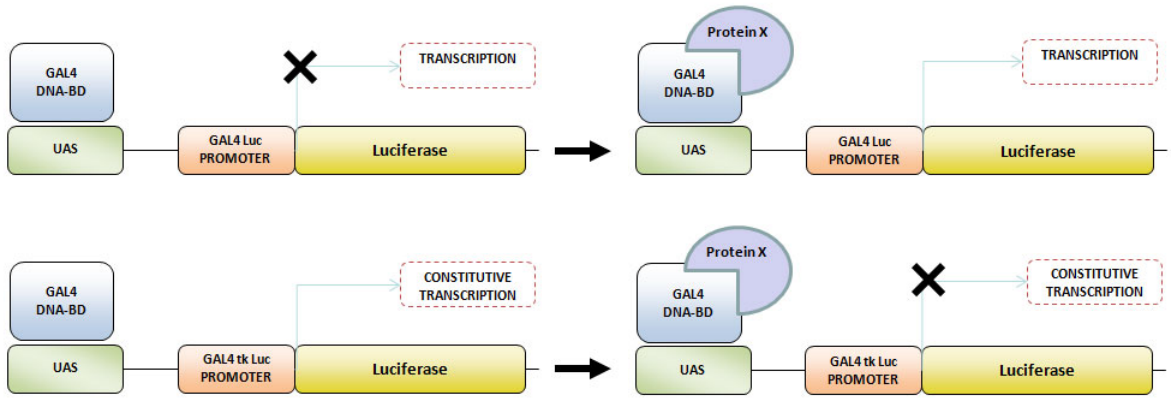


Figure 2.2 Reporter gene assay. In mammalian cells the GAL4-DBD fusion protein is expressed. The GAL4-DBD of the fusion protein binds to the $GAL4_{UAS}$ in the promoter region of the reporter gene. (A) If the expressed protein is an activator of transcription the transcription of the luciferase reporter gene, from the $pGAL4_{LUC}$ plasmid, will be induced. (B) If the protein is a repressor of transcription the constitutive expression of the luciferase reporter gene, from the $pGAL45_{tk}LUC$ plasmid will be repressed.

Transfection of the human embryonal kidney 293T cells was performed as described in 2.2.16. 24 hrs after the transfection, cells were washed with 1 ml PBS and 120 µl of passive lyses buffer (PLB) was added to the cells ($5x10^4$ cells). The cells were incubated for 15 min at RT shaking vigorously. After incubation, the cell lysates were mixed by pipetting and 10 µl of the lysate was applied to 96-well luminometry plates (Promega). The Dual-Luciferase® Reporter Assay System (Promega) was used because it provides the possibility to measure the two reporter genes firefly *Photinus pyralis* and *Renilla*

Renilla reniformis, sequentially from a single sample. The assay was processed by Orion Microplate Luminometer as follows: 50 µl Luciferase Assay Reagent II (LARII) was injected to the samples and the firefly luciferase activity was measured. Next, Stop & Glo® Reagent was injected to the samples and the *Renilla* luciferase activity was measured. The program was performing a 2-sec pre measurement delay, followed by a 10-sec measurement period for each reporter assay. *Firefly* luciferase expression was normalized to the *Renilla* luciferase expresssion.

2.3 Micro array analysis

2.3.1 Expression analysis of FHL2 in leukemia samples

Micro array data from 139 samples, 129 leukemia samples and 10 normal bone marrow samples, were used to analyze the expression levels of FHL2. The HG-U133A and HG-U133B Affymetrix chips were used. The leukemic patient samples were from 13 different leukemia subgroups; CML, CALM/AF10 positive leukemias, AML_nk: AML with normal karyotype, AML_comp: AML with complex aberrant karyotype, AML_MLL: AML with MLL rearrangement,s AML_M4: AML with CBFB/MYH11 fusion, AML_M3: AML with PML/RARA fusion, AML_M2: AML with AML1/ETO fusion, AML_FLT3: AML with normal karyotype and with a FLT3 internal tandem duplication, ALL_Ph: ALL with BCR/ABL fusion, c-ALL (common ALL) and pro-ALL. The data set was normalized using the VSN (Variance stabilization and calibration for microarray data) and presented as boxes using the “boxplot” function. The expression signal intensities are displayed on a logarithmic scale.

3 Results

3.1 Identification of CALM interacting proteins using the yeast two hybrid system

In order to identify CALM-interacting proteins, yeast two-hybrid screens were performed. Previously, different CALM deletion mutants had been cloned in frame into the pGBKT7 and pAS2 yeast expression vectors as fusions with the GAL4 DNA binding domain (GAL4-DBD). They had been tested for transcriptional activity in yeast with respect to different reporter genes (HIS3, ADE2 beta-GAL) (2.2.16.2). These experiments revealed the presence of at least two independent transcriptional transactivation domains in CALM between aa 408 and 572. The longest C-terminal CALM deletion mutant without transactivation activity, the pGBKT7-CALMΔ10 (fig 3.1), was used as bait for the yeast two hybrid screens (Fröhlich-Archangelo L. et al., 2006)

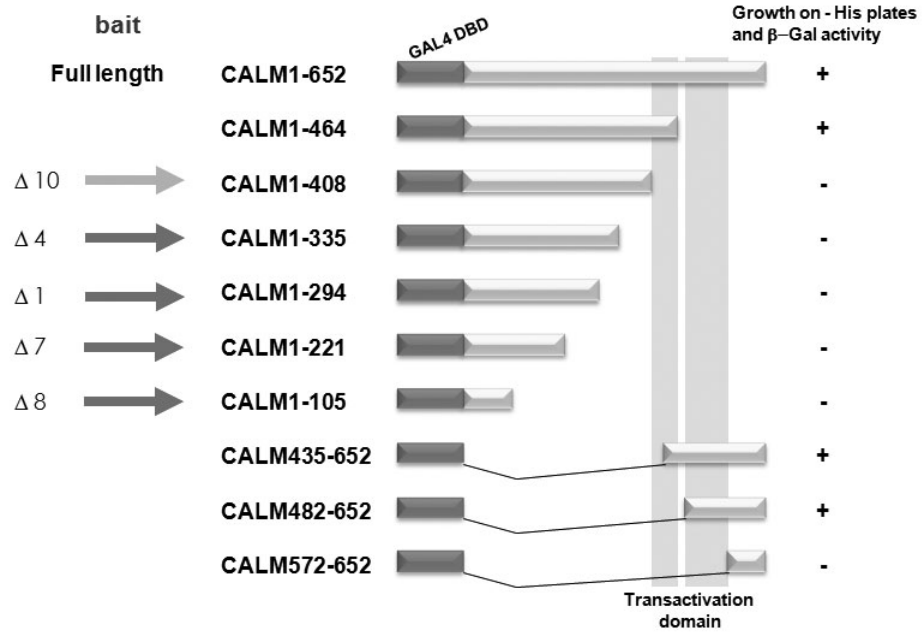


Figure 3.1 Diagrammatic representation of GAL4-DBD-CALM deletion constructs. "+" and "-" indicates growth and no growth of AH109 yeast cells transformed with the CALM deletion constructs and then plated on SD plates lacking tryptophan and histidine (Fröhlich-Archangelo L. et al., 2006).

Two human cDNA libraries expressing proteins as GAL4 activation domain fusion proteins (prey) were screened with the pGBKT7-CALMΔ10 bait plasmid: a HeLa (HeLa S3) and a Thymus cDNA library.

If the bait and prey proteins interact the GAL4-DBD and GAL4-AD are brought into proximity. The transcriptional activator complex is then reconstituted and activates the expression of reporter genes in the yeast. The yeast strain used was AH109 which includes four reporter genes ADE2, HIS3 and lacZ/MEL1 genes.

For the yeast two hybrid screen using the HeLa cDNA library, a high stringency screen of $2x10^6$ transformants yielded 2 and 3 colonies in two independent screens. These clones were able to grow as blue colonies on highly stringent selection plates lacking histidine, adenine, leucine and tryptophan (-His, -Ade, -Leu, -Trp) supplemented with X-α-GAL (2.2.16). The product of MEL1 reporter gene converts the colorless X-α-GAL substrate into blue color. The prey plasmid (pGAD-GH) inserts of these colonies were sequenced with vector primers Y2H2 and Y2H3'AD (2.1.12). A BLAST nucleotide similarity search of the insert sequences revealed homologies with four genes: 1) CALM interacting protein expressed in thymus and spleen (CATS); 2) poly(rC)-binding protein 1 (PCBP1); 3) Tumor susceptibility gene 101 protein (TSG101), 4) Clathrin assembly lymphoid myeloid leukemia protein (CALM). Table 3.1 summarizes the potential interacting proteins of CALM from the screens with the HeLa cDNA library and gives a brief description of each interaction protein.

Protein name	Sub Cellular localization	Protein description*	No of independent clones	NCBI Accession Nummer
CATS	Predominantly in nucleus	**C**ALM interacting protein expressed in **t**hymus and **s**pleen **[1]**	1	NM_019013
PCBP1	Nucleus. Cytoplasm	**p**oly(r**C**)-**b**inding **p**rotein **1**. Single-stranded nucleic acid binding protein that binds preferentially to oligo dC **[2]**	1	NM_006196.2
TSG101	Cytoplasm	**T**umor **s**usceptibility **g**ene **101** protein. Regulator of vesicular trafficking process. **[3]**	1	BC002487
CALM	Cytoplasm	**C**lathrin **a**ssembly **l**ymphoid **m**yeloid leukemia protein. Involved in CALM/AF10 translocation. Recruites clathrin and adaptor protein complex 2 (AP2) to cell membranes..**[4]**	1	AB210017.1

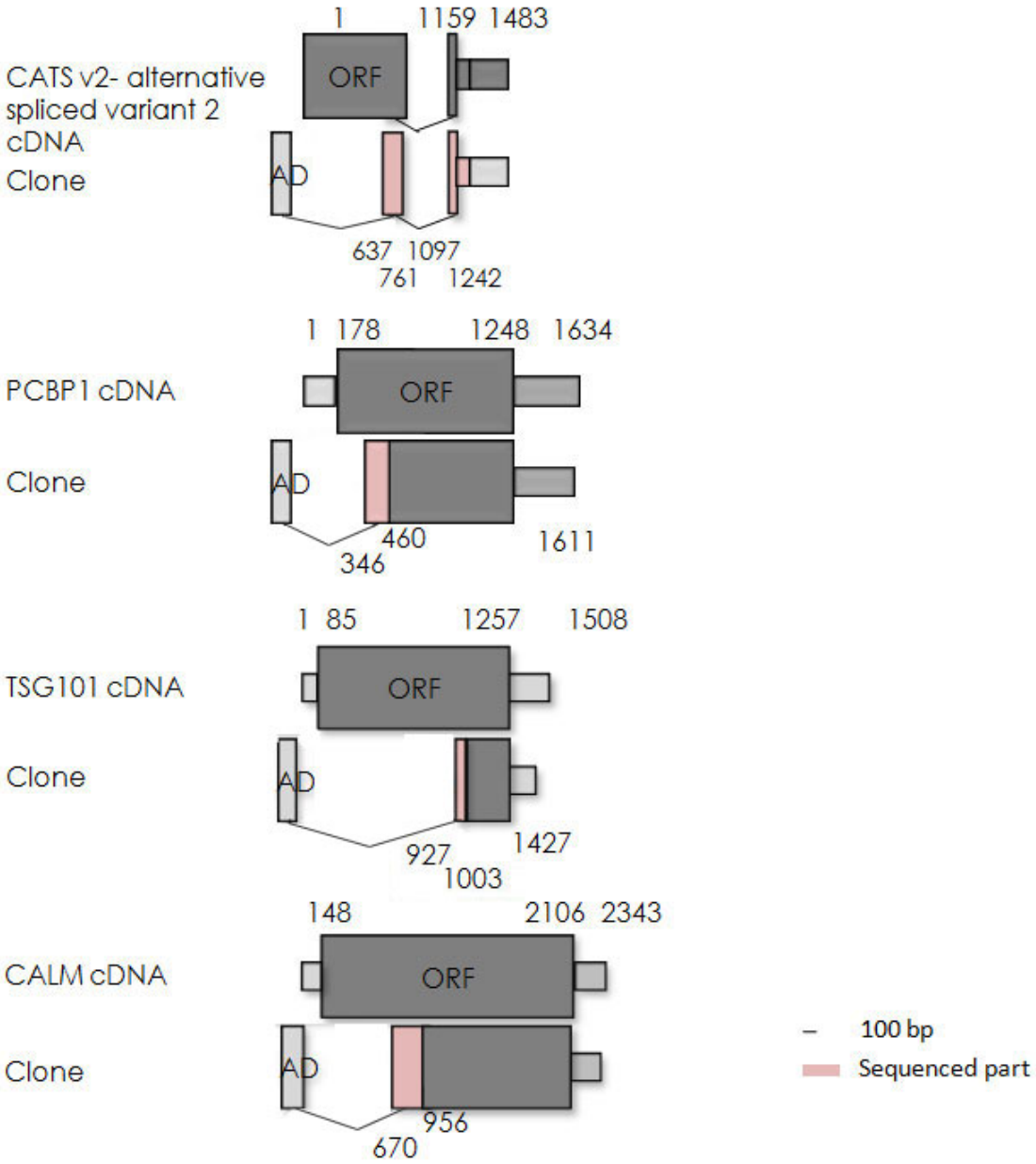
Table 3.1 Putative CALM interacting partners obtained from the HeLa S3 cDNA library

In the thymus cDNA library high stringency screen of a total of $2x10^6$ transformants in two screens 4 independent colonies were obtained in the first screen and 3 independent clones in the second screen. These 7 clones grew as blue colonies when plated on -His, -Ade, -Leu, -Trp selection plates supplemented with X-α-GAL. The inserts of the prey plasmids (pACT2) from these clones were sequenced, and the BLAST analysis revealed homologies to five different cDNAs. For one clone no sequence could be obtained. Two clones were coding for Filamin A (FLNA). One clone each was identical to: 1) Fatty acid binding protein 4- (FABP4); 2) Poly(rC) binding protein 1 (PCBP1); 3) Dipeptidyl-peptidase 7 (DPP7); 4) Four and a half LIM domain protein 2 (FHL2). Table 3.2 summarizes the potential interacting proteins of CALM from the screen with Thymus cDNA library and gives a brief description of each interaction-protein.

Protein name	Sub Cell localization	Protein description*	Nr of independent clones	NCBI Accession Nummer
FLNA	cytoplasmic	**Fil**amin **A**, actin binding protein 280, promotes orthogonal branching of actin filaments and links actin filaments to membrane glycoproteins **[5]**	2	NM_001456
FABP4	cytoplasmic	**F**atty **a**cid **b**inding **p**rotein **4**, adipocyte. Lipid transport protein in adipocytes. Binds both long chain fatty acid and retinoic acid [6]	1	BC003672.1
PCBP1	Nucleus. Cytoplasm	**p**oly(r**C**) **b**inding **p**rotein **1**, Single-stranded nucleic acid binding protein that binds preferentially to oligo dC , [7]	1	NM_006196.2
DPP7	Lysosome. Cytoplasmic vesicle	**dip**eptidyl-**p**eptidase **7**, Plays an important role in the degradation of some oligopeptides [8]	1	NM_013379
FHL2	Nucleus. Cytoplasm	**F**our and a **h**alf **L**IM domain protein **2**. Transcriptional co activator of β-catenin, integrin-signaling pathway.	1	L42176.1

Table 3.2 Putative CALM interacting partners from the Thymus cDNA library.

Figure 3.2 schematically depicts the full length cDNA sequence (protein coding region as bigger dark grey box) of the CALM interacting proteins (top in each drawing) and the prey clones (bottom) obtained from the library screens. The activation domain (AD) encodes for 113 amino acids of the yeast GAL4 transcription factor which is part of the prey vector. The pink colored box denotes the sequenced part of the clones. The clones were sequenced from the 5' as well as the 3' ends using Y2H2 and Y2H3'AD primers (2.1.12). From our yeast two hybrid experiment we obtained the C-terminus of the alternative spliced variant 2 coding for isoform 2 of CATS. The prey clones of PCBP1, CALM, FABP4 and DPP7 do not contain the N terminus. The FHL2 prey clone was full length. Note that FLNA is drawn at half scale compared to the other clones.


CATS v2- alternative spliced variant 2 cDNA
Clone
1
1159
1483
ORF
AD
637
761
1097
1242
PCBP1 cDNA
Clone
1
178
1248
1634
ORF
AD
460
346
1611
TSG101 cDNA
Clone
1
85
1257
1508
ORF
AD
927
1003
1427
CALM cDNA
Clone
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2343
ORF
AD
956
670
100 bp
Sequenced part

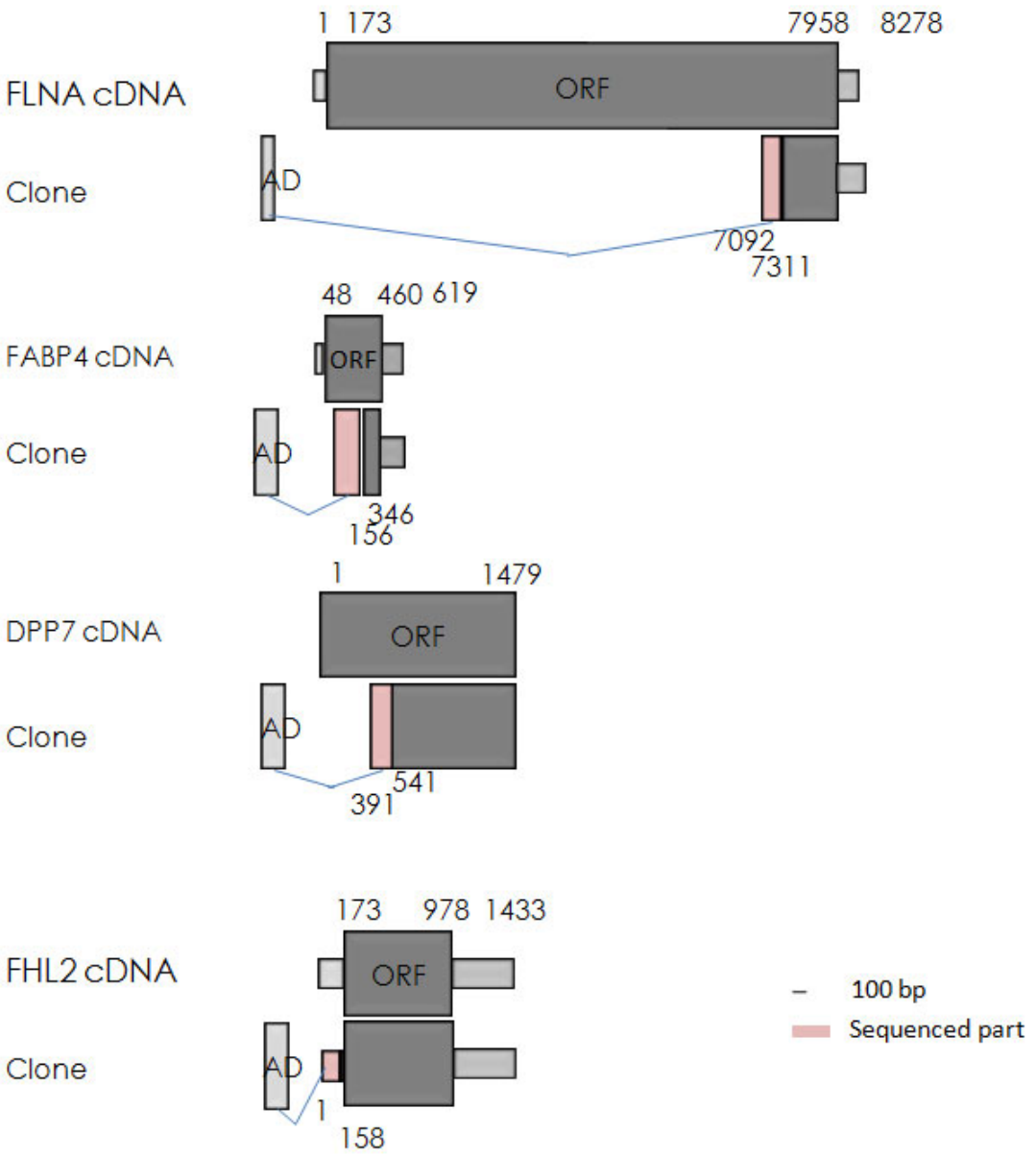


Figure 3.2 Map of cDNA, Open Reading Frames (ORFs) of full length proteins and the extent of the DNA sequence in the prey clones isolated in the yeast two hybrid screens with pGBKT7-CALMΔ10. Activation domain (AD) is coding for 113 amino acids. The pink colored box denotes the sequenced part of the clones. The clones were sequenced from the 5' as well as the 3' ends using Y2H2 and Y2H3'AD primers (2.1.12). From our yeast two hybrid experiment we obtained the alternative spliced variant 2 coding for isoform 2 of CATS. CALM, FABP4 and DPP7 sequences are all missing the N-terminal part, however the missing parts are of different sizes. TSG101 and FLNA obtained from the yeast two hybrid are containing the C-terminal part of the full length sequence. FHL2 obtained from the yeast two hybrid screen

is the full length FHL2. Because of the size, FLNA is depicted in half size in comparison to the rest of the proteins

3.2 Confirmation of potential protein interactions

As an initial test to confirm that the proteins identified in the screen are indeed CALM interacting partners, we co-transformed the CALM bait plasmid with the individual prey plasmids into yeast strain AH109. Figure 3.3 shows the growth of the co-transformed yeast clones containing the CALM bait and one of the prey plasmids of the interacting partners: FHL2, FABP4, PCBP1, DPP7, CALM, TSG101, CATS and FLNA (FLJ00343) on selective plates lacking the essential nutrients histidine (His, H), adenine (Ade, A), leucine (Leu, L) and tryptophan (Trp, W). These results indicate that all the clones initially identified in the screen could be confirmed as potential interacting partners.

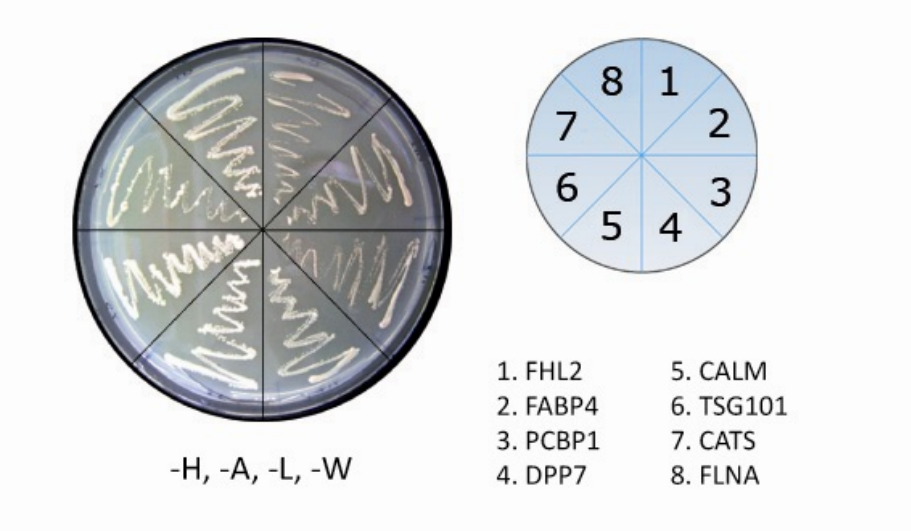


Figure 3.3 Interaction of CALM with 8 different proteins. Yeast strain AH109 was cotransformed with pGBKT7-CALMΔ10 and the prey plasmids from the screen**.** Transformed cells were streaked on high stringency SD plates lacking His, Ade, Trp and Leu.

The different interaction partners of CALM suggest that CALM is involved in several different cellular processes. The CALM interaction partner FHL2, which had been shown to be important in several leukemia-associated cellular pathways, was chosen for further detailed analysis including colocalization, co-immunoprecipitation experiments and reporter gene assays.

3.3 Mapping of the CALM-FHL2 interaction in yeast

3.3.1 Construct of FHL2 bait protein

For mapping the CALM interaction domain of FHL2, various portions of the *FHL2* cDNA were inserted into the pGBKT7 vector to produce a series of GAL4 DBD-FHL2 deletion mutants. The appropriate portions of the *FHL2* cDNA were amplified with the primers FHL2-T, FHL2-LIM1/2, FHL2-LIM1/2-1, FHL2-LIM1/2-2 and FHL2-LIM1/2-3 (2.1.12) and inserted into the *NcoI* and *BamHI* restriction sites of the pGBKT7 vector (fig 3.4 A). As a full length FHL2 clone the prey clone (fused to GAL4 AD) obtained from the two hybrid screen was used.

The FHL2 deletion mutants were transformed into yeast strain AH109. The expression of the different FHL2 deletion mutants was analyzed with Western blot using a GAL4-DBD antibody. Lanes 1- 6 show pGBKT7-LIM1/2, pGBKT7-LIM1, pGBKT7-LIM2, pGBKT7-LIM3, pGBKT7-CALMΔ10 and pGBKT7 empty, respectively (fig 3.4 B). The full length FHL2 fused to the GAL4-AD (original prey plasmid) was detected with a GAL4-AD antibody as seen in lane 1 in fig 3.4 C.

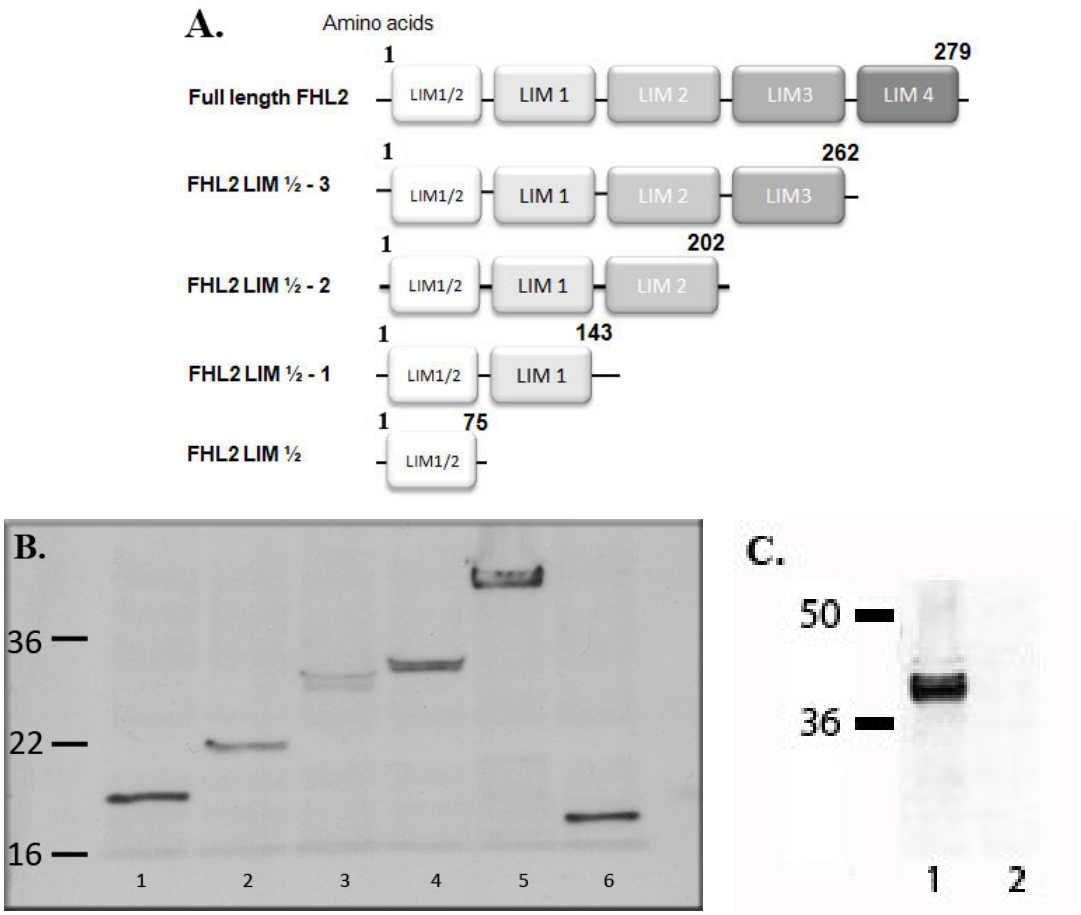


Figure 3.4 A) Diagram of the FHL2 deletion constructs cloned into pGBKT7. The deletion constructs were cloned into pGBKT7 vector to produce GAL4-DBD-fused FHL2 deletion proteins. The full length FHL2 clone in the pACT2 vector was obtained from the initial yeast two hybrid screen. **B) Western blot of FHL2 deletion mutants fused to the GAL4 DBD.** Protein was isolated from AH109 cells transformed with pGBKT7-LIM ½ (Lane 1), pGBKT7-LIM ½-1 (lane 2), pGBKT7-LIM ½-2 (lane 3), pGBKT7-LIM ½-3 (lane 4), pGBKT7-CALMΔ10 (lane 5) and pGBKT7 alone in lane 6. GAL4-DBD antibodies were used for detection**. C) Western blot of FHL2 fused to the GAL4-AD.** pACT2-FHL2 (lane 1) and pGBKT7 alone in lane 2 were transformed in AH109 yeast cells. GAL4-AD antibodies were used for detection.

3.3.2 Mapping of the CALM interaction domain of FHL2

The different FHL2 deletion mutants had been tested for transcriptional activity with respect to different reporter genes in AH109 (HIS3, beta-GAL) (2.2.16.2). These experiments did not reveal a transcriptional transactivation domains in FHL2 (data not shown). To map the CALM interaction domain of FHL2, pGAD-GH-CALM (aa 114-652) was cotransformed into AH109 with the deletion mutants of FHL2 (described in 1.3.1). Note that the full length FHL2 (fused to GAL4-AD obtained from the screen) was transformed with pGBKT7-CALMΔ10 (2.1.11). Co transformants were plated on selective plates lacking His, Ade, Leu and Trp. Growth occurred only when full length FHL2 was co transformed with pGBKT7-CALMΔ10, fig 3.5.

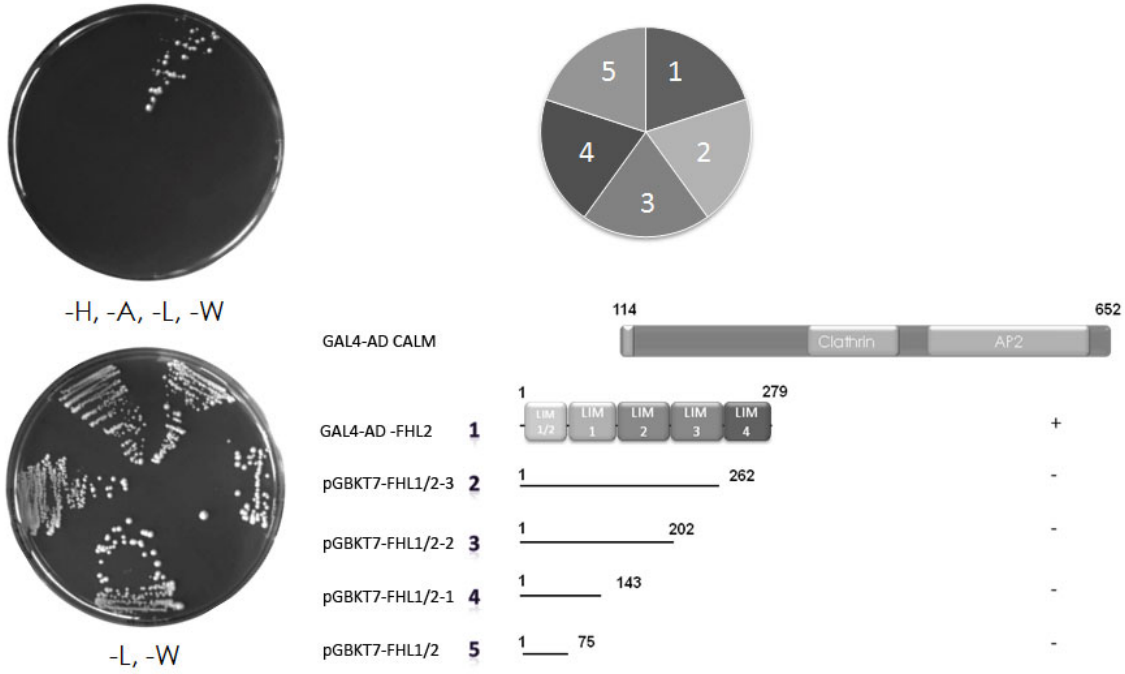


Figure 3.5 Full length FHL2 is needed for the interaction with CALM. The yeast strain AH109 was cotransformed with the various pGBKT7-FHL2 deletion mutants and the pGAD-GH-CALM (expressing aa 114-652) clone or with the pGBKT7-CALMΔ10 (fig 3.1) and the pACT2-FHL2 clone. Transformed cells were streaked out on selective plates lacking tryptophan, leucine, histidine and adenine. The interaction occurs only between the full length FHL2 fused to GAL4 AD and pGBKT7-CALMΔ10.

3.3.3 Mapping the FHL2 interaction domain of CALM

To map the FHL2 interaction domain on CALM, the various CALM deletion mutants fused to the GAL4-DBD (in the pGBKT7 and pAS2 plasmid) and FHL2 fused to GAL4-AD (in pACT2) shown in fig 3.1, were used in the yeast two hybrid assay. The co-transformants were assayed for growth on selective plates as seen in fig 3.6. CALMΔ4 (aa 1-335) and CALMΔ10 (aa 1-408) show an interaction with FHL2 in contrast to CALMΔ1 (aa 1-294), CALMΔ7 (aa 1-221) and CALMΔ8 (aa 1-105) which are not displaying any interaction. These data suggest that FHL2 requires amino acids 294-335 of CALM for interaction.

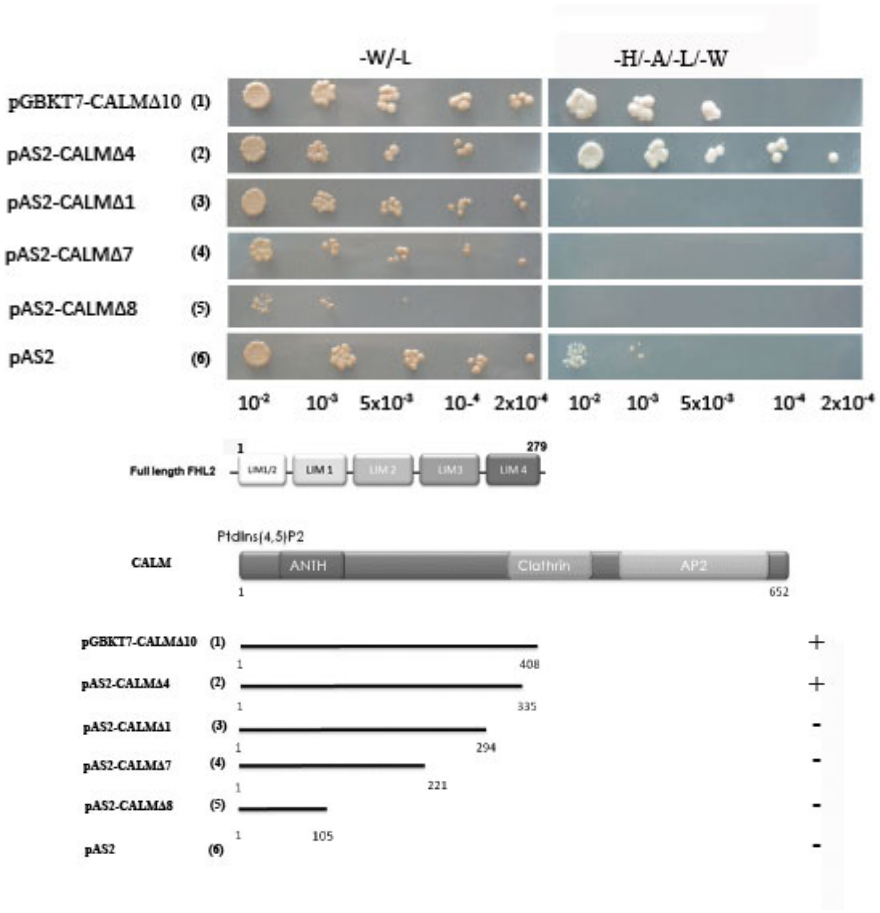


Figure 3.6 FHL2 interacts with amino acids 294 to 335 of CALM. The yeast strain AH109 was cotransformed with plasmids expressing GAL4 DBD-CALM deletion mutants and a plasmid (pACT-FHL2) expressing the GAL4 AD-FHL2 fusion. Transformed cells were serially diluted (1:100, 1:1000, 1:10000 and 1:20000) and plated on medium lacking histidine, adenine, leucine and tryptophan (-H/ -A/-L/-W). The interaction occurs only between the CALM deletion mutants CALMΔ10 and CALMΔ4, and

the full length FHL2 indicating that the FHL2 interaction domain in CALM lies between 294 and 335 of CALM.

3.4 CALM-FHL2 GST pulldown assay

To confirm the direct interaction between CALM and FHL2, a glutathione S-transferase (GST) pulldown assay was carried out.

3.4.1 Expression of FHL2

For the GST pulldown assay, full length FHL2 fused to GST expressed from the pGEX-4T1-FHL2 plasmid was used (2.1.11) (fig 3.7) (Müller J. et al., 2000). Bacterially expressed GST-FHL2 protein was purified using glutathione sepharose beads.

Figure 3.7 Diagram of FHL2 fused to GST. The FHL2 was cloned into pGEX-4T1 vector (Müller J. et al., 2000).

Subsequently the samples were loaded on a SDS PAGE gel and stained with Coomassie blue, fig 3.8. 10 µl of the proteins were loaded per lane.

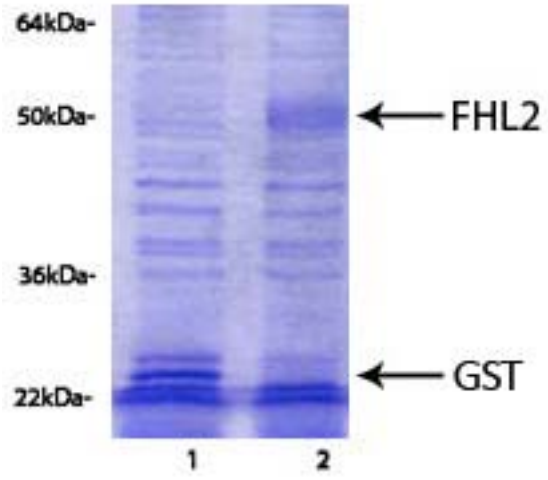


Figure 3.8 GST and the GST-FHL2 fusion protein on a Coomassie stained PAGE gel. Lane 1: protein expressed from empty pGEX-4T1. Lane 2) GST-FHL2. In each lane 10 µl protein lysate was loaded. The total amount of lysate was obtained from a 50 ml culture.

3.4.2 CALM interacts with FHL2 *in vitro*

To analyze the CALM-FHL2 interaction *in vitro*, a GST pulldown assay with the full length FHL2 fused to GST (GST-FHL2) and a S-35 methionine labeled CALMΔ10 protein (expressed from pGBKT7 in a TNT reaction) was performed. Only GST-FHL2 construct bound to glutathione beads was able to retain the S-35 methionine-labeled CALM, fig 3.9. Input reflects 10% of the protein incubated with the beads.

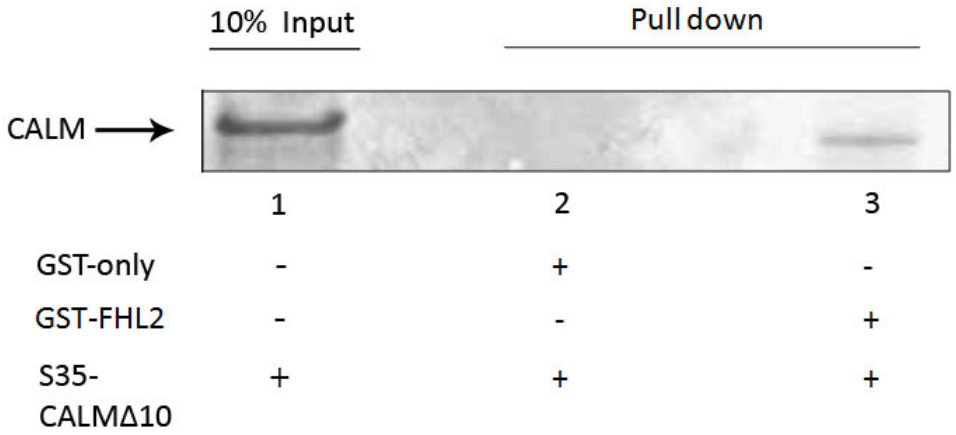


Figure 3.9 GST pulldown assay. Lane 1) 10% input of the S^{35}-labeled CALMΔ10 protein. Lane 2) no unspecific interaction occurs between GST only and S^{35}-labeled CALMΔ10 protein. Lane 3) Direct interaction between full length FHL2 fused to GST and CALMΔ10 proteins is confirmed.

3.5 CALM interacts with FHL2 *in vivo*

To establish whether CALM and FHL2 interact *in vivo,* a co-immunoprecipitation experiment was performed. Human endothelial kidney (HEK) 293T cells were transiently co-transfected (2.2.14) with pEYFP-C1-CALM and pcDNA6/V5-FHL2 (2.1.11). Immunoprecipitation was performed using CALM antibodies. After immunoprecipitation and Western blotting, the mixture of polyclonal anti-CALM antibodies detected the immunoprecipitated YFP-CALM fusion protein and the two bands with the expected size of endogenous CALM (66 and 72 kDa), (fig 3.10). The membrane was stripped and reprobed with a monoclonal anti-V5 antibody. In lane 2 a band can be seen which corresponds to the co-immunoprecipitated V5-FHL2 protein. A weak band is also seen in lane 1, suggesting that endogenous CALM interacts with overexpressed V5-FHL2. In lanes 4, 5 and 6 the input lysates are run as controls for protein expression. These results clearly show that the overexpressed YFP-CALM protein interacts with the overexpressed V5-FHL2 protein and that it is very likely that endogenous CALM is also interacting with the overexpressed V5-tagged FHL2 protein.

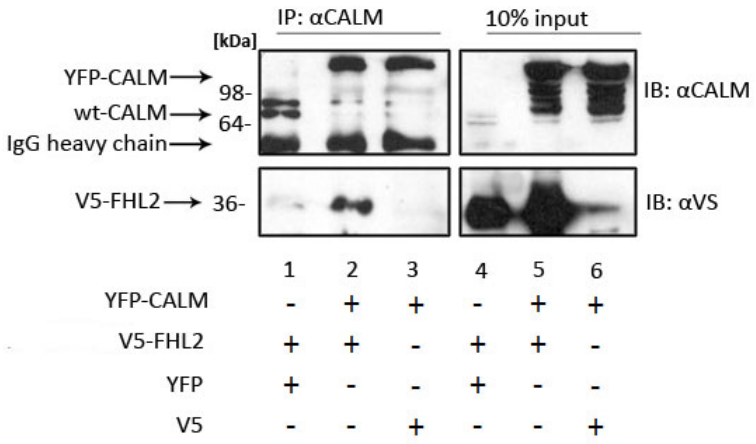


Figure 3.10 CALM interacts with FHL2 *in vivo*. Lane 1) Precipitation of endogenous CALM with CALM antibodies co-precipitates small amounts of the overexpressed V5-FHL2, seen as a weak band. Lane 2) Precipitation of YFP-CALM with CALM antibodies co-precipitates V5-FHL2. FHL2 is detected with a V5 antibody. Lane 3) V5 alone and the precipitated YFP-CALM detected with CALM antibody.

Lanes 4), 5) and 6) loaded with the input lysates. The amounts of heavy chain detected in lane 1, 2 and 3 demonstrate that the amount of antibodies used in the experiment is constant.

To exclude nonspecific binding between YFP and FHL2 we performed the co-immunoprecipitation also using the GFP-binder protein, which is a single chain camelid antibody highly specific for GFP (Rothbauer U. et al., 2008). While precipitation of YFP-CALM results in co-precipitation of FLAG-FHL2, precipitation of YFP alone does not co-precipitate FLAG-FHL2, (fig 3.11) YFP and YFP-CALM are detected by rabbit monoclonal anti GFP antibodies, while FHL2 is detected with the mouse monoclonal anti-FLAG antibody.

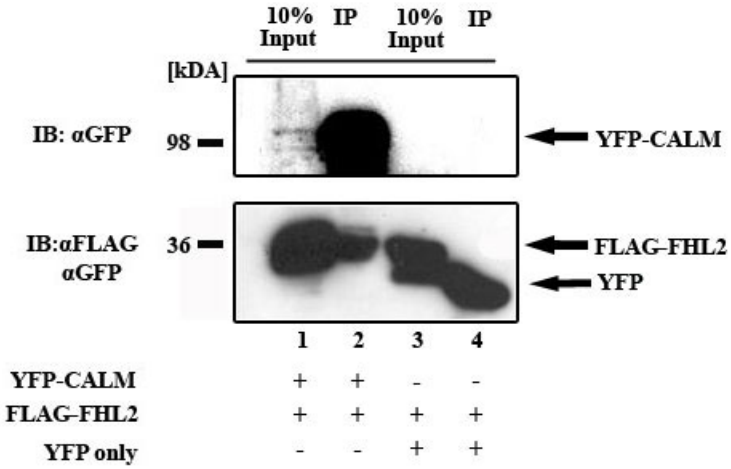


Figure 3.11 CALM interacts with FHL2 in vivo. The precipitation was performed with GFP-binder (Rothbauer U. et al., 2008) Lane 1) 10% of the input lysate from 293T cells expressing YFP-CALM and FLAG FHL2. Lane 2) The precipitated YFP-CALM co-precipitates FLAG-FHL2. Lane 3) 10% of the lysate input, from 293T cells expressing YFP and FLAG FHL2. Lane 4) The precipitated YFP does not co-precipitate FHL2. YFP-CALM is detected with rabbit anti- GFP antibody and FLAG-FHL2 is detected with mouse monoclonal anti-FLAG antibody.

3.6 Co-localization of CALM and FHL2

To further explore the CALM-FHL2 interaction and examine whether the expression of one of the proteins has an influence on the subcellular localization of the other protein, a YFP-tagged CALM and CALM/AF10 protein (YFP-CALM, YFP-CALM/AF10) and a FLAG-tagged FHL2 (FLAG-FHL2) were co-expressed in U2OS (Human Osteosarcoma cells). The transfected cells were fixed and then stained with FLAG antibody before being analyzed with a confocal microscope. As previously reported, YFP-CALM and YFP-CALM/AF10 are predominantly located in the cytoplasm (fig 3.12). It has also been shown that CALM and CALM/AF10 shuttle between the cytoplasm and nucleus (Fröhlich-Archangelo L. et al., 2006). YFP-CALM is rather homogenously distributed in the cytoplasm, while the cells expressing YFP-CALM/AF10 have a "speckled" pattern in both the cytoplasm and to a lesser extent also in the nucleus. In particular, it was noticable that cells expressing YFP-CALM/AF10 are partially forming cytoplasmic inclusions in contrast to cells expressing YFP-CALM or YFP-FHL2. As previously reported, FHL2 is localized in both the cytoplasm and the nucleus (fig 3.12) (Lee S-W. et al., 2005; Ng E.K. et al., 2002). After incubation with FLAG antibodies and Alexa 555 conjugated secondary antibodies, no signal is detected in the Cy3 channel in cells that were only transfected with YFP-CALM, YFP-CALM/AF10 or YFP-FHL2. Only minimal background signal is detected in the Cy3 channel at the maximum level of laser stimulation (600 V). Thus we exclude cross reactivity between the FLAG antibody and YFP-fusion-proteins in the immunofluorescence experiments described below.

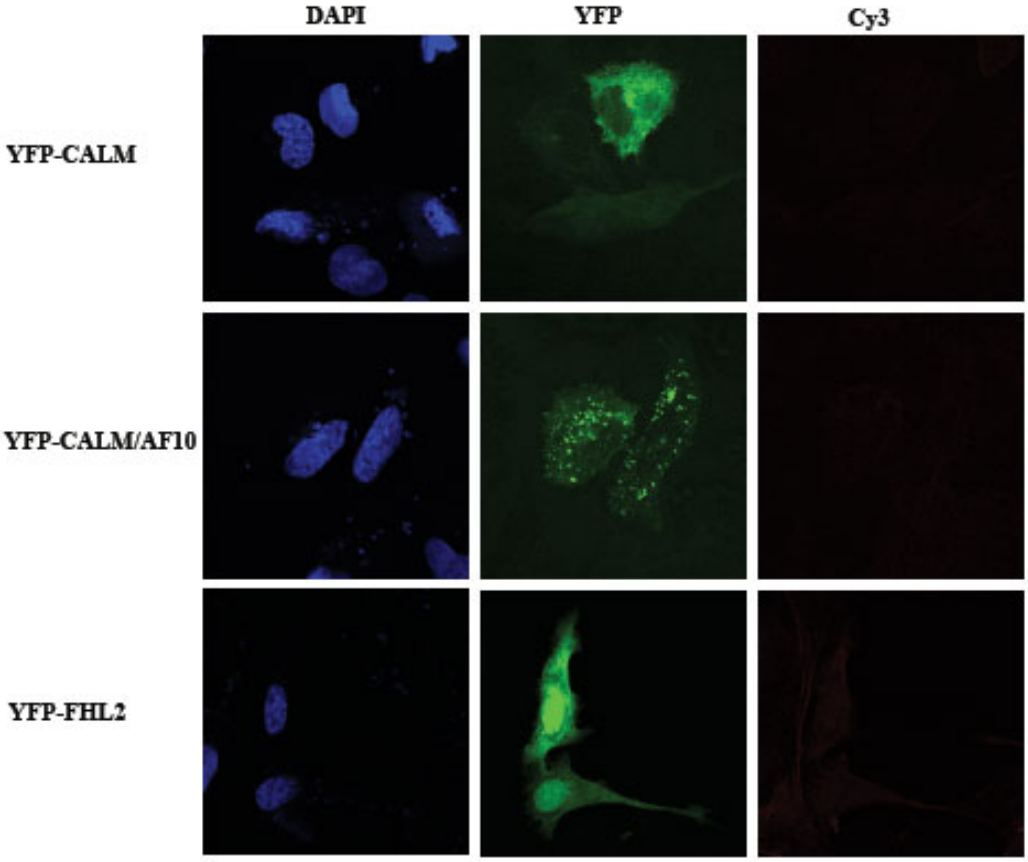


Figure 3.12 YFP-CALM, YFP-CALM/AF10 and YFP-FHL2 overexpressed in U2OS cells. YFP-CALM and YFP-CALM/AF10 are mainly located in the cytoplasm. In cells expressing YFP-CALM/AF10, inclusion bodies are visualized, in contrast to the cells expressing YFP-CALM and YFP-FHL2. YFP-FHL2 is located in both nucleus and cytoplasm. Since only minimal background signal is seen in the Cy3 channel the cross reaction between the FLAG antibody and the three different YFP-fusion proteins used in co-localization experiments was excluded.

In cells co-expression YFP-CALM and FLAG-FHL2, co-localization of the two proteins can be observed in the cytoplasm and also in several nuclear spots. Co-localization is indicated by yellow color in the merged image and overlapping peak intensities in the line scan (fig 3.13). Thus, shuttling YFP-CALM might be retained in the nucleus through interaction with FLAG-FHL2.

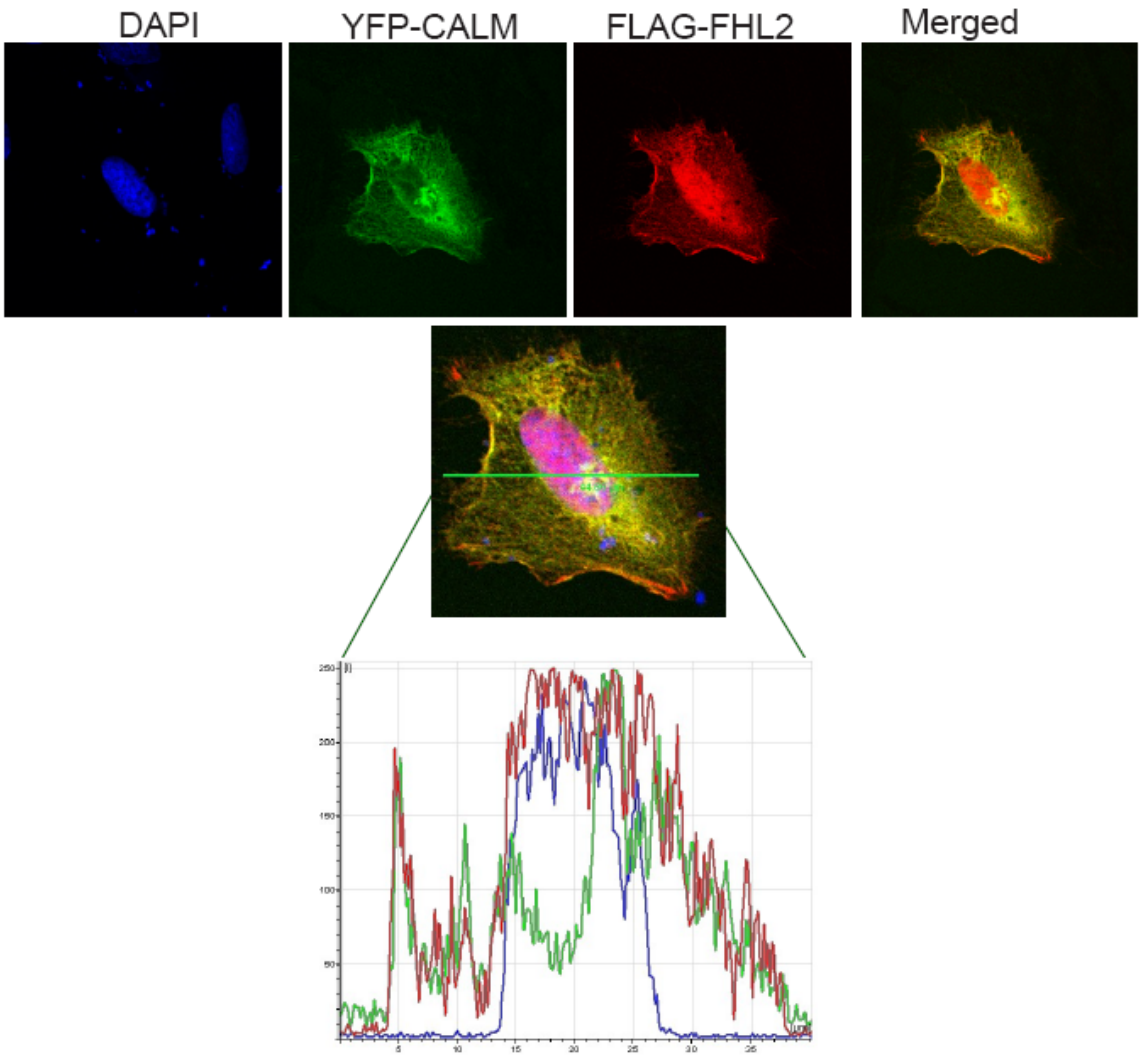


Figure 3.13 CALM and FHL2 colocalize in the nucleus and the cytoplasm. A series of confocal images of U2OS cells co-expressing YFP-CALM and FLAG-FHL2. YFP-CALM is mainly located in the cytoplasm. FLAG-FHL2 is located in both the cytoplasm and nucleus. The confocal line scan shows overlapping signal intensity peaks of YFP-CALM and FLAG-FHL2 in the cytoplasm as well as in nuclear spots. The green line corresponds to YFP-CALM, red to FLAG-FHL2 and the blue line corresponds to the DAPI staining.

3.7 Co-localization of CALM/AF10 and FHL2

In order to examine if FHL2 has an effect on the subcellular distribution of CALM/AF10 or vice versa, we performed the colocalization experiments with FHL2 and CALM/AF10.

In initial experiments, we observed that the expression of YFP-CALM/AF10, but not of YFP-CALM or of YFP-FHL2, leads to the formation of partial intracellular inclusions (fig 3.12). In the cells that co-express YFP-CALM/AF10 and FLAG-FHL2 the "speckled" pattern of YFP-CALM/AF10 remains unchanged, however, in addition to its normal distribution FLAG-FHL2 appears to be enriched in the cytoplasmic CALM/AF10 spots (fig 3.14). This co-localization is indicated by yellow color in the merged image and by overlapping peak intensities in the line scan (fig 3.14).

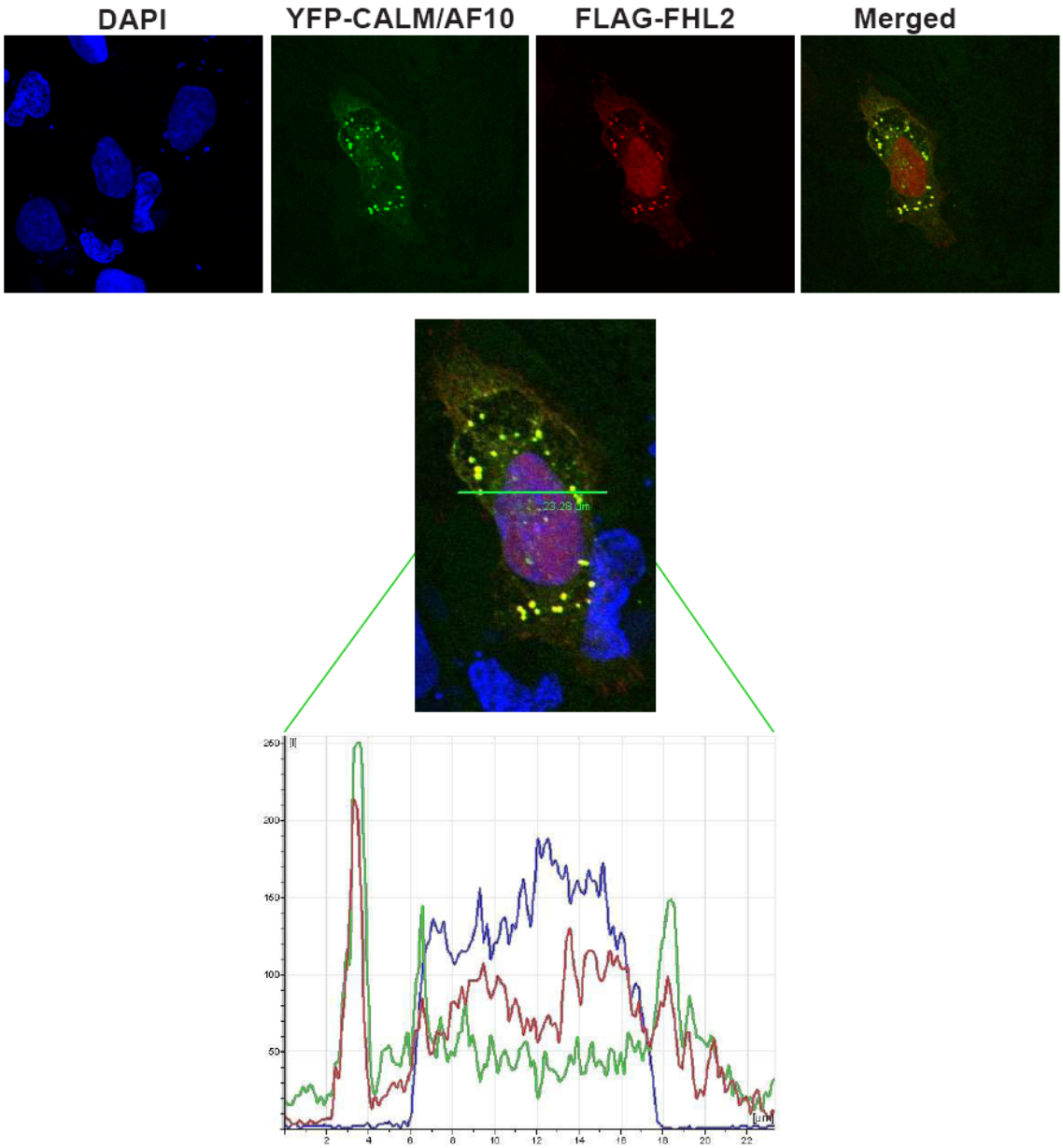


Figure 3.14 YFP-CALM/AF10 and FLAG-FHL2 co-localize mainly in the cytoplasm. While both proteins show their original distribution, with a speckled CALM/AF10 pattern and FHL2 both in the nucleus and the cytoplasm, the signals strongly overlap in cytoplasmic spots, seen in yellow color in the merged image and overlapping peak intensities in the confocal line scan. The green line corresponds to YFP-CALM/AF10, red to FLAG-FHL2 and the blue line corresponds to the DAPI staining.

To examine whether the distribution pattern of CALM/AF10 is influenced by the relatively large green fluorescent protein (GFP) fluorescent tag (27 kDa) we also used another CALM/AF10 expressing construct with a short FLAG-tag to transfect U2OS cells. FLAG-CALM/AF10 showed a similar mainly cytoplasmic localization like the YFP-CALM/AF10 protein. However, the distribution appears to be slightly more homogenous with fewer speckles. This might be due to a more stable protein expression or changes in solubility. Furthermore, we did not observe differences in the localization of FLAG-FHL2 and YFP-FHL2 when expressed alone. Co-expression of YFP-FHL2 together with FLAG-CALM/AF10 led to similar results like the co-expression of the YFP-CALM/AF10 and FLAG-FHL2 proteins, with co-localization in the cytoplasm (fig 3.15). Thus we could exclude any major influence of the fluorescent tags on the subcellular localization of the proteins we examined.

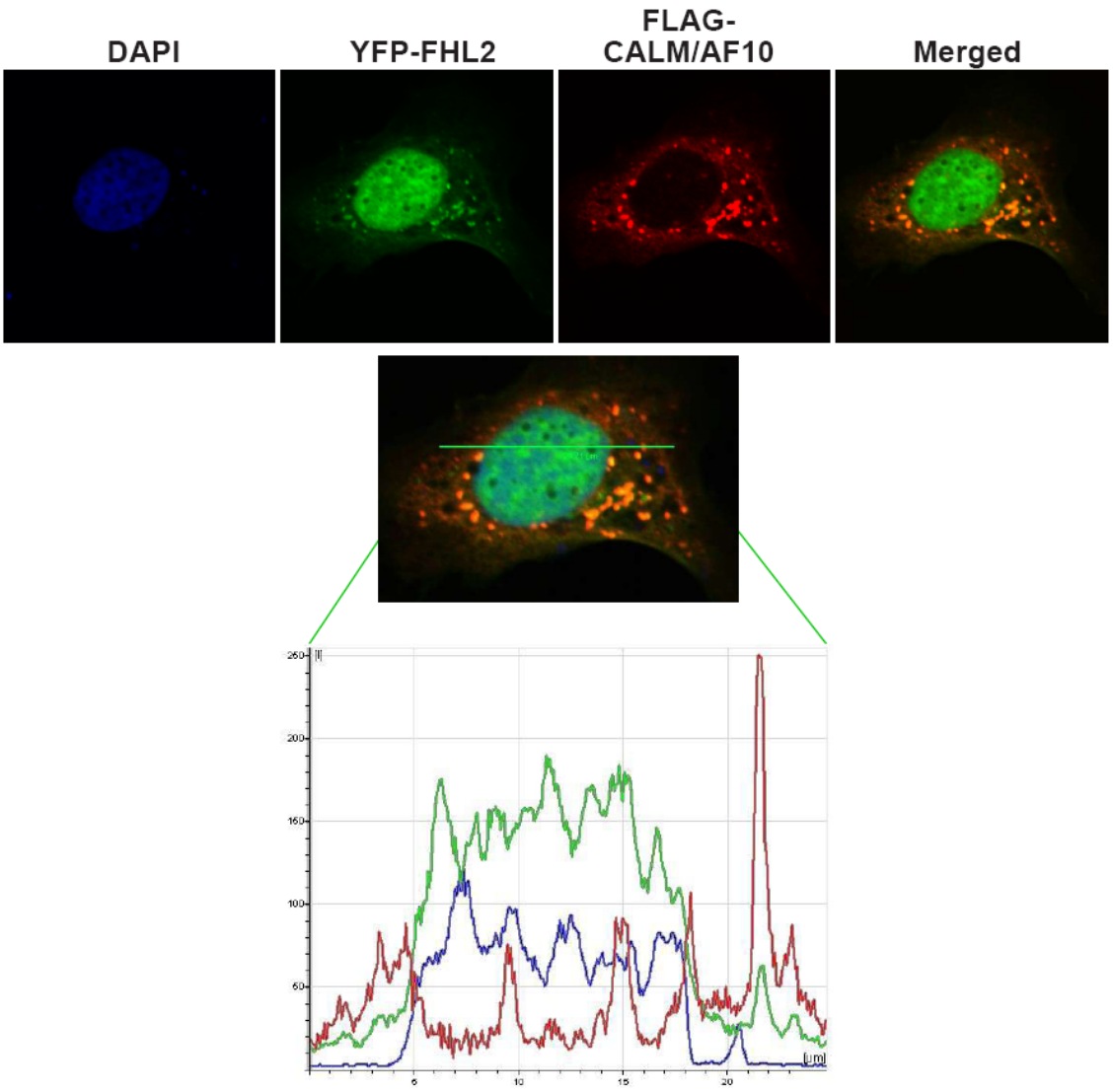


Figure 3.15 FLAG-CALM/AF10 and YFP-FHL2 colocalize in the cytoplasm. Confocal images of U2OS cells cotransfected with YFP-FHL2 and FLAG-CALM/AF10. The FLAG-CALM/AF10 is localized in the cytoplasm, while YFP-FHL2 is found both in the cytoplasm and in the nucleus. YFP-FHL2 and FLAG-CALM/AF10 colocalize in the cytoplasm which is seen in in orange color of the merged image and overlapping peak intensities in the confocal line scan. The green line corresponds to YFP-FHL2, red to FLAG-CALM/AF10 and the blue line corresponds to the DAPI staining.

3.8 Transcriptional activation assays

After demonstrating that CALM and FHL2 interact both *in vitro* and *in vivo*, we asked what the functional consequences of this interaction would be. Vecchi *et al.*, (2001) reported that CALM activates the transcription of a GAL4 promoter driven luciferase reporter gene when fused to the GAL4 DNA-binding domain (GAL4 DBD) in mammalian cells. In addition, it is known that FHL2 can act as a co-activator or co-repressor for a number of transcription factors. In fact, it was shown by Müller *et al.*, (2000), that FHL2, when fused to the GAL4 DNA-binding domain, will activate a GAL4 promoter driven luciferase reporter gene. We therefore used a luciferase reporter gene assay to examine the possible functional consequences of FHL2 on CALM-mediated transcriptional activation.

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

Figure 3.16 shows a diagram with the possible effects of CALM when activating or repressing the transcription of a luciferase reporter gene.

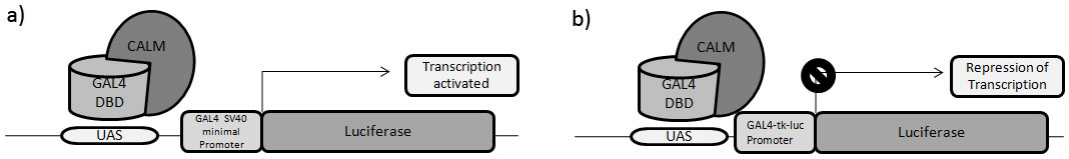


Figure 3.16 Diagram of CALM in GAL4-based transactivation assay. a) If CALM is an activator of transcription the transcription of the luciferase reporter gene is activated. To examine transcriptional activation, a reporter plasmid was used in which the luciferase reporter gene was under the control of 5 GAL4 UAS sites and an SV40 minimal promoter (Gal4-Luc) b) If CALM is a repressor of transcription the constitutive expression of the luciferase reporter gene will be reduced. Constitutive expression of the luciferase reporter gene is achieved with a reporter plasmid in which the luciferase reporter gene is under the control of 5 GAL4 UAS sites and the Herpes Simplex Virus thymidine kinase promoter (Gal4-tk-luc).

To examine if CALM is a transcriptional activator, CALM together with the GAL4 Luc plasmid was transfected into HEK 293T cells. In the plasmid GAL4 Luc, the firefly luciferase reporter gene is under the control of 5 GAL4 UAS sites and a GAL4 SV40 minimal promoter. If CALM is a transcriptional activator the transcription of the

luciferase reporter gene is activated. To achieve constitutive expression of the luciferase reporter gene and examine if CALM acts as a transcriptional repressor, the Gal4-tk-Luc plasmid is transfected into the cells. In the Gal4-tk-Luc plasmid the luciferase reporter gene is under the control of 5 GAL4 UAS sites and the Herpes Simplex Virus thymidine kinase promoter. If CALM acts as a repressor the transcription the constitutive expression of the luciferase reporter gene will be reduced.

We were unable to reproduce the CALM-mediated transcriptional activation reported by Vecchi *et al.*, (2001) and decided not to use GAL4 DBD-CALM for further experiments.

3.8.2 FHL2 acts as transcriptional activator in a GAL4 based transactivation assay

Müller *et al.*, (2000) reported that GAL4 DBD-FHL2 can act as a transcriptional activator. Fig 3.17 shows a diagram of this experiment.

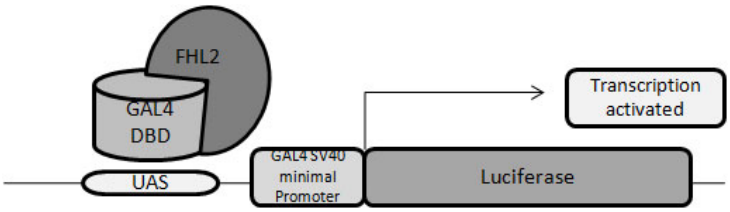


Figure 3.17 FHL2 transcription assay model. When GAL4DBD-FHL2 binds to upstream activating sequence the transcription of the luciferase reporter gene is activated (Müller *et al.*, 2001).

GAL4 DBD-FHL2 induced up to 40 fold increase in reporter gene activity in comparison to GAL4 DBD alone (Fig 3.18). The positive control, GAL4 DBD-STAT 2, activated the transcription of the luciferase reporter gene on average 3520 fold (data not shown). We confirmed that FHL2 activates the transcription of the luciferase gene, as reported previously. The experiment was performed four times (fig 3.18.)

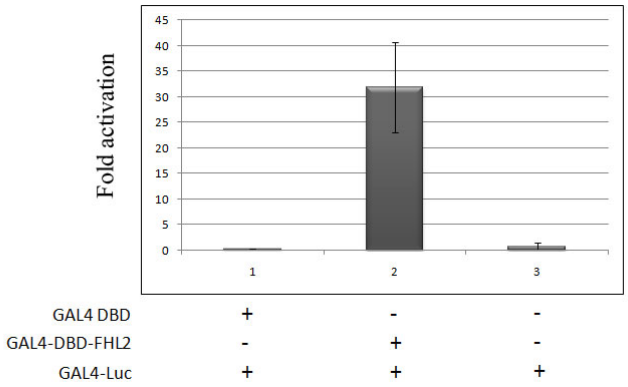


Figure 3.18 FHL2 fused to GAL4DBD acts as activator of transcription. The GAL4 DBD-FHL2 activated the transcription of the luciferase up to 40 fold in 293T cells. The cells were transfected with GAL4 DBD and GAL4 Luc as negative controls. The positive control, GAL4 DBD-STAT 2, activated the transcription of the luciferase reporter gene on average 3520 fold (data not shown). Firefly luciferase expression was normalized to Renilla luciferase expression.

3.8.3 CALM interferes with the transcriptional activation of GAL4 DBD-FHL2

Since we had shown that FHL2 interacts with CALM and can also affect the subcellular localization of CALM, we asked whether CALM would affect the transcriptional activity of FHL2 (fig 3.19).

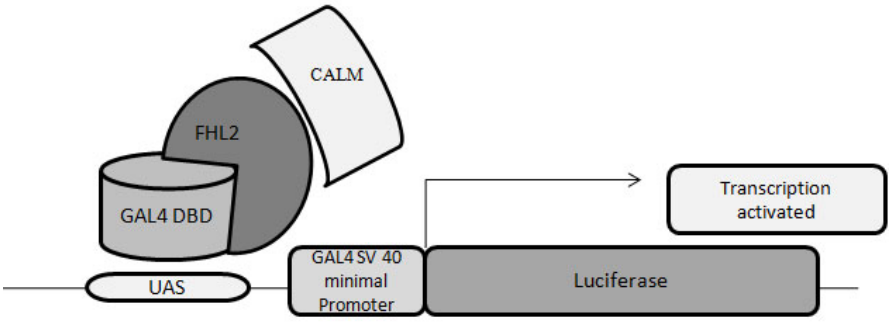


Figure 3.19 Model of CALM-FHL2 interaction. The interaction between FHL2 and CALM might affect the transcriptional activation of the luciferase reporter gene.

We could show that GAL4 DBD-FHL2 significantly (up to 40 fold) activates the transcription of the luciferase reporter gene (Fig 3.18 and 3.20). Interestingly, when GAL4 DBD-FHL2 was cotransfected with YFP-CALM (100 ng, 200 ng and 400 ng) in 293T cells, the transcriptional activation mediated by GAL4 DBD-FHL2 was reduced by about 50% (fig 3.20). These results were very consistent in four independent experiments with three measurements per sample. The positive control, GAL4 DBD-STAT 2, activated the transcription of the luciferase reporter gene 981 fold (on average, data not shown).

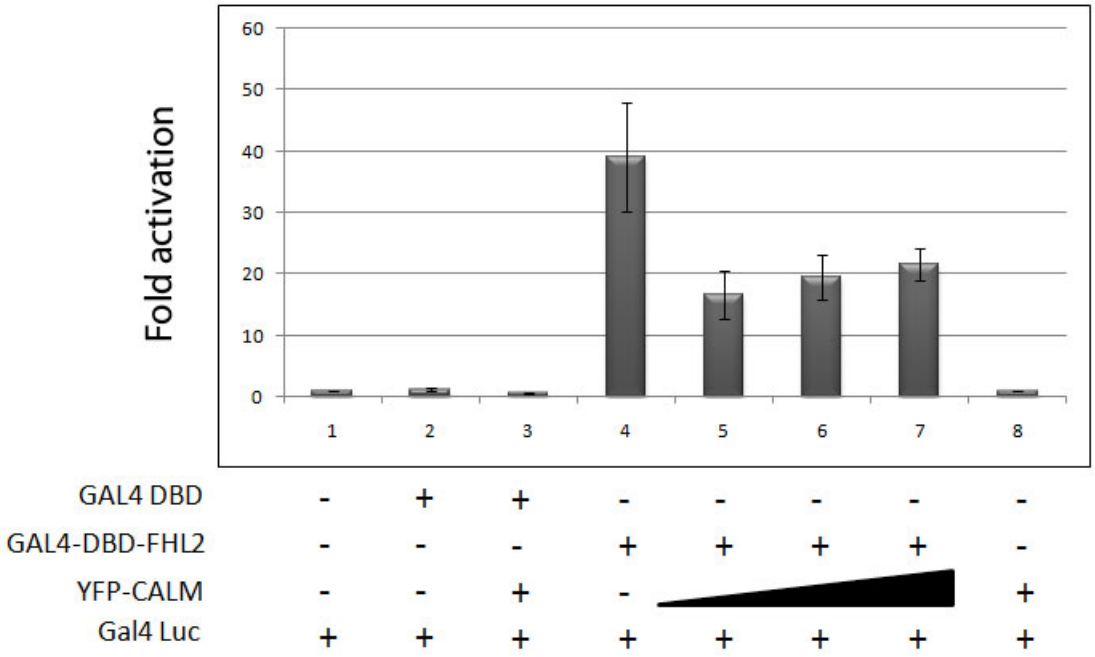


Figure 3.20 CALM disturbs the activation of GAL4DBD-FHL2. GAL4DBD-FHL2 alone activates the transcription of luciferase significantly. Co-transfecting (100 ng, 200 ng and 400 ng) YFP-CALM results in significant inhibition of transcriptional activation. The error bars represent standard deviations. 4 independent experiments with 3 measurements per sample were performed. The positive control, GAL4 DBD-STAT 2, activated the transcription of the luciferase reporter gene to an average of 981 fold (data not shown). Firefly luciferase expression is normalized to Renilla luciferase expression.

3.8.4 CALM/AF10 does not affect FHL2 activation

We then explored the effect of co-expressing YFP-CALM/AF10 on the transcriptional activation function of FHL2 (fig 3.22).

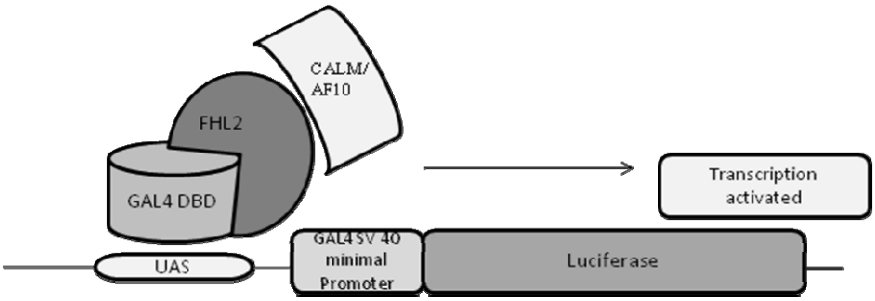


Figure 3.21 Model of GAL4-DBD-FHL2 co-transfected with CALM/AF10. If CALM/AF10 has an effect on FHL2 the transcription of luciferase gene will be either activated or disturbed

GAL4 DBD-FHL2 alone activates the transcription of the luciferase reporter gene about 30 fold. Cotransfection of YFP-CALM/AF10 (100ng, 200 ng and 400 ng) does not affect the transcriptional activation of GAL4 DBD-FHL2 (fig 3.22). This is in striking contrast to the effect of co-transfecting YFP-CALM which led to a 2 fold reduction of GAL4 DBD-FHL2 mediated transcriptional activation.

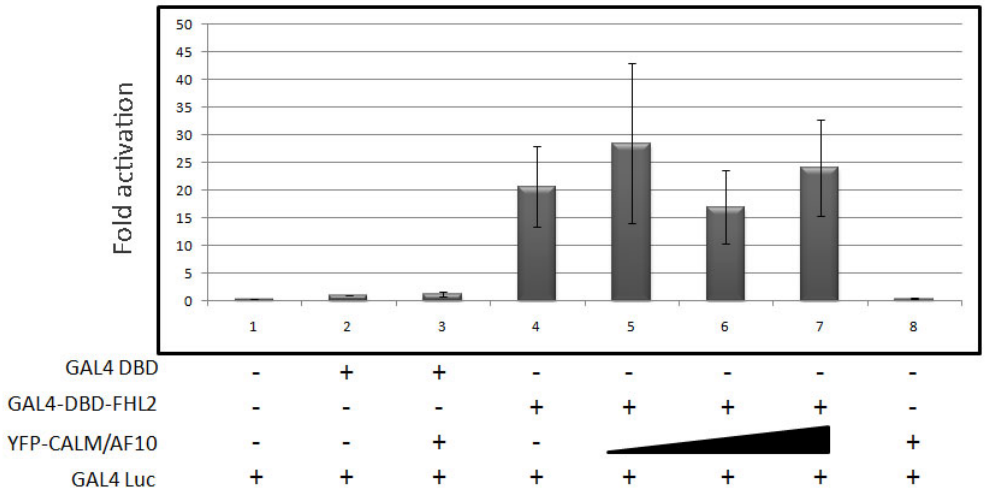


Figure 3.22 YFP-CALM/AF10 does not affect GAL4 DBD-FHL2-mediated transcriptional activation in 293T cells. No significant difference between GAL4 DBD-FHL2 alone and GAL4 DBD-FHL2 co transfected with (100 ng, 200 ng and 400 ng) CALM/AF10 is seen.

3.8.5 FHL2 disturbs GAL4DBD-CALM/AF10-mediated transcriptional activation

Since we could show that GAL4 DBD-FHL2 is affected by YFP-CALM but not by YFP-CALM/AF10, we wondered whether there would be any effect of FHL2 on GAL4 DBD-CALM/AF10 (figure 3.23).

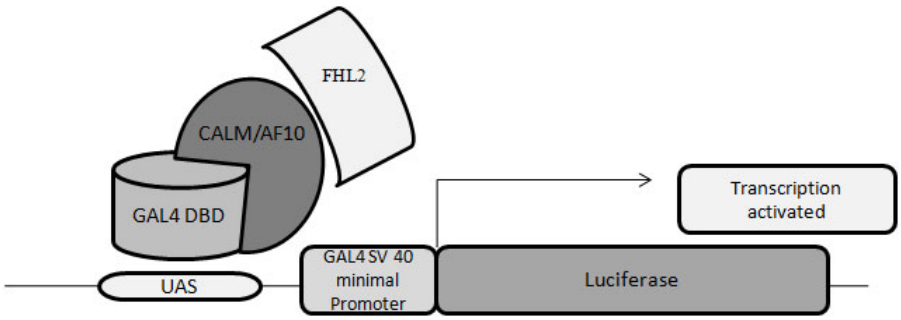


Figure 3.23 Cartoon of GAL4DBD-CALM/AF10 influenced by FHL2.

In pilot experiments we determined that GAL4 DBD-CALM/AF10 is indeed an activator of transcription of the luciferase reporter gene, leading to a 50-fold activation of transcription compared to the expression of GAL4 DBD alone. Interestingly, when a FLAG-FHL2 expressing plasmid was cotransfected in this system the transcriptional activation of GAL4 DBD-CALM/AF10 was reduced to only 20-fold (fig 3.24).

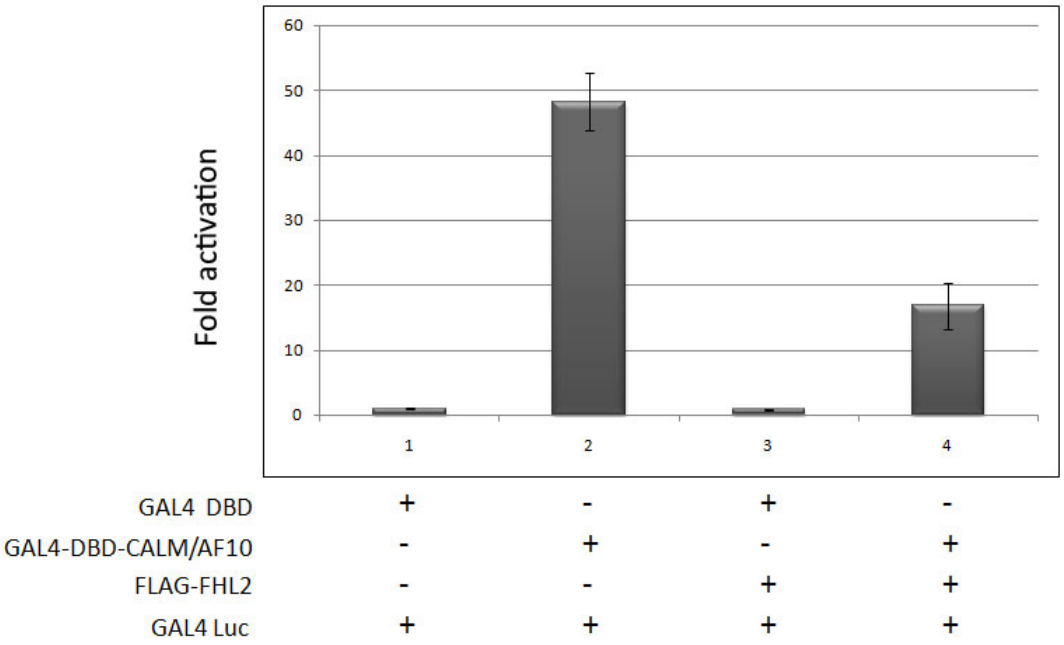


Figure 3.24 Transcriptional activation by GAL4 DBD-CALM/AF10 is disturbed by FLAG-FHL2. A clear reduction in transcriptional activation of GAL4 DBD-CALM/AF10 is observed when FLAG-FHL2 is cotransfected in 293T cells. Three independent experiments were performed and measured in duplicates.

3.9 Expression analysis of the FHL2 protein

3.9.1 Expression analysis of FHL2 in hematopoietic cell lines

Western blot analysis was used to examine the expression of the FHL2 protein in different hematopoietic cell lines (fig 3.25). High expression of FHL2 was found in the human chronic myeloid leukemia in blast crisis (CML) cell line K-562. Lower expression of FHL2 can be seen in the human B cell lymphoma cell line Karpas 422 and the Epstein Barr-virus (EBV) transformed B-cell cell line LCL 3.1. No FHL2 expression was found in DG-75, which is a human Burkitt lymphoma cell line**.** None of the AML cell lines, T-cell lines or the B-cell lines NCEB-1, Jeko-1, Granta 519 and Nalm-6 express FHL2 at the protein level (fig 3.25b).

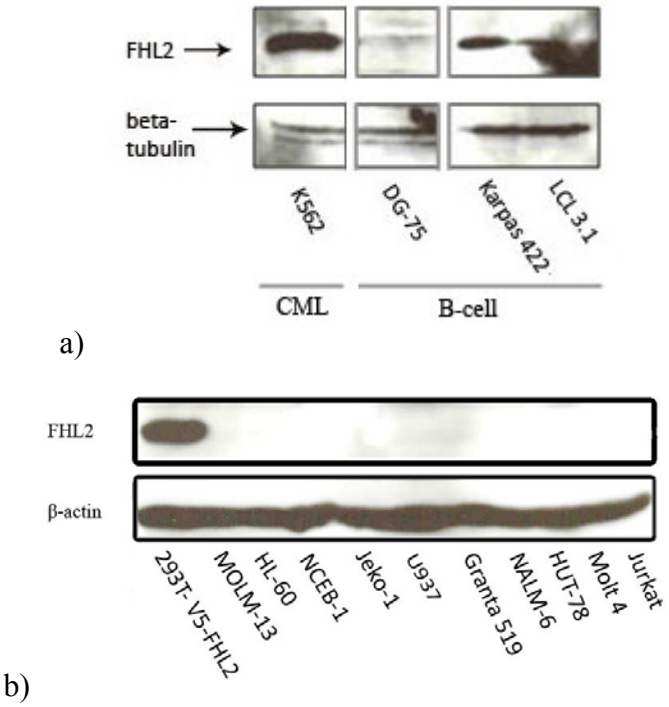


Figure 3.25 Expression of FHL2 in hematopoietic cell lines. a) Strong expression of FHL2 is seen in K562, weaker expression in Karpas 422 and LCL 3.1 but no expression in DG-75. In a) 80 µg of protein lysate was loaded in each lane. b) No protein expression of FHL2 could be detected in several other cell lines. The lysate from 293T cells overexpressing V5-FHL2 was used as a positive control. In b) 100 µg protein was loaded in each lane. FHL2 was detected with a mouse monoclonal anti-FHL2 antibody (2.1.13)

3.9.2 Expression analysis of FHL2 in normal and solid tumor cell lines

We also examined the expression of FHL2 in cell lines of normal tissue and solid tumors. Western blot analysis revealed expression of FHL2 in a cervical cancer cell line (HeLa), an osteosarcoma cell line (U2OS) and human embryonic kidney cells (293T). In the human lung fibroblast cell line (Wi38) and the osteosarcoma cell line (SaOS) no expression of FHL2 could be detected.

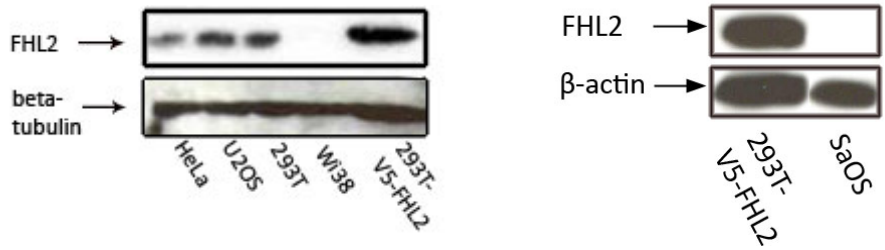


Figure 3.26 Western blot analyses of FHL2 in normal and solid tumor cell lines. FHL2 is expressed in HeLa, U2OS, and the 293T cell lines but not in Wi38 or the SaOS cell line. 100 µg protein was loaded in each lane and FHL2 was detected with a mouse monoclonal anti-FHL2 antibody.

3.9.3 Expression analysis of Fhl2 in rodent cell lines

Since the monoclonal anti human FHL2 antibody cross-reacts with the mouse and rat FHL2 proteins we examined the expression of Fhl2 in several rodent cell lines. For expression analyses of murine cell lines 100 µg protein was used and Fhl2 was detected with the monoclonal FHL2 antibody (2.1.13). Fig 3.27 shows that the mouse pro B-cell (BA/F3) cell line does not express FHL2 in contrast to the mouse embryonic fibroblast cell line NIH3T3 and the rat fibroblast cell line TGR which show strong expression.

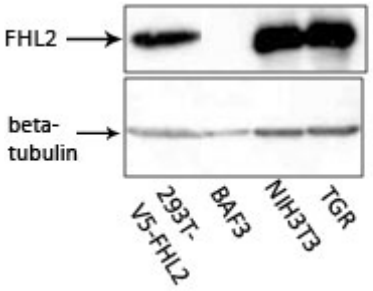


Figure 3.27 Expression of FHL2 in rodent cell lines. Fhl2 is strongly expressed in a mouse embryonic fibroblast cell line (NIH3T3) and in a rat fibroblast cell line (TGR). The murine pro B-cell cell line Ba/F3 does not express FHL2. As positive control V5-FHL2 expressed in 293T cells was used. 100 µg of protein was loaded in each lane.

3.10 Expression analysis of FHL2 mRNA in leukemia samples

3.10.1 Microarray

We used Affymetrix microarray data to analyze the expression levels of FHL2 in 129 leukemia patient samples and 10 normal bone marrow samples. The leukemic patient samples were from 13 different leukemia subgroups including 10 samples from CALM/AF10 positive leukemias. The other leukemias included chronic myeloid leukemia (CML), several subtypes of acute myeloid leukemia (AML) and of acute lymphoblastic leukemia (ALL). For the analysis of the VSN normalized data set the dChip program was used. A comparison of different subtypes of leukemia and normal bone marrow revealed a higher expression of FHL2 in CML and in AML samples with complex aberrant karyotype (fig 3.28). The expression level of FHL2 was relatively low in all the other subgroups examined including the CALM/AF10 positive leukemias. The higher expression of FHL2 in CML and AML patients with complex aberrant karyotypes did not reach statistical significance at the 0.05 level but suggested a trend, which needs to be examined in a larger patient cohort.

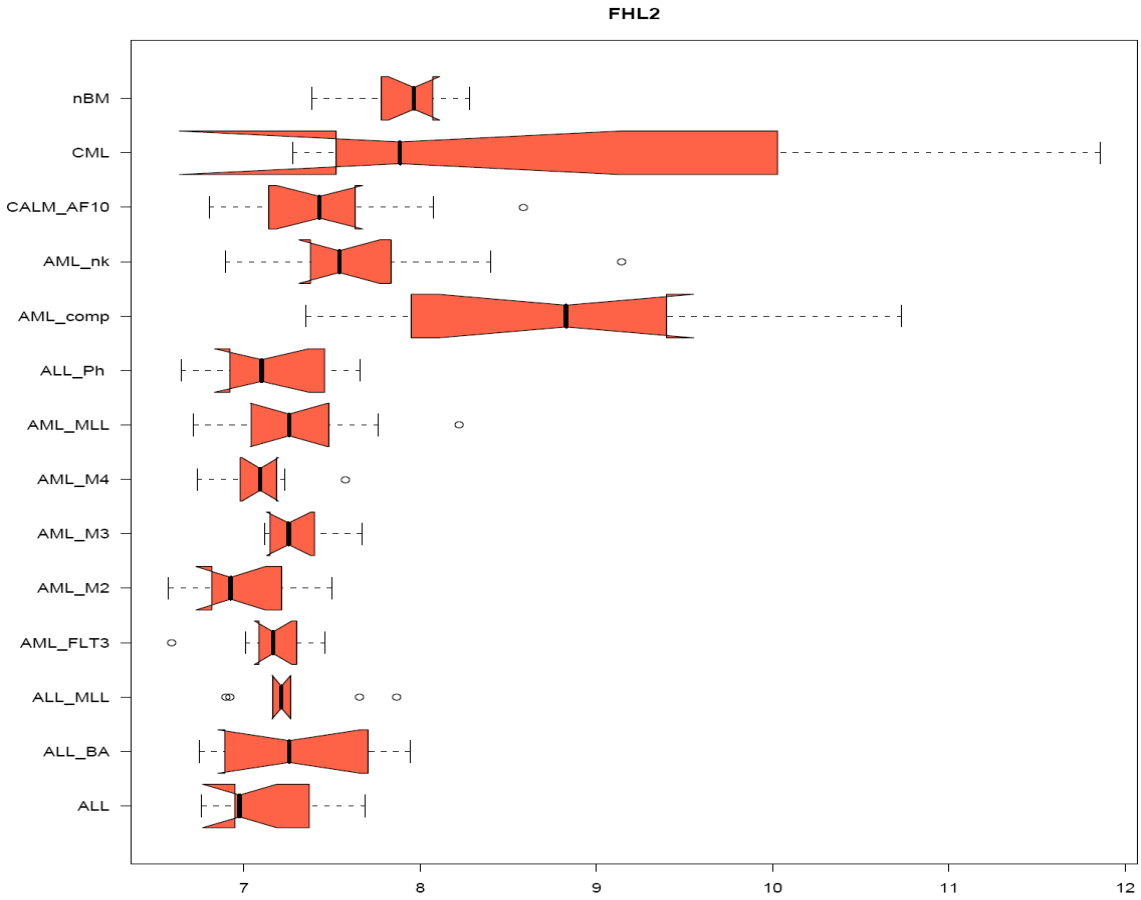


Figure 3.28 FHL2 is expressed at higher levels in CML (Chronic myeloid leukemia) and AML (Acute myeloid leukemia) with complex aberrant karyotype. A microarray analysis was performed on 129 patient samples and 10 normal bone marrow samples. Boxplots depicts the mRNA expression levels of FHL2 in normal bone marrow (nBM), CML, CALM/AF10 positive leukemias, in 7 different AML subtypes (AML_nk: AML with normal karyotype, AML_comp: AML with complex aberrant karyotype, AML_MLL: AML with MLL rearrangement, AML_M4: AML with CBFB/MYH11 fusion; AML_M3: APL with PML/RARA fusion; AML_M2: AML with AML1/ETO fusion; AML_FLT3: AML with normal karyotype and with a FLT3 internal tandem duplication), ALL_Ph: ALL with BCR/ABL fusion; c-ALL: (common ALL) and pro-ALL. Expression intensities are depicted on a logarithmic scale. The box presents 50% of the dataset and is called the interquartile range (IQR). It is obtained by subtracting the first quartile from the third quartile. An expression value was considered to be an outlier when it lies more than 1.5 IQR lower or higher than the first or third quartile, respectively. Outliers are represented as circles. The smallest and largest values that are not outliers are indicated by a vertical tic mark or "whisker" which are connected to the box via a horizontal line.

4 Discussion

The t(10;11)(p13;q14) translocation is a chromosomal abnormality observed in patients with acute myeloid and lymphoblastic leukemias (AML and ALL) (Dreyling M. et al., 1996). The t(10;11)(p13;q14) translocation results in the fusion of *CALM* on chromosome 11 band q14 to *AF10* on chromosome 10 band p13. The CALM/AF10 fusion protein encompasses nearly all the protein domains of both CALM and AF10 (Fig 1.7) (Dreyling M. et al., 1996). Deshpande *et al.* showed that expression of CALM/AF10 induces leukemia in a murine bone marrow transplant model (Deshpande A. et al., 2006). To learn more about CALM/AF10, the physiological functions of CALM and AF10 need to be understood. In order to elucidate the function of CALM we searched for CALM interacting proteins using a yeast two hybrid screen.

The *CALM*-Gene (Clathrin Assembly Lymphoid Myeloid Leukemia Gene) encodes a 652 amino acid long protein that is ubiquitously expressed and has a high homology with the neuronal specific protein AP180. CALM is a clathrin assembly protein and has been demonstrated to play an essential role in clathrin-mediated endocytosis (CME). CALM is involved in protein-protein and protein-lipid interactions that lead to the stable recruitment of clathrin coats (Evans R. and Owen J., 2002; Hinrichsen L. et al., 2006; Legendre-Guillemin V. et al., 2004). CALM is involved in two different translocations and fusion proteins: the CALM/AF10 and the MLL/CALM. This together with the finding that several other endocytic proteins have been identified in leukemia-associated gene fusion, suggests that alterations of the CME play a hitherto underappreciated role in leukemia development (Bohlander S.K. et al., 2000; Dreyling M. et al., 1996; Floyd S. and De Camilli P., 1998; Polo S. et al., 2004; Wechsler D.S et al., 2003).

4.1 Identification of CALM interacting proteins

By identifying protein interaction partners of CALM, we would be able to connect CALM with cellular pathways and place it in a functional context within the living cell. We performed two yeast two hybrid screens using the N-terminal portion of CALM (aa 1-408) as a bait. In a HeLa cDNA library we identified the following CALM interacting

partners: the CALM interacting protein expressed in thymus and spleen (CATS), the poly(C)-binding protein 1 (PCBP1), the Tumor susceptibility gene 101 (TSG101) and CALM itself. The proteins obtained from the screen of the thymus cDNA library were filamin actin binding protein (FLNA), adipocyte fatty acid binding protein 4 (FABP4), dipeptidyl-peptidase 7 protein (DPP7) and four and a half LIM domain protein 2 (FHL2).

4.1.1 CATS

The CALM interactor CATS was first identified in a previous yeast two hybrid screen of our group as a CALM interacting protein. The CATS protein does not contain any known conserved protein motifs. The CATS transcript is highly expressed in thymus, spleen and colon (Fröhlich-Archangelo L. et al., 2006). From our yeast two hybrid experiment we obtained the alternative spliced variant 2 coding for isoform 2 of CATS. The CALM-CATS interaction had previously been confirmed with GST-pulldown experiment, co-immunoprecipitation assay as well as in immunofluorescence assay. It is known that the localization of a protein is critical for its function. Intriguingly, it was demonstrated that in mouse fibroblasts cells (NIH3T3) the presence of CATS can affect the subcellular localization of CALM and CALM/AF10. When CATS was coexpressed with CALM/AF10, most of the CALM/AF10 protein was drawn to the nucleus. In contrast to CALM which shifted only partially to the nucleus upon co-expression with CATS (Fröhlich-Archangelo L. et al., 2006). Interestingly, CATS was shown to be highly expressed in lymphoid tissues, which indicate that the CATS-CALM interaction might be important in CALM/AF10 mediated leukemogenesis.

4.1.2 PCBP1

The CALM interactor poly(C)-binding protein 1 (PCBP1) is a member of the PCBP1 protein family. PCBP1 is characterized by three K-homologous (KH) domains which may be involved in RNA binding (Leffers H. et al., 1995). PCBP1 is a RNA binding protein that has been shown to be involved in mRNA stabilization, translational activation and silencing, and is therefore likely to play a role in a diverse set of post-transcriptional control pathways (for review see (Makeyev A. and Liebhaber S., 2002). Together with the poly(C)-binding protein 2 (PCBP2), PCBP1 functions as translational

coactivator of poliovirus RNA (Blyn L.B et al., 1996). PCBP1 has also been implicated in the translational control of the human papilloma virus (Collier B. et al., 1998). The PCBP1 clone obtained from our yeast two hybrid screen lacks the first KH domain completely. Interestingly, PCBP1 has also been shown to interact with CATS (Fröhlich Archangelo L., 2006). Thus, it is tempting to speculate that CALM and CATS are competing for the binding on PCBP1 or they might form a trimeric complex. Since PCBP1 is a shuttling protein (Makeyev A. and Liebhaber S., 2002) it is possible that the interaction with CALM might keep PCBP1 in the cytoplasm. Considering the fact that CATS completely relocated the CALM/AF10 protein (and not CALM) to the nucleus it is tempting to speculate that the CALM/AF10 fusion protein could disrupt the physiological function of the CALM-PCBP1 interaction.

4.1.3 TSG101

The tumor susceptibility gene 101, which was found to interact with CALM by us, was first identified in a screen for potential tumor suppressors (Li L. and Cohen S., 1996). TSG101 is an endocytic adaptor protein that has been shown to be crucial in cell proliferation, cell survival (Krempler A. et al., 2002), and has a role in the ubiquitin-controlled endosomal sorting pathways (Bishop N. and Woodman P.G., 2000). Normally, TSG101 functions in the cellular vacuolar protein sorting (VPS) pathway, coordinating the sorting of membrane associated proteins. This pathway is composed of a series of endosomal compartments, and eventually the proteins are delivered to the lysosomes (Dupre S. et al., 2001; Piper R. and Luzio J., 2001). Impairment of TSG101 protein function disturbs the endosomal trafficking which results for example in prolonged signaling of growth factor receptors. This effect is believed to contribute to the tumorigenic phenotype exhibited by the TSG101 mutant fibroblasts (Babst M. et al., 2000).

The TSG101 clone obtained in our yeast two hybrid screen lacked the N-terminal domain. This suggests that only the C-terminal part of TSG101 (aa 280-390) is needed for an interaction with CALM. The C-terminal portion of TSG101 which was identified in our screen contains part of the putative coiled coil domain (~37 aa), the PTAP motif

and the steadiness box (S-Box). It has been shown that the expression of the C-terminal fragment of TSG101 blocks the budding of PTAP- and PPPY dependent retroviruses like HIV (Johnson M. et al., 2005). Depletion or overexpression of TSG101 arrested or decreased the release of HIV-1, thereby functionally linking TSG101 to virus budding and release (Demirov D.G. et al., 2002; Garrus et al., 2001).

Clathrin-coated vesicles mediate endocytosis of transmembrane receptors and the transport of newly synthesized lysosomal hydrolases from the trans-Golgi network (TGN) to the lysosomes (Schmid S., 1997). Clathrin and various adaptor protein complexes play also an important role in shaping the budding vesicles as well as in the packaging of cargo (Ritter B. and McPherson P.S., 2004). This demonstrates that protein sorting and vesicle budding are functionally integrated. Interestingly, clathrin has been described as the major binding partner of CALM. After overexpressing CALM, inhibition of endocytosis and disruption of clathrin localization in the TGN was observed. This resulted in perturbance in the TGN-endosome trafficking (Tebar F. et al., 1999).

Despite the unknown biological function of the CALM-TSG101 interaction, it is quite apparent that these two proteins are involved in the same pathways, including the endosomal sorting pathway. Thus, it is conceivable that the CALM/AF10 fusion protein might disrupt the endosomal sorting pathway through protein relocalization.

4.1.4 CALM

The CALM gene was first identified as fusion partner of AF10 in the t(10;11)(p13;q14) translocation, which was observed in acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and malignant lymphoma (Dreyling M.H. et al., 1998). We identified CALM as a CALM interacting protein suggesting that CALM might dimerize or multimerize with itself. The prey clone of CALM which we obtained in our yeast two hybrid screened lacked the first 114 amino acids. The biological function of CALM dimerization or multimerization is still unknown and remains to be explored.

4.1.5 FLNA

The CALM interactor FLNA is a homodimeric F-actin crosslinker organizing actin filaments in parallel arrays or three dimensional webs linking them to the cell membrane

(Gorlin J. et al., 1990). FLNA contributes to the stability of the membrane by anchoring membrane proteins to the actin cytoskeleton. There is increasing evidence that FLNA also plays a role in the regulation of nuclear functions. Previous findings indicate that FLNA is processed by proteolysis. The proteolytic products of FLNA seem to play a role as signaling molecules integrating nuclear and cytosolic pathways (Garcia E. et al., 2006; Uribe R. and Jay D., 2007). Our FLNA prey clone contained only the C-terminal part (aa 2306 - 2595) of FLNA. FLNA and the adapter protein complex 2 (AP-2) are competing for the binding sites on the prostate specific membrane antigen (PSMA) (Rajasekaran A. et al., 2005). Interestingly, CALM has also been shown to interact with AP-2 (Owen D. and Luzio P., 2000). It has been suggested that the dissociation of FLNA facilitates the binding of AP-2 to PSMA (Ghosh A. and Heston W., 2007). PSMA is then endocytosed via CME (Hinrichsen L. et al., 2006; Rajasekaran A. et al., 2005). Depletion of AP-2 weakens the association between CALM and the plasma membrane (Meyerholz A. et al., 2005). It is therefore possible that the detached CALM relocates to the spot where the endocytic cycle starts. The exact function of the CALM-FLNA interaction in this complicated process remains to be explored.

4.1.6 FABP4

Adipocyte fatty acid binding protein (FABP4) is a member of the intracellular fatty acid binding protein (FABP) family. It plays a role in lipid metabolism, fatty acid uptake, transport, metabolism and homeostasis in adipocytes (Michal J. et al., 2006). For the CALM-FABP4 interaction the C-terminal part (aa 36-153) of FABP4 seems to be required, since this portion of FABP4 was contained in our prey clone. The biological function of CALM-FABP4 interaction is unknown. One possibility is that CALM has an unknown role in the regulation of lipids and glucose homeostasis.

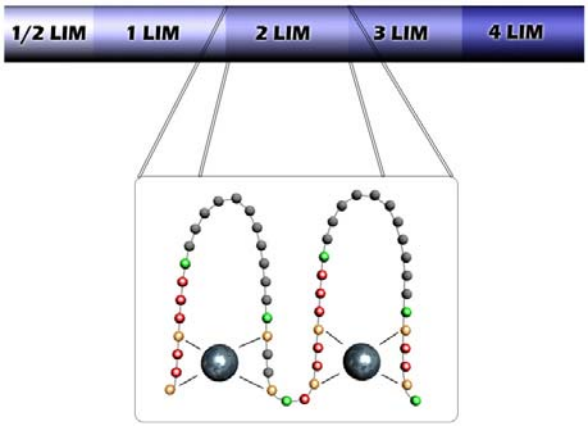
4.1.7 DPP7

Dipeptidyl-peptidase 7 protein (DPP7) is a post-proline cleaving aminopeptidase expressed in quiescent lymphocytes. These quiescent lymphocytes are protected from programmed cell death by a cellular program that suppresses apoptosis. Downregulation of DPP7 inhibits the suppression of the apoptosis and resting lymphocytes become active

(Chiravuri M. et al., 1999). Our DPP7 prey clone lacks the first 130 amino acids. The interaction between CALM and DPP7 suggest a possible role of CALM in the regulation of quiescence of lymphocytes. The leukemogenic CALM/AF10 fusion protein might disrupt the function of DPP7 for example through relocalization. The CALM-DPP7 interaction might thus play an important role in CALM/AF10-mediated leukemogenesis.

4.2 FHL2

We chose to examine the interaction between CALM and the four and a half LIM protein 2 (FHL2) in more detail. FHL2, also called DRAL for Downregulated in Rhabdomyosarcoma LIM protein, was first identified because of its differential expression between normal human myoblasts and their malignant counterparts, the rhabdomyosarcoma cells (Genini M. et al., 1997). The FHL2 protein is composed of 4 complete LIM domains and a half LIM domain at its N-terminus (figure 4.1).
LIM is an acronym for Lin-11, Isl-1 and Mec-3 which are the first three proteins in which the LIM motif was found (Freyd G. et al., 1990; Karlsson O. et al., 1990; Way J. and Chalfie M., 1988). LIM domains are characterized by a highly conserved double zinc finger motif. The cysteine and histidine residues bind two zinc atoms as shown in figure 4.1 (Michelsen J.W. et al., 1993; Schmeichel K.L. and Beckerle M.C., 1997).



Figur 4.1 Domain structure of the FHL2 protein. FHL2 is composed of 9 zinc fingers in total**.** Note that the half LIM domain on N-terminus contains just one zinc finger**.** One LIM domain consists of two zinc fingers. Zinc atoms (grey spheres) in the middle, are bound by the cystein and histidine residues (yellow).

The FHL2 prey clone isolated from our yeast two hybrid assay contains the complete coding sequence of the FHL2 protein. The *FHL2* gene is located on chromosome 2 band q12-q13 and encodes a protein of 279 amino acids (Chan K. et al., 1998).
FHL2 is a protein interacting with many different proteins belonging to diverse functional classes including steroid receptors like estrogen receptor alpha (ERalpha), androgen receptor (AR), integrin receptor a7ß1, structural proteins, signal transducers, transcription factors and cofactors, splicing factors, DNA replication-, repair- and diverse other enzymes (Kobayashi S. et al., 2004; Müller J. et al., 2000; Samson T. et al., 2004).

Intriguingly, it has been shown that transcription of the FHL2 gene can be stimulated by p53. P53 induces FHL2 expression during apoptosis (Scholl F. et al., 2000). p53 mutations or deletions are found in nearly all tumor types. It is estimated that they can be found in more than 50% of all cancers (Berkson R. et al., 2005). In addition, it has been observed that increased nuclear FHL2 expression correlates with progression to a highly malignant phenotype in prostate carcinoma (Müller J. et al., 2002). Strong nuclear FHL2 immunoreactivity was also observed in lung tumor biopsies, while no FHL2 was detected in healthy lung tissue (Borczuk A. et al., 2004). Compared to normal tissue, elevated expression of FHL2 was also detected in human epithelial ovarian cancer (Gabriel B. et al., 2004). In expression analyses, FHL2 mRNA levels were increased in patients with AML-FAB M7 subtype, compared to other AML's (Lotem J. et al., 2005). The finding of increased expression of FHL2 in various types of cancers made it an interesting candidate for further characterization.

4.2.1 FHL2 interacts with CALM and influences the subcellular localization of CALM

The CALM and FHL2 interaction was confirmed in a GST pulldown assay and was further corroborated *in vivo* by co immunoprecipitation. We showed that the full length FHL2 was necessary for the CALM-FHL2 interaction to take place. The FHL2 interacting domain in CALM was determined to be between amino acids 294 and 335. Interestingly, FHL2 binds to β-integrin (Samson T. et al., 2004), which together with clathrin, a major binding partner of CALM, has been shown to be involved in endocytosis

of bacteria (Tebar F. et al., 1999; Van Nhieu G.T. et al., 1996). This suggests a possible role for the CALM–FHL2 interaction in the complex interaction network of endocytosis. The hypothesis that FHL2 and CALM act together in the cytoplasm in the endocytic machinery is supported by the fact that expression of YFP-CALM and FLAG-FHL2 in U2OS cells result in co-localization of both proteins in the cytoplasm and at the cell membrane. In addition, there was also co-localization of FHL2 and CALM in the nucleus of the U2OS cell when CALM and FHL2 were cotransfected, suggesting a nuclear function for these proteins.

FHL2 has previously been shown to localized to both the nucleus and the cytoplasm (Hill A. and Riley P., 2004; Labalette C. et al., 2004; Scholl F. et al., 2000). We performed immunofluoroscence experiments with YFP tagged CALM/AF10 and FLAG tagged FHL2 to examine the possible colocalization of FHL2 with CALM/AF10. However, the cells expressing the YFP-CALM/AF10 protein formed inclusion bodies of YFP-CALM/AF10 making it difficult to interprete the results. The same experiments were performed using YFP tagged FHL2 and FLAG tagged CALM/AF10. These experients revealed FLAG-CALM/AF10 mainly in the cytoplasm while FHL2 was predominantly seen in the nucleus. A minor fraction of the FHL2 co-localized with CALM/AF10 in the cytoplasm. It could be shown by several investigators that increased nuclear FHL2 expression correlates with progression to a highly malignant phenotype of prostate carcinoma, lung tumor and human epithelial ovarian cancer (Borczuk A. et al., 2004; Gabriel B. et al., 2004; Müller J. et al., 2002). Overexpression of FHL2 has also been observed to induce apoptosis in human rhabdomyosarcoma (RD) cells, transformed monkey kidney (COS-1) cells, and normal mouse fibroblast (NIH 3T3) cells (Scholl F. et al., 2000).

When fused to the GAL4 DNA binding domain (GAL4-DBD), FHL2 is activating the transcription of the luciferase reporter gene (Müller J. et al., 2000). We could show that CALM co transfected with GAL4-DBD-FHL2 significantly disturbed the transcriptional activation induced by FHL2. Since the FHL2 interaction domain on CALM is present in the CALM portion of the leukemogenic CALM/AF10 we also tested whether

CALM/AF10 would influence the transcriptional activation function of GAL4 DBD-FHL2. Surprisingly, this was not the case. The fact that the transcriptional activation mediated by FHL2 was inhibited by CALM but not by CALM/AF10 could mean that in the presence of the CALM/AF10 fusion protein the normal negative regulatory role of CALM on FHL2 is reduced thereby increasing the tumor promoting activity of FHL2.

Since Vecchi *et al*., had shown that a GAL4-DBD-CALM fusion acts as a transcriptional activator in a reporter gene assay we were interested to see which influence the co-expression FHL2 would have in this system (Vecchi M. et al., 2001). However, we were unable to reproduce the results of Vecchi et al. Intrestingly, we were able to show that a GAL4-DBD-CALM/AF10 fusion behaved as a potent transcriptional activator in our reporter gene assays. Co transfection of FHL2 and GAL4 DBD-CALM/AF10 led to a marked reduction of the CALM/AF10-mediated transcriptional activation of the luciferase reporter gene. In contrast, there was no influence of CALM/AF10 on the transcriptional activiation mediated by GAL4 DBD-FHL2. One explanation for this lack of influence could be that CALM/AF10, being a protein of about 170 kDa, is expressed at a much lower molarity than the small FHL2 protein (~ 33 kDa). The CALM/AF10 would therefore not be able to effectively bind to and influence the much more abundant GAL4 DBD-FHL2. On the other hand, when GAL4-DBD-CALM/AF10 is expressed, the FHL2 protein binds to all the GAL4-DBD-CALM/AF10 protein molecules thus inhibiting or quenching the transcription induced by CALM/AF10. Another explanation might be that the CALM interacting domain of FHL2 is masked when FHL2 is fused to the GAL4-DBD protein.

4.2.2 FHL2 is expressed in tumor cell lines

FHL2 has been shown to be expressed in heart (Kong Y. et al., 2001; Müller J. et al., 2000; Scholl F. et al., 2000), brain, liver, lung (Tanahashi H. and Tabira T., 2000), human skeletal muscle (Genini M. et al., 1997; Morgan MJ. , 1996), as well as in human osteoblasts (Chu P. et al., 2000). In expression analyses of different normal and tumor cell lines, we observed FHL2 expression in the cervical cancer cell line HeLa, the osteosarcoma cell line U2OS and the human embryonal kidney cell line 293T. No

expression was seen in the human lung fibroblast cell line Wi38 and the osteosarcoma cell line SaOS. It has been shown that enhanced levels of p53 stimulate the expression of FHL2 (Johannessen M. et al., 2006), conversely one would expect very low levels of FHL2 in cells with low p53 expression. Since the osteosarcoma cell line SaOs does not express p53 this might be the explanation for the absence of FHL2 expression in this cell line. Expression of FHL2 has been observed in the MRC-5 cell line, which is a normal lung fibroblast cell line (Tanahashi H. and Tabira T., 2000). Therefore we expected to see expression of FHL2 in the normal lung fibroblast cell line Wi38 but in our experiments no FHL2 expression was detected. The Wi-38 cell line was derived from a female fetus of about 3 months gestational age while the MRC-5 cell line is derived from a 14 week male fetus. We thus lack a possible explanation for the absence of FHL2 in Wi38 cells. Expression studies on rodent cell lines revealed strong expression of FHL2 in the mouse embryonic fibroblast cell line NIH3T3 and the rat fibroblast cell line TGR. No FHL2 expression was detected in the murine pro B cell line Ba/F3. Except for the EBV-transformed B-cell cell line LCL 3.1, no expression of FHL2 could be detected in human B-cell cell lines . None of the T-cell nor AML cell lines were showing any expression of the FHL2. FHL2 expression was observed in the B-cell lymphoma cell line Karpas 422 and the BCR/ABL expressing CML cell line K562. The explanation for the expression of FHL2 in K562 cells might be that these cells express the BCR-ABL fusion gene. It has been reported that expressing BCR-ABL in Ba/F3 cells resulted in an increased expression of the p53 (Wendel H-G. et al., 2006). In addition, it was shown that p53 can stimulate the expression of FHL2 (Scholl F. et al., 2000). Combining these findings suggest the following picture: BCR/ABL induces the expression of p53 in K562 cells, which in turn stimulates the expression of FHL2. The fact that the cell lines Karpas 422 and LCL 3.1 also express p53 and FHL2 supports this conclusion.

4.2.3 FHL2 is highly expressed in CML and AML with complex aberrant karyotypes

We also analyzed the expression levels of FHL2 in a series of cytogenetically and genetically well-characterized AML, ALL and CML samples for which Affymetrix micro-array expression data were available. Interestingly, the results from the K562 cell

line that FHL2 expression might be high in CML were also mirrored in the patient samples. FHL2 showed higher expression in CML patients and patients with AML with complex aberrant karyotypes compared to other leukemia subgroups. However, the standard deviations of the FHL2 expression levels in the CML and complex aberrant AML leukemia subgroups were quite high. This might indicate that these groups are heterogeneous with respect to their FHL2 expression levels (containing subgroups of FHL2 high and low expressions). An alternative explanation for this observation is the fact that we only examined a very small number of patient samples in each group (n = 10).

One of the characteristics of AML with complex aberrant karyotypes is the frequent loss of chromosome 5 as well as a high incidence of *TP53* deletions and/or mutations resulting in an overall unfavorable prognosis (Schoch C. et al., 2002). Interestingly, Qian et al. showed that therapy related myelodysplastic syndrome (t-MDS) and acute myeloid leukemia (t-AML) characterized by a loss of a whole chromosome 5 or a deletion of the long arm of chromosome 5 (-5/del(5q)) showed elevated FHL2 expression levels (Qian Z. et al., 2005). These issues need to be addressed by analyzing the FHL2 expression in a larger number of patients with CML and AML with complex aberrant karyotypes (with and without -5/del(5q)).

If the expression levels of FHL2 are significantly different in these leukemia subgroups it might even be possible to use FHL2 as a marker for these groups. These observations are also consistent with the fact that a high FHL2 expression in solid tumors is associated with an adverse prognosis.

FHL proteins have been described as coregulators of transcription by integrating the activity of different transcription factors (Fimia GM. et al., 2000; Johannessen M. et al., 2006). Despite their structural similarity, they seem to have evolved specialized functions by regulating distinct transcription factors (Fimia G-M. et al., 2000). Interestingly, it was recently shown that another member of the FHL family, FHL1, plays a role in AML prognosis. High expression of FHL1 correlated with a poor prognosis in cytogenetically normal AML (Metzeler K. et al., 2007). Intriguingly, FHL1 was found to be overexpressed in MLL wild type versus MLL knock out cells (Schraets D. et al., 2003). It

was shown that the homeobox gene HOX11 is a transcriptional regulator for FHL1 (Rice KL. et al., 2008). HOX11 has also been implicated to function as an oncogenic transcriptional activator in leukemogenesis (Zhang N. et al., 1996). Together these findings suggest a hitherto underappreciated role of four and a half LIM domain proteins in leukemogenesis.

4.3 Possible mechanisms of CALM/AF10-mediated leukemogenesis

It is intriguing to speculate that the dimerization potential of CALM plays a critical role in the malignant transformation potential of the CALM/AF10 fusion protein. The mixed lineage leukemia gene (MLL) is involved in more than 50 chromosomal translocations associated with human acute leukemias (Popovic R. and Zeleznik-Le N.J., 2005). One of the chromosomal translocations involving MLL is the MLL-AF10 translocation. MLL-AF10 was found in AML (Chaplin T. et al., 1995). It is known that certain MLL fusion proteins dimerize and then bind to DNA, and that the dimerization domains are supplied by the fusion partner. It is thus conceivable that the dimerization domain provided by CALM is very critical in the malignant transformation mediated by the CALM/AF10 fusion protein.

Alternatively, the critical domain supplied by the CALM moiety in the CALM/AF10 fusion protein might be the transcriptional activation domain of CALM. A transcriptional activation domain could be mapped to the C-terminal portion of the CALM protein which is also contained in the CALM/AF10 fusion protein. This activation domain mapping of CALM was performed in the yeast system. In our hands, however, this transcriptional activation domain of CALM was not very apparent in mammalian cells (luciferase reporter assays). An argument against CALM developing the same transcriptional activity in CALM/AF10 fusions as MLL in the MLL/AF10 fusion is the structural difference between CALM and MLL. In contrast to CALM, which lacks DNA binding domains, the N-terminal of MLL has two DNA binding domains: the AT hook motifs and the DNA methyltransferase homology region. These two domains guide MLL to the specific chromosomal sites. (Martin M.E. et al., 2003; Slany R., 2005). The C-terminal domain of CALM has been described to behave as a long fishing line (Evans R. and

Owen J., 2002). We propose that CALM dimerization occurs when these "fishing lines" anchor to each other thus providing a platform for recruitment of additional clathrin assembly proteins and proteins involved in endocytosis and membrane trafficking. In the presence of the CALM/AF10 fusion this platform normally provided by CALM might be disturbed**.**

However, we can not exclude that CALM/AF10 also leads to a disruption of normal AF10 function. AF10 has a Drosophila homologue called *Alhambra* that has been shown to regulate transcription through *Polycomb* group-responsive elements (PREs) (Perrin L. et al., 2003). These elements maintain transcriptional decisions to ensure correct cell identity during development and differentiation. Together with the findings that AF10 is a putative transcription factor it is possible that a disruption of AF10 function by CALM/AF10 leads to a transcriptional deregulation of the many genes that are regulated by *polycomb* and *trithorax* complexes. Interestingly, in support of this idea we and others could show that CALM/AF10 fusion-positive patients show overexpression of the polycomb group gene BMI1, the homeobox gene MEIS1 and the HOXA cluster genes, (Caudell D. et al., 2007; Krause A. et al., 2004). In addition, it was shown that patients with MLL rearrangements had a very similar HOX expression pattern as CALM/AF10 positive patients (Krause A., 2006). The important role of HOX genes in acute leukemias is well established (Bergeron J. et al., 2006; Rice K. and Licht J.D., 2007; Soulier J. et al., 2005; Thorsteinsdottir U. et al., 2001).

A structure/function analysis of AF10 in the leukemogenic fusion proteins MLL/AF10 and CALM/AF10 showed that the octapeptide motif (OM) and the leucine zipper (LZ) of AF10 are required for malignant transformation mediated by both proteins (DiMartino JF. et al., 2002; Okada Y. et al., 2005). Interestingly, the AF10 interaction domain for the lymphoid transcriptional regulator Ikaros was mapped to the OMLZ domain of AF10, suggesting an important role of Ikaros in CALM/AF10 and MLL/AF10 induced leukemias (Greif P. et al., 2007). AF10 has also been shown to interact with the histone methyl transferase DOT1L (disruptor of telomeric silencing 1), as well as with the GAS41 which is a component of the chromatin remodeling complex (Debernardi S. et al., 2002; Okada Y. et al., 2005).

Alternatively to disrupting the normal AF10 function, the CALM/AF10 fusion protein might mediate some of its effects by using the AF10 moiety to disrupt other cellular functions. It was shown that CATS can recruit the CALM/AF10 fusion protein to the nucleolus. It is thus possible that the AF10 portion of CALM/AF10 might affect the function of nucleolar proteins (Fröhlich-Archangelo L. et al., 2006). Nucleolar proteins regulate cell proliferation and growth by ribosome biogenesis and are very important in controlling p53 function (Horn H. and Vousden K., 2004; Maggi l. and Weber J., 2005; Olson MO., 2004; Rubbi CP. and Milner J., 2003). Deregulation of these mechanisms is the key event in the initiation and progression of malignant transformation (Horn H. and Vousden K., 2004; Lohrum M. and Vousden K., 2000).

It has become clear during the course of this discussion that there are many different possible mechanisms by which CALM/AF10 might induce leukemogenesis. The careful dissection of CALM/AF10 protein interactions, like the CALM-FHL2 interaction, will provide new entry points for unravelling the complicated mechanisms which are responsible for the leukemogenic potential of the CALM/AF10 fusion protein.

5 Summary

The Clathrin Assembly Lymphoid Myeloid gene (*CALM*) was first found in the fusion gene *CALM/AF10* as the fusion partner of AF10 resulting from the t(10;11)(p13;q14)-translocation. The CALM/AF10 fusion protein has been shown to play a crucial role in CALM/AF10 associated leukemogenesis. In order to gain insight into the function of CALM yeast two hybrid screens of HeLa and Thymus cDNA library were performed using the N-terminal half of CALM as bait. Eight different putative CALM interacting proteins were obtained including the four and a half LIM domain protein (FHL2). The interaction between CALM and FHL2 was confirmed with co-immunoprecipitation, GST pulldown assay and colocalization experiments. The FHL2 interaction domain in CALM was mapped to amino acids 294 to 335. This domain is also contained in the CALM/AF10 fusion protein.. Luciferase reporter gene assays were performed to analyze the effect of FHL2, CALM and CALM/AF10 on each other as transcriptional regulators. Interestingly we could show that the GAL4DBD-FHL2-induced activation of transcription was inhibited when CALM was coexpressed, and that the GAL4DBD-CALM/AF10-induced activation of transcription was strongly inhibited by FHL2. Western blot analyses showed no expression of FHL2 in any of the AML, T-cell and B-cell cell lines tested except for the EBV-transformed B-cell cell line L.C.L 3.1 and the B-cell lymphoma cell line Karpas 422. FHL2 was highly expressed in cervical cancer, osteosarcoma and CML cell lines. Micro array analyses of *FHL2* expression levels in 139 leukemia and normal bone marrow samples showed that *FHL2* is highly expressed in CML and in AML complex aberrant karyotypes. The inhibitory function of CALM on FHL2 in our reporter gene assays as well as the high expression levels of *FHL2* in CML and AML with complex aberrant karyotypes suggests that *FHL2* might play an important role in leukemogenesis not only in CALM/AF10 positive leukemias but also in a broader spectrum of leukemias.

The identification of the CALM-FHL2 interaction provides an interesting new entry point to dissect the mechanisms by which the CALM/AF10 fusion protein mediates leukemogenesis.

6 Zusammenfassung

Das *Clathrin Assembly Lymphoid Myeloid* Gen (*CALM*) wurde zuerst als Fusionspartner von AF10 in dem Fusionsgen CALM/AF10, bei der t(10;11)(p13;q14) Translokation entdeckt.. Das CALM/AF10 Fusionsprotein spielt eine entscheidende Rolle in der CALM/AF10 assoziierten Leukämieentstehung. Um einen Einblick in die Funktion von CALM zu erlangen, sind *Yeast Two Hybrid Screens* von HeLa und Thymus cDNA Bibliotheken mit der N-terminalen Hälfte von CALM als Köder durchgeführt worden. Dabei konnten acht potentielle CALM Interaktionspartner identifiziert werden, einschließlich des *four and a half LIM domain* Proteins (FHL2). Die Interaktion zwischen CALM und FHL2 wurde durch Koimmunopräzipitations-, GST-*pulldown*- und Kolokalisationsexperimente bestätigt. Die FHL2 Interaktionsdomäne von CALM wurde den Aminosäuren 294 bis 335 zugeordnet. Diese Domäne ist auch in dem CALM/AF10 Fusionsprotein enthalten. Die Expression von FHL2 führt zu einer Anreicherung von CALM in dem Zellkern, aber die vorwiegend zytoplasmatische Lokalisation von dem leukämischen Fusionsprotein CALM/AF10 wird nicht beeinflusst. Um den Einfluss von FHL2, CALM und CALM/AF10 aufeinander als Transkriptionsregulatoren zu analysieren, sind Luciferase Reportergenexperimente durchgeführt worden. Die Ergebnisse zeigen eine Hemmung der GAL4DBD-FHL2-induzierten Transkriptionsaktivierung durch gleichzeitige Expression von CALM. Ebenso wurde die GAL4DBD-CALM/AF10-induzierte Transkriptionsaktivierung stark durch Koexpression von FHL2 gehemmt. Western-Blot Analyse zeigt, dass FHL2 in weder in AML, noch in T- oder B-zell Zelllinien experimiert wird. Exprimiert wird FHL2 hingegen in der EBV-transformierten B-Zell Zelllinie L.C.L 3.1, in der B-Zell Lymphom -Zelllinie Karpas 422 exprimiert sowie in den Cervixkarzinom-, Osteosarkom- und CML-Zelllinien. Die *Micro Array* Analyse von dem *FHL2* Expressionsniveau in 139 Proben von Leukämiepatienten und normalen Knochenmark zeigen auf, dass *FHL2* in AML mit komplexem Karyotyp sowie in CML hochexprimiert ist.

Sowohl die hemmende Funktion von CALM auf FHL2 in unserem Reportergenexperiment, als auch das hohe Expressionsniveau von *FHL2* in CML und

AML mit komplexem Karyotyp, deuten an, dass FHL2 nur bei der Leukämieentstehung in *CALM/AF10* positiven Leukämien sondern auch in einem breiteren Spektrum von hämatologischen Tumoren eine wichtige Rolle spielt.

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Pasalic Z., Tizazu B, Bohlander S. The Four and a Half LIM domain protein FHL2 interacts with CALM, 16. Jahrestagung der Deutschen Gesellschaft für Humangenetik gemeinsam mit der Österreichischen Gesellschaft für Humangenetik und der Schweizerischen Gesellschaft für Medizinische Genetik,
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Workshops

ReMaT-Research management training — Sweden *2008*

Nationales Genomforschungsnetz-NGFN, Course in Practical DNA Microarray analysis — Germany *2008*

European School of Genetic medicine in Bertinoro di Romagna,
7th course in Bioinformatics for Molecular Biologists — Italy *2007*

EUCOMM/GSF training event:
Protection and Commercialization of Intellectual Property — Germany *2006*

Experience — University of Skövde, Sweden

Student Ambassador *1999-2000*
The primary task was to promote the University at education fairs, job centres etc. all over Sweden, but also receive assemblies of academic visits who arrive in order to get more information about the university

Voluntary work — University of Skövde, Sweden

- Department of Computational Science, University of Skövde, Sweden
  Elected class representative *2000-2002*
- Studentkåren i Skövde (student´s union) *2000*
  Elected member of authorized representative (1 mandate)
- Department of Nature Science, University of Skövde *1999-2000*
  Elected member of "institutionsnämnden". Institutionsnämnden is the executive group of the department and consists of 5 professors and 2 students. The student representatives pass on the opinions of students to theprofessors and vice versa. Among the fields where institutionsnämnden operates is the distribution of the budget of the department or forming the educational programs that will be offered at the department.
- Dataföreningen vid Högskolan i Skövde (Student´s computer Association)
  Elected member of the party committee *1997-1998*

Other work experiences

Eriksdalskolan, Primary and secondary school, Classes 1-10 — Skövde,Sweden
Worked as a substitute teacher *2002-2003*

Bosnisk-Hercegovinska Kvinnoförbundet, — Skövde, Sweden
(Bosnia-Herzegovinas National Assocation for Women) *1994-2000*
Co-worker in the magazine "Kvinna-Zena" (Woman Swe-Bos)