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ORIGINAL ARTICLE

FHL2 interacts with CALM and is highly expressed in acute erythroid leukemia

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The t(10;11)(p13;q14) translocation results in the fusion of the CALM (clathrin assembly lymphoid myeloid leukemia protein) and AF10 genes. This translocation is observed in acute myeloblastic leukemia (AML M6), acute lymphoblastic leukemia (ALL) and malignant lymphoma. Using a yeast two-hybrid screen, the four and a half LIM domain protein 2 (FHL2) was identified as a CALM interacting protein. Recently, high expression of FHL2 in breast, gastric, colon, lung as well as in prostate cancer was shown to be associated with an adverse prognosis. The interaction between CALM and FHL2 was confirmed by glutathione S-transferase-pulldown assay and co-immunoprecipitation experiments. The FHL2 interaction domain of CALM was mapped to amino acids 294–335 of CALM. The transcriptional activation capacity of FHL2 was reduced by CALM, but not by CALM/AF10, which suggests that regulation of FHL2 by CALM might be disturbed in CALM/AF10-positive leukemia. Extremely high expression of FHL2 was seen in acute erythroid leukemia (AML M6). FHL2 was also highly expressed in chronic myeloid leukemia and in AML with complex aberrant karyotype. These results suggest that FHL2 may play an important role in leukemogenesis, especially in the case of AML M6.

Introduction

Chromosomal translocations play a crucial role in the development of many types of leukemia, lymphomas, sarcomas and solid tumors.[1] In translocations, normal gene function can be altered in two different ways: either by the formation of fusion genes or by transcriptional deregulation of genes adjacent to the breakpoints.[2] The t(10;11)(p12;q14) translocation results in the fusion of *CALM* (*clathrin assembly lymphoid myeloid leukemia protein*) on chromosome 11 band q14 with *AF10* (*MLLT10*) on chromosome 10 band p12.[3] The CALM/AF10 fusion is observed in acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL) and malignant lymphoma, and has a poor prognosis[4,5] (Figure 1a).

The *AF10* gene was first identified as the fusion partner of *MLL* in AML patients carrying a t(10;11)(p12;q23) translocation.[6] *AF10* codes for a 1027-amino-acid-long putative zinc-finger transcription factor. The *Drosophila* homolog of *AF10*, *alhambra*,

has been suggested to play a role in heterochromatin-mediated transcriptional silencing.[7] We could recently show that AF10 interacts with Ikaros, an important regulator of lymphoid development, and that CALM/AF10 influences the subcellular localization of Ikaros.[8]

The *CALM* gene is located on chromosomes 11 band q14 and encodes a 652-amino-acid-long protein. CALM is ubiquitously expressed and homologous to the neuron-specific clathrin assembly protein AP180.[9] CALM is mainly located in the cytoplasm and along the membrane in clathrin-coated pits.[9] CALM does also shuttle between the cytoplasm and the nucleus, permitting CALM and the nucleolar protein CATS to interact.[10]

CALM promotes the assembly of clathrin into clathrin cages and takes part in the initial stage of clathrin-coated pit formation and invagination together with clathrin, AP-2 and PtdIns(4,5) P_2.[9,11–13] Both overexpression and downregulation of CALM have been shown to inhibit clathrin-mediated endocytosis and impair the trafficking of receptors between the trans-Golgi network and endosomes.[9,12] Interestingly, point mutations in the mouse *CALM* homolog *Picalm* were shown to cause abnormalities in hematopoiesis, iron metabolism and bone growth.[14]

The CALM/AF10 fusion protein has been shown to be strongly leukemogenic. It causes an aggressive acute leukemia in a murine bone marrow transplantation model[15] and leads to the development of acute leukemia in a transgenic mouse model after a median latency of about 12 months.[16]

To study the function of CALM and the CALM/AF10 protein, we searched for protein interaction partners of CALM using a yeast two-hybrid (Y2H) approach. The four and a half LIM domain protein 2 (FHL2) was identified as one of several CALM interacting partners. FHL2 has been shown to be involved in several important cellular processes like transcriptional regulation, DNA replication and signal-transduction pathways.[17] FHL2 plays an important role in Wnt signaling.[18,19] Recently, high expression of *FHL2* has been associated with adverse prognosis in breast and prostate cancer,[20–22] and high expression of *FHL2* is found in ovarian and gastrointestinal cancer.[23,24] In this study, we show that FHL2 interacts with CALM and that it is highly expressed in acute erythroid leukemia (AML M6), suggesting an important role for FHL2 in leukemogenesis, especially in the development of erythroleukemia.
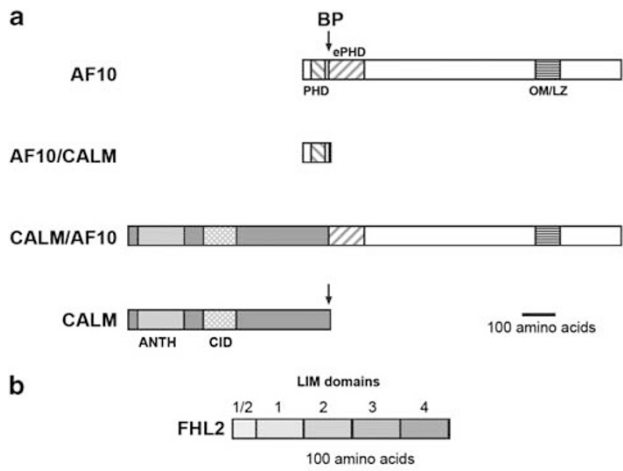
Materials and methods

Plasmid construction
The bait plasmids for the Y2H screen were constructed by inserting the full-length CALM cDNA, as well as the CALM deletion mutants in frame into pGBKT7 vector (Clontech, Heidelberg, Germany), to be expressed as GAL4-DBD fusion proteins. The following fragments of CALM were cloned in

Figure 1 (**a**) Structure of AF10, AF10/CALM, CALM/AF10 and CALM. BP: breakpoint; PHD: plant homeo domain; ePHD: extended plant homeo domain; OM/LZ: octapeptide/leucine zipper; ANTH: AP180 N-terminal homology domain; CID: CATS interaction domain. The BP in AF10 and CALM is indicated by arrows. (**b**) Structure of the FHL2 protein. FHL2 is composed of four and a half LIM domains.

frame with the GAL4-DBD (aa 1–147) into the yeast expression vector pGBKT7: (1) CALM (1–408); (2) CALM (1–294); (3) CALM (1–335); (4) CALM (1–221); and (5) CALM (1–105). For mapping the CALM interaction domain of FHL2, the following FHL2 fragments were cloned in frame into the pGBKT7 vector: (1) LIM domains $\frac{1}{2}$–3; (2) LIM domains $\frac{1}{2}$–2; (3) LIM domains $\frac{1}{2}$–1; and (4) LIM domain $\frac{1}{2}$, all PCR amplified using the following forward primer: 5′-ATTACCATGGATGGCTGGCATTTTGACTTTGGG-3′ and the following backward primers: LIM $\frac{1}{2}$ (5′-CGGGATCCCTGACGAACAGGGTCTCAAAGCACAC-3′), LIM $\frac{1}{2}$-1 (5′-CGGGATCCCGCATTCCTGGCACTTGGATGAG-3′), LIM $\frac{1}{2}$-2 (5′-CGGGATCCCTGACGCACT-GCATGGCATGTTG-3′) and LIM $\frac{1}{2}$-3 (5′-CGGGATCCCTGACCCAGCACACTTCTTGG-CATAC-3′).

For the GST-pulldown assay, the GST-Flirt 1 (full-length FHL2) construct was used (a gift from Dr Judith Müller, Freiburg, Germany). The FLAG-FHL2 construct for co-immunoprecipitation was provided by Dr Paul Riley (London, UK). Dr Judith Müller (Freiburg) provided the GAL4-DBD-FHL2 construct for the luciferase assay. The pGAL4-DBD-CALM/AF10 plasmid was constructed by inserting the full-length CALM/AF10 open reading frame, fused to GAL4-DBD (aa 1–47), into pcDNA3. Plasmids expressing YFP-FHL2 fusion protein was constructed in the pEYFP-C1 vector (Clontech).

Y2H screen
A co-transformation of the yeast strain AH109 (Clontech), using the lithium-acetate procedure, was performed with the pGBKT7-CALM (1–408) bait plasmid and a thymus library cloned into pACT2 (Clontech). The resulting colonies were plated on selective plates lacking histidine, adenine, leucine and trypto-phan, and colonies exhibiting a positive β-galactosidase reaction were selected for further analysis.

Mapping of interacting domains
The interaction domain mapping was performed by co-transformation of the CALM deletion mutants and pACT-FHL2

prey plasmid into yeast strain AH109. Growth was assayed on selective plates lacking tryptophan and leucine (–W, –L: transformation control) and interaction was scored on plates lacking histidine, adenine, leucine and tryptophan (–H, –A, –L, –W: interaction assay).

Transfection of mammalian cells and preparation of cytoplasmic extracts
A total of 1×10^6 HEK293T cells grown in 100 mm Petri dishes were transiently transfected with 8 μg of plasmid DNA and 12 μg polyethyleneimine (Sigma-Aldrich, Steinheim, Germany). After 24 h, the cells were harvested and nuclear extract was prepared using 400 μl hypotonic buffer (10 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM dithiothreitol, 4 μl protease inhibitor cocktail (Sigma-Aldrich)) as lysis buffer for cytoplasmic extracts. Cytoplasmic extracts were then used freshly for immunoprecipitation experiments.

GST-pulldown
The GST-Flirt 1 (full-length FHL2) was expressed in the *Escherichia coli* strain XL1 blue (Agilent Technologies, Böblingen, Germany) and purified from bacterial proteins by incubation with glutathione Sepharose 4B beads (GE Healthcare Bio-Sciences, Munich, Germany). The CALM protein was obtained from the *in vitro* TNT translation (Promega, Mannheim, Germany) of the pGBKT7-CALM (aa 1–408) in the presence of ^{35}S-methionine. A measure of 20 μl of the ^{35}S-labeled CALM lysate was incubated with 10 μl of Sepharose-bound GST fusion protein or Sepharose-bound GST as control in 100 μl binding buffer (100 mM NaCl, 25 mM Tris (pH 7.5), 0.1% NP-40). After incubation, the beads were washed five times with the binding buffer and subsequently boiled and separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The CALM protein was then detected by autoradiography.

Immunoprecipitation
A measure of 200 μl of freshly prepared cytoplasmic extract from HEK293T cells, co-transfected with pcDNA-FLAG-FHL2 and YFP-CALM, was used for the immunoprecipitation experiment. The cytoplasmic extract was incubated in 200 μl protein binding buffer (Dulbecco's phosphate-buffered saline (DPBS), 0.1% NP-40) together with 15 μl GFP-binder beads. The reaction was incubated for 30 min at 4 °C on a rotating platform, whereafter the beads were washed extensively with the binding buffer. The beads were then resuspended in SDS sample buffer, boiled and separated on a 12% SDS-PAGE gel. Subsequent western blot analysis was carried out with a monoclonal rabbit anti-GFP antibody (Life Technologies, Darmstadt, Germany), to detect the precipitated YFP-CALM and YFP, and the monoclonal mouse anti-FLAG M2 antibody (Sigma-Aldrich) to detect the co-immunoprecipitated FHL2.

Immunoblotting analysis
Immunocomplexes and cellular lysates were electrophoresed on 10–12% SDS-PAGE gel and transferred to PVDF membrane (Hybond P; GE Healthcare). The membranes were blocked for 1 h with 5% non-fat dried milk at room temperature and probed with the described primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase. Proteins were detected with an enhanced chemiluminescence reagent (ECL; GE Healthcare).

Immunostaining and confocal laser scanning fluorescence microscopy

For localization studies, U2OS (bone sarcoma) cells were grown on coverslips and co-transfected with FLAG-FHL2 and YFP-CALM or FLAG-CALM/A10 and YFP-FHL2. After 24 h, the cells were fixed for 10 min with PBS 2% paraformaldehyde, and then permeabilized with PBS 0.1% Triton X-100 for 10 min and blocked with PBS 10% fetal calf serum for 1 h. Coverslips were incubated with monoclonal mouse anti-FLAG M2 antibody (Sigma-Aldrich). After extensive washing with PBS 0.1% Tween, the coverslips were incubated with secondary Cy3-conjugated antibody for 1 h in room temperature. After further washing, step cells were stained with 4′,6-diamidino-2-phenylindole for 2 min and then mounted with Cytomation medium (Dako, Hamburg, Germany). The preparations were analyzed with a confocal fluorescence laser scanning system (TCS-SP2 scanning system and DM IRB inverted microscope; Leica, Solms, Germany).

Reporter gene assays

A total of 5×10^4 HEK293T cells were seeded in 24-well plates and co-transfected with 100 ng GAL4 SV40 minimal promoter reporter plasmid,[10] 20 ng of p-RL co-reporter vector (Promega), 200 ng of GAL4-DBD-FHL2- or GAL4-DBD-only expression plasmids, 100–400 ng of YFP-CALM and YFP-CALM/AF10, respectively, and as a control the pEYFP-C1 (Clontech) only. For the CALM/AF10 experiment, the cells were co-transfected with 100 ng GAL4 SV40 minimal promoter reporter plasmid, 20 ng of p-RL co-reporter vector (Promega), 100 ng GAL4-DBD-CALM/AF10 or GAL4-DBD empty expression plasmid, 100 ng pcDNA-FLAG-FHL2 and empty pcDNA vector. The cells were harvested after 24 h and assayed for firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter Assay System (Promega). The *Renilla* luciferase activity measurements were used for normalization. Experiments were carried out four times and the samples were measured three times.

Expression analysis of FHL2 in leukemic samples

For the first set of expression analysis, patient bone marrow samples were hybridized to Affymetrix HG-U133A and HG-U133B chips as described previously.[25] The HG-U133A, HG-U133B and HG-U133plus2 CEL files were first normalized separately using robust multi-array normalization and then combined into one matrix and normalized altogether using an empirical Bayes method as described previously.[26] The normalized expression data were analyzed with the R software package.[27] The expression signal intensities are given on a logarithmic scale.

For the second analysis, 308 expression profiles from AML leukemia patients were analyzed on Affymetrix HG-U133A, HG-U133B and HG-U133plus2 chips. The data were depicted as boxplot. The Wilcoxon–Mann–Whitney test was used to calculate the statistical significance.

Quantitative real-time PCR

qPCR was used to determine the mRNA expression levels of FHL2 in patients with AML M6, normal and complex karyotypes. The endogenous control RPL10A was selected from the microarray data. Individual qPCR reactions were set up in triplicates in 96-well plates and were carried out in 10 μl reactions with TaqMan Gene Expression Assay according to the manufacturer's protocol (Life Technologies). In all, 100 ng of patient cDNA was used for each reaction. The plates were run and analyzed on an ABI PRISM 7900HT Sequence Detection System (SDS 2.1; Applied Biosystems) according to the manufacturer's protocol (Applied Biosystems). The $2^{-\Delta\Delta CT}$ relative quantification method was used to analyze the qPCR experiment.[28]

Results

Identification of FHL2 as a CALM interacting protein

To identify interaction partners of the CALM protein, the N-terminal 408 amino acids of CALM were used as a bait to perform a Y2H screen of a human thymus cDNA library. The N-terminal 408 amino acids of CALM are contained within the CALM/AF10 fusion protein and do not show any transcriptional activation properties in yeast.[10] Under high stringency screening conditions of 1×10^6 primary transformants, six clones were identified that grew in the absence of histidine and adenine and were β-galactosidase positive. These six clones encoded five different proteins. One of these clones contained the complete open reading frame of *FHL2* (Figure 1b). *FHL2* encodes for a 279-amino-acid-long protein with four and a half LIM (Lin11, Isl-1, Mec-3) domains, reported to be overexpressed in several different cancer types.

CALM interacts with FHL2 in vitro

To confirm the interaction between CALM and FHL2 *in vitro*, a glutathione *S*-transferase (GST)-pulldown assay was performed. FHL2 was expressed as a GST fusion protein in bacteria and immobilized on glutathione beads. The beads were then incubated with ^{35}S-methionine-labeled CALM protein (aa 1–408). FHL2, but not GST alone, retained the radiolabeled CALM protein (Figure 2a). These results indicate a direct physical interaction between CALM and FHL2.

CALM interacts with FHL2 in vivo

The *in vivo* interaction between CALM and FHL2 was confirmed by co-immunoprecipitation experiments. After transient transfection of FLAG-FHL2- and YFP-CALM-expressing plasmids in HEK293T cells, and immunoprecipitation of YFP-CALM using GFP-binder beads,[29] the co-precipitated FHL2 protein could be detected with anti-FLAG antibodies (Figure 2b). We were also able to co-precipitate small amounts of overexpressed FHL2 by immunoprecipitation of endogenous CALM in HEK293T cells (Supplementary Figure S1).

Mapping of the FHL2 interacting domains of CALM

To map the minimal FHL2 interaction domain of CALM, yeast cells were co-transformed with several CALM deletion mutants fused to the GAL4-DNA-binding domain (DBD) and the FHL2 prey clone (Figure 3a). Only transformants with CALM mutants containing amino acids 294–335 grew on selective plates indicating protein–protein interaction (Figure 3b). These results show that amino acids 294–335 of CALM are required for its interaction with FHL2.

Mapping of the CALM interacting domains of FHL2

To map the minimal CALM interaction domain of FHL2, yeast cells were co-transformed with several FHL2 deletion mutants fused to GAL4-DBD and with a CALM prey clone, obtained in the Y2H screening using the CALM bait (Figure 3c). Only transformants with full-length FHL2 grew on selective plates
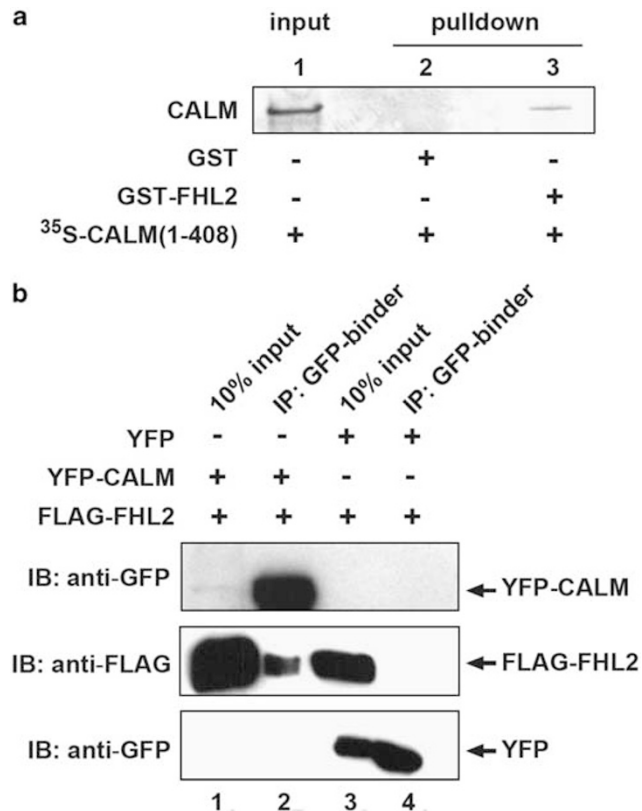
Figure 2 (**a**) GST-pulldown experiment: bead-bound GST-FHL2 was incubated with ^{35}S-labeled CALM (1–408), and the beads were washed and analyzed by SDS-PAGE. Only GST-FLH2 (lane 3), but not GST alone (lane 2), is able to retain ^{35}S-labeled-CALM (1–408). (**b**) Co-immunoprecipitation (co-IP) of FLAG-FHL2 and YFP-CALM. HEK293T cells were transiently co-transfected with plasmids expressing FLAG-FHL2 and YFP-CALM or FLAG-FHL2 and YFP. In lanes 1 and 3, 10% of the protein extract that was used for each co-IP reaction is loaded. GFP-binder beads were used for the precipitation, and only YFP-CALM (lane 2), but not YFP alone (lane 4), is able to precipitate FLAG-FHL2.

indicating protein–protein interaction (Figure 3c). These results show that amino acids 206–279 of FHL2 are required for its interaction with CALM.

CALM/AF10 alters the subcellular localization of FHL2
To learn more about the interaction between CALM and FHL2, colocalization experiments were performed. YFP-CALM, YFP-CALM/AF10 and YFP-FHL2 were transiently expressed in the osteosarcoma cell line U2OS (Figures 4a–i). YFP-CALM is localized mainly in the cytoplasm (Figures 4a and b), as is YFP-CALM/AF10 (Figures 4d and e). In contrast to YFP-CALM, the CALM/AF10 protein is enriched in speckles in the cytoplasm (Figure 4e). These cytoplasmic speckles might be CALM/AF10 protein inclusions in clathrin-coated pits due to disturbed endocytosis or because of an altered solubility of the CALM/AF10 protein. YFP-FHL2 is expressed in both the cytoplasm and the nucleus (Figures 4g and h). When FLAG-FHL2 was co-expressed with YFP-CALM, the distribution of YFP-CALM changed with more YFP-CALM found in the nucleus (compare Figures 4b and k). However, the distribution of FHL2 did not change noticeably upon co-expression of YFP-CALM (compare Figures 4h and l). There was also colocalization of YFP-CALM and FLAG-FHL2 in the nucleus and especially in the cytoplasm (Figures 4j–m), as it can be visualized in line scan images in

Figure 5a. When FLAG-FHL2 was co-expressed with YFP-CALM/AF10 (Figures 4n–q), the subcellular localization of YFP-CALM/AF10 did not change markedly (compare Figures 4e and o), but there were pronounced changes in the cytoplasmic localization of FLAG-FHL2 (compare Figures 4h and p), which colocalized with YFP-CALM/AF10 in distinct cytoplasmic speckles (Figures 4n–q). The colocalization of FLAG-FHL2 and YFP-CALM/AF10 was also clearly visible in the line scans of the confocal images (Figure 5b). The same pattern of colocalization of CALM/AF10 and FHL2 was also observed when a FLAG-tagged CALM/AF10 and YFP-FHL2 fusion protein were co-expressed (Figures 4r–u and 5c).

CALM reduces the transcriptional activation of FHL2
Since FHL2 is known to act as an activator of transcription in reporter gene assays when fused to a GAL4-DBD (GAL4-DBD-FHL2),[30] we tested the influence of CALM and CALM/AF10 on FHL2-mediated transcriptional activation using a GAL4-UAS minimal SV40 promoter luciferase reporter construct (GAL4luc) in transiently transfected HEK293T cells. Expression of GAL4-DBD-FHL2 resulted in a 40-fold activation of the reporter gene. Co-expression of YFP-CALM and GAL4-DBD-FHL2 reduced the FHL2-mediated activation from approximately 40- to 20-fold (Figure 6a). However, co-expression of YFP-CALM/AF10 had no effect on FHL2-mediated activation (Figure 6b). One explanation for this result could be the fact that the AF10 portion of CALM/AF10 contains an activating domain (see Figure 6c) that over-rides the repressive effects seen with YFP-CALM. Interestingly, when CALM/AF10 was expressed as a GAL4-DBD fusion (GAL4-DBD-CALM/AF10), a strong transcriptional activation of about 50-fold over background of the reporter gene was seen (Figure 6c). Co-expression of FLAG-FHL2 led to a threefold reduction of the CALM/AF10-mediated activation (Figure 6c).

FHL2 is highly expressed in AML M6 and is a marker for erythroleukemia
To obtain an overview of *FHL2* expression levels in leukemia cells, we analyzed the expression of *FHL2* in bone marrow samples from 129 leukemia patients and 10 normal bone marrows using Affymetrix expression chips. High expression of *FHL2* was observed in AML samples with complex aberrant karyotypes and in chronic myeloid leukemia (CML) patient samples. The expression levels of *FHL2* were relatively low in all the other subgroups examined, including CALM/AF10-positive leukemias (Figure 7). The higher expression levels of *FHL2* in CML patients and AML patients with complex aberrant karyotypes did not reach statistical significance at the 0.05 level, but suggested a trend, which was then examined in a larger group of AML patients.

The second analysis was performed with a total of 308 leukemia patient samples using Affymetrix expression chips that were divided into three groups. Group 1 consisted of 167 AML patients with normal karyotypes and excluding any samples with erythroleukemia (AML-M6). Group 2 was composed of 115 AML patients with complex aberrant karyotypes, which included 46 patients with monosomy 5 or 5q deletions (subgroup '−5/5q−'), seven patients with monosomy 7 or 7q deletions (subgroup '−7/7q−'), 47 patients with aberrations of both chromosomes 5 and 7 (subgroup '5 + 7') and 15 patients with no aberration of either chromosome 5 or 7 (subgroup 'neither'). Group 3 contained 26 patients with AML M6. This second analysis clearly showed that *FHL2* is significantly higher expressed in AML M6 patients than in patients with normal
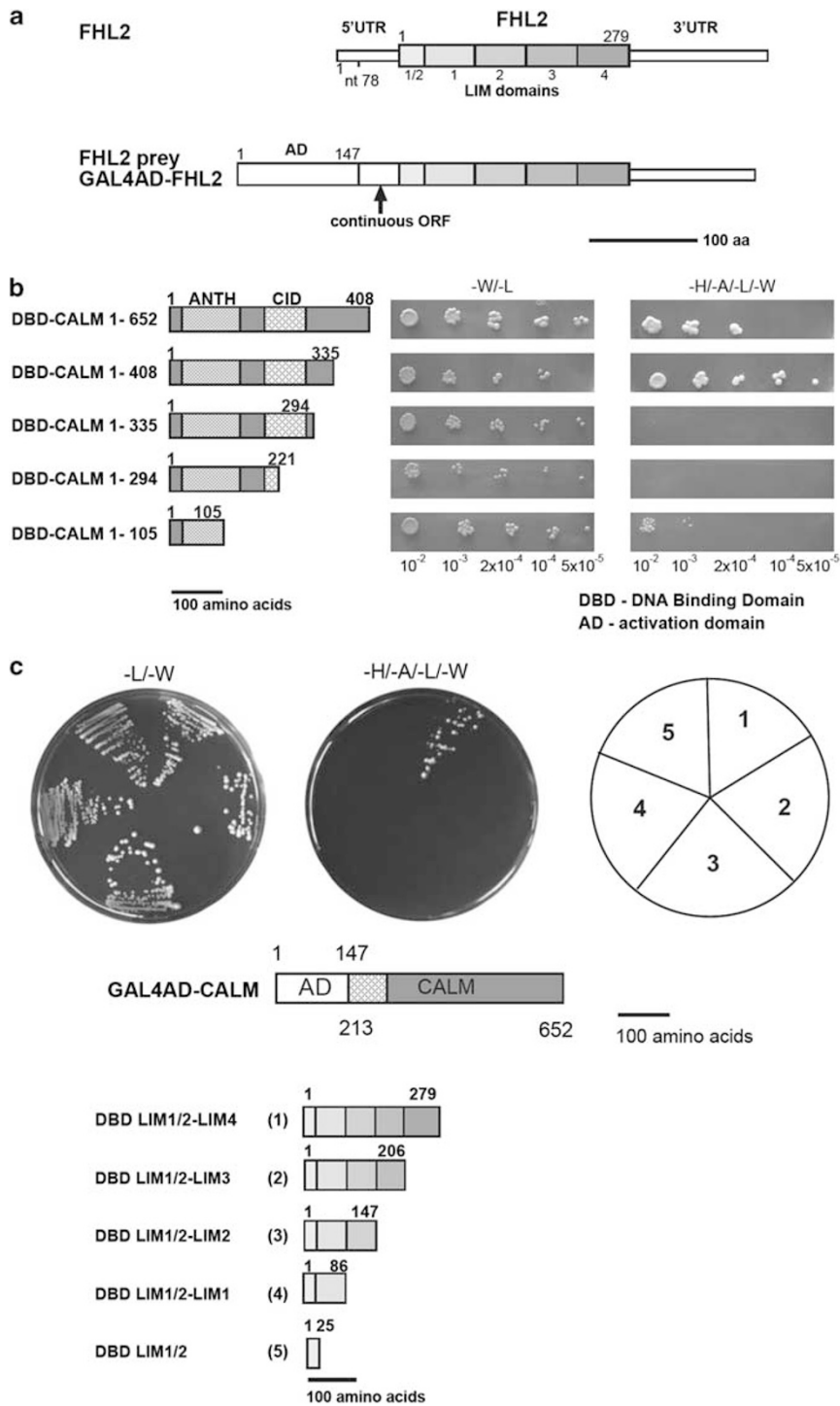
Figure 3 (**a**) FHL2 prey clone: alignment of the FHL2 prey clone with FHL2 (GAL4AD-FHL2). UTR: untranslated region; ORF: open reading frame; AD: activation domain; nt: nucleotide. Numbers above the diagrams refer to amino acids, and that below refer to nucleotides. (**b**) Mapping of the FHL2 interaction domain of CALM. A series of CALM deletion mutants was expressed as bait proteins in yeast together with the FHL2 prey protein (GAL4AD-FHL2). Dilutions of the transformants were spotted on yeast synthetic drop-out medium lacking tryptophan and leucine (–W/–L) to control for double transformant status or lacking tryptophan, leucine, histidine and adenine (–H/–A/–L/–W) to assay the activation of the reporter genes. Only the yeast clones with the CALM mutants CALM (1–408) and CALM (1–335) grow on the –H/–A/–L/–W plates, indicating that amino acids 294–335 of CALM are necessary for its interaction with FHL2. (**c**) Mapping of the CALM interaction domain of FHL2. Structure of CALM prey clone obtained from the yeast two-hybrid assay, GAL4 AD-CALM. AD: activation domain. A series of FHL2 deletion mutants was expressed as bait proteins in yeast together with the CALM prey protein (GAL4AD-CALM). Yeast double transformants were streaked on yeast synthetic drop-out medium lacking tryptophan and leucine (–W/–L) to control for double transformant status or on SD plates lacking tryptophan, leucine, histidine and adenine (–H/–A/–L/–W) to assay the activation of the reporter genes. Only the yeast clones with the full-length FHL2 (clone (1)) grow on the –H/–A/–L/–W plates, indicating that amino acids 206–279 of FHL2 are required for interaction with CALM.
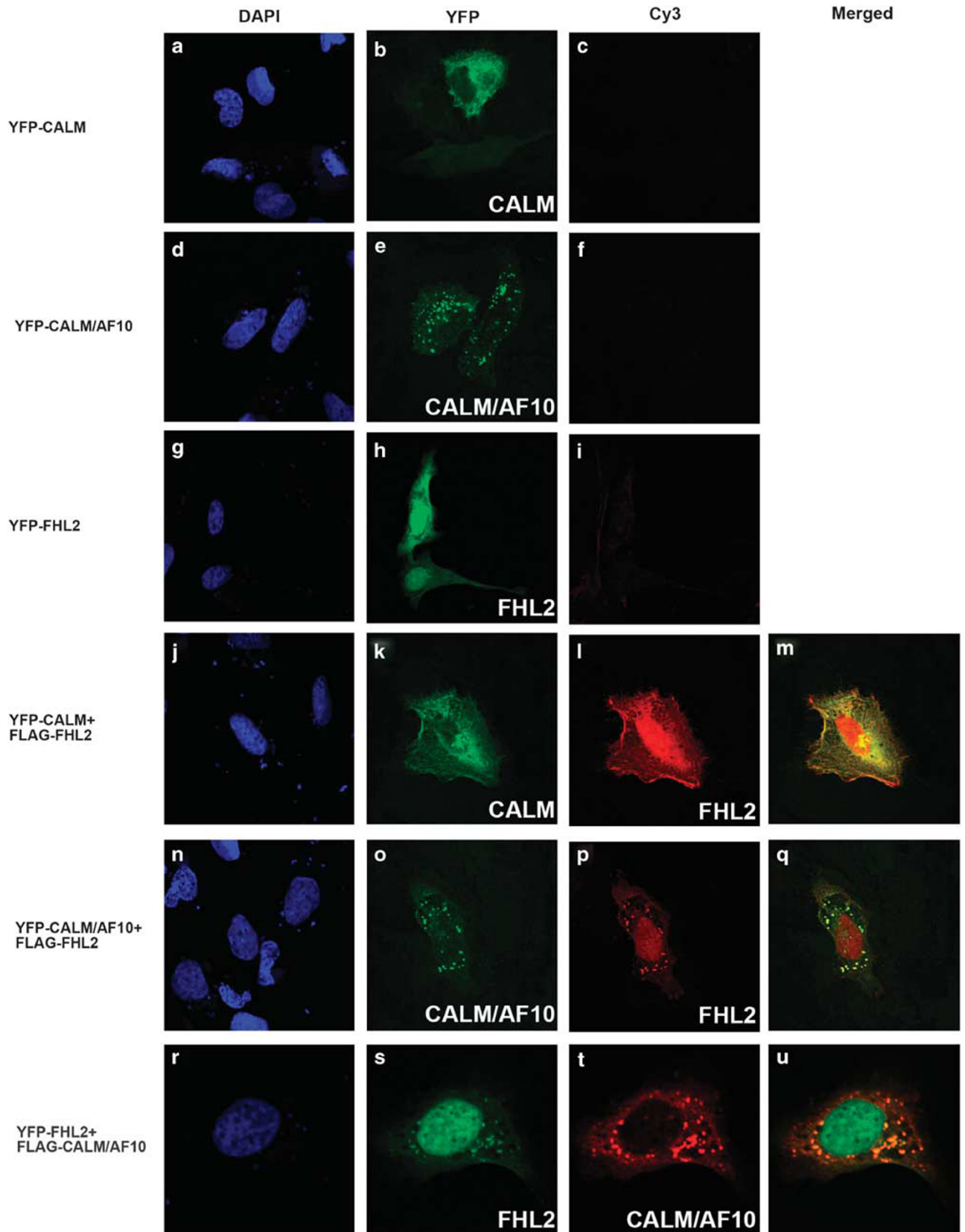
Figure 4 Subcellular localization of FHL2, CALM and CALM/AF10. Confocal images of U2OS cells transiently transfected with plasmids expressing the proteins indicated on the left-hand side. Channels are indicated above the images (4′,6-diamidino-2-phenylindole, YFP, Cy3 and merged). For a detailed explanation of this figure, see the Results section.
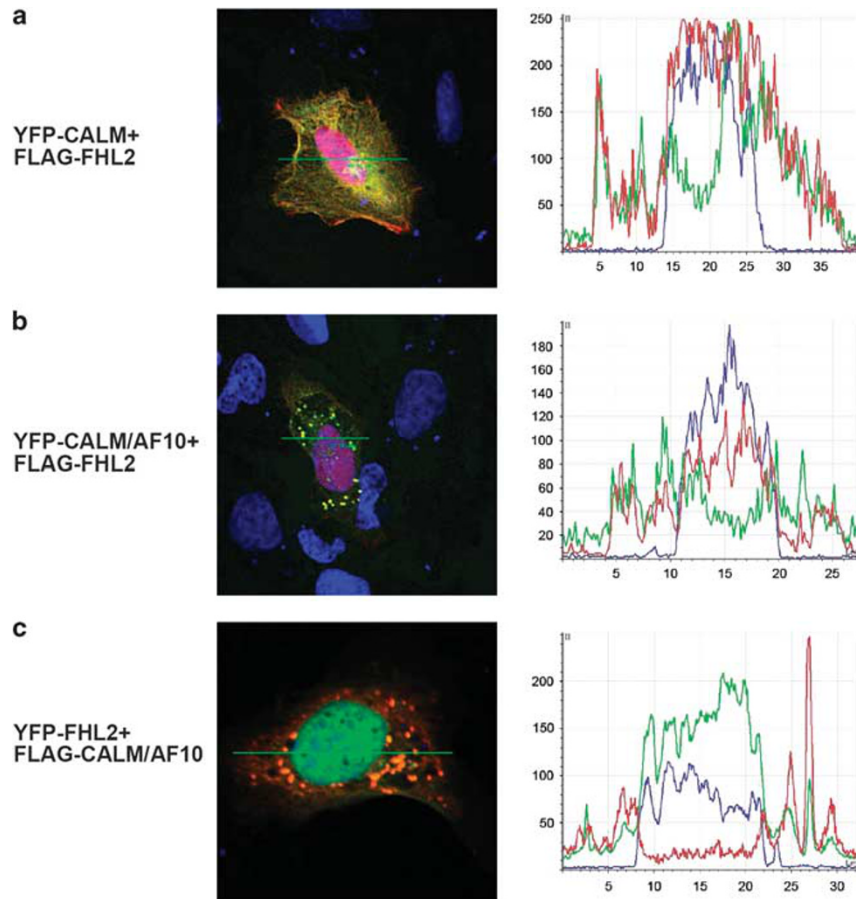
Figure 5 Line scans of the confocal images m, q and u from Figure 5 to demonstrate colocalization of (**a**) FLAG-FHL2 with YFP-CALM; (**b**) FLAG-FHL2 with YFP-CALM/AF10; and (**c**) YFP-FHL2 with FLAG-CALM/AF10.

karyotype or in the patients with complex aberrant karyotypes (P-value $= 5.256 \times 10^{-13}$ and $= 7.543 \times 10^{-7}$, respectively) (Figure 8). There were no significant differences in the level of *FHL2* expression between the different subgroups of patients with complex aberrant karyotypes (data not shown).

High expression of FHL2 mRNA transcript in patients with AML M6

To confirm the results obtained from the microarray experiment, a quantitative real-time-polymerase chain reaction (qPCR) was performed on 45 patient samples. The 45 patients were divided into three groups: patients with AML M6 ($n = 15$), patients with complex karyotype ($n = 15$) and AML patients with normal karyotype ($n = 15$). The qPCR revealed that the mRNA expression of FHL2 in patients with AML M6 was significantly higher than that in patients with complex karyotype (P-value: 0.000 with (2369, 5379) 95% confidence interval) or normal karyotype (P-value: 0.000 (2064, 5156) with 95% confidence interval). No significant difference was detected between the mRNA expression of FHL2 in patients with complex karyotype or normal karyotype (P-value: 0.685 (-0.906, 0.378) with 95% confidence interval) (Figure 9).

Discussion

The CALM/AF10 fusion is found in myeloid and lymphoid lineage acute leukemias. In contrast to many other leukemia-associated fusion genes (for example, AML1/ETO), expression of CALM/AF10 leads to the development of an acute leukemia with a latency of only 9 weeks in a murine bone marrow transplantation model.[15] One of the critical domains in the CALM/AF10 fusion protein that is required for malignant transformation is the octapeptide/leucine zipper domain in the AF10 portion of the fusion, which interacts with the histone methytransferase DOT1L.[31] The octapeptide/leucine zipper domain is also critical for the transformation potential of MLL/AF10 fusion.[32] However, much less is known about the domains in CALM that contribute to the transforming potential of the CALM/AF10. We therefore used a Y2H approach to identify CALM protein interactors.

Using the N-terminal 408 amino acids of CALM as bait, a member of the FHL subfamily of LIM-only proteins, FHL2, was identified as a protein interactor of CALM. FHL2 interacts with proteins belonging to different functional classes, including nuclear receptors, structural proteins, transcription factors and cofactors, splicing factors, DNA replication and repair enzymes, metabolic enzymes and signal transducers.[17,30,33–38]

The FHL2 interacting domain in CALM was mapped to amino acids 294–335 of CALM. The N-terminal domain (aa 22–138) of CALM contains the ANTH domain, which specifically binds to lipids (PtdIns(4,5)P$_2$) in the plasma membrane.[11,39–41] The three-dimensional structure of the N-terminal 280 amino acids of CALM has been solved and consists of 10 α-helices, which aggregate to form a globular protein.[11] The C-terminal half of CALM, on the other hand, has most likely a poorly ordered structure that contains multiple short protein interaction motifs,

functioning like a flexible 'fishing line'.[42] This composition of CALM permits the establishment of interactions during clathrin-coated pit assembly.[40] Interestingly, FHL2 binds to β-integrin,[43] which together with clathrin, a major binding partner of CALM, has been shown to be involved in endocytosis.[9,44] Taken together, these data suggest a role for the CALM–FHL2 interaction in the complex interaction network in endocytosis. The notion that FHL2 and CALM may act together in the cytoplasm in the endocytic machinery is supported by the fact that the expression of YFP-CALM and FLAG-FHL2 in U2OS cells result in colocalization of both proteins in the cytoplasm and at the cell membrane. In addition, CALM and FHL2 also colocalized in the nucleus of the U2OS cells, suggesting a

nuclear function for these proteins. FHL2 has previously been shown to be localized to both the nucleus and cytoplasm.[45–47]

Our immunofluorescence experiments with YFP-tagged FHL2 and FLAG-tagged CALM/AF10 showed that CALM/AF10 was located mainly in the cytoplasm, while the FLAG-tagged FHL2 was predominantly seen in the nucleus. Interestingly, a minor fraction of the FHL2 also colocalized with CALM/AF10 in distinct spots in the cytoplasm and there was a pronounced change in the distribution pattern of the cytoplasmic FHL2 when CALM/AF10 was co-expressed with FHL2 colocalizing with CALM/AF10 in distinct speckles in the cytoplasm. We suggest that these speckles might be inclusions of CALM/AF10 into clathrin-coated pits due to CALM/AF10-induced aberrant endocytosis. CALM/AF10 may tether its interaction partners into these endocytic vesicles and thereby disturb their physiological function.

FHL2 interacts with transcription factors like SKI and the promyelocytic leukemia zinc-finger protein (PLZF). PLZF has been shown to be a transcriptional repressor with a role in the control of cellular proliferation and Hox gene regulation,[48,49] and FHL2 has been shown to enhance PLZF-mediated repression.[50] PLZF is involved in the PLZF/RARα translocation found in patients with acute promyelocytic leukemia.[51] Through the interaction with PLZF, FHL2 may mediate the activation of Hox genes by CALM/AF10, which was previously observed in patient samples and murine models.[31,52] Interestingly, the FHL2 interactors FOXO1 and SIRT1[37] link FHL2 to the histone methyltransferase DOT1L, which interacts with AF10 and CALM/AF10 and methylates histone H3 at lysine 79. CALM-AF10-positive patient samples and cell lines show marked histone H3 K79 hypomethylation.[53] Therefore, CALM/AF10 may strongly influence the levels of H3K79 methylation by interacting with both FHL2 and DOT1L; however, it will be very challenging to understand the complex interplay between these multiple interacting proteins involved in the regulation of histone marks. The SKI–FHL2 interaction potentiates the function of β-catenin in melanoma cells, inducing proliferation, cell cycle alterations and tumor progression.[54] FHL2 is also a crucial member of the transmembrane glycoprotein EpCAM's signaling complex, which regulates transcription in the nucleus through its association with components of the Wnt signaling pathway, like β-catenin and Lef1.[55]
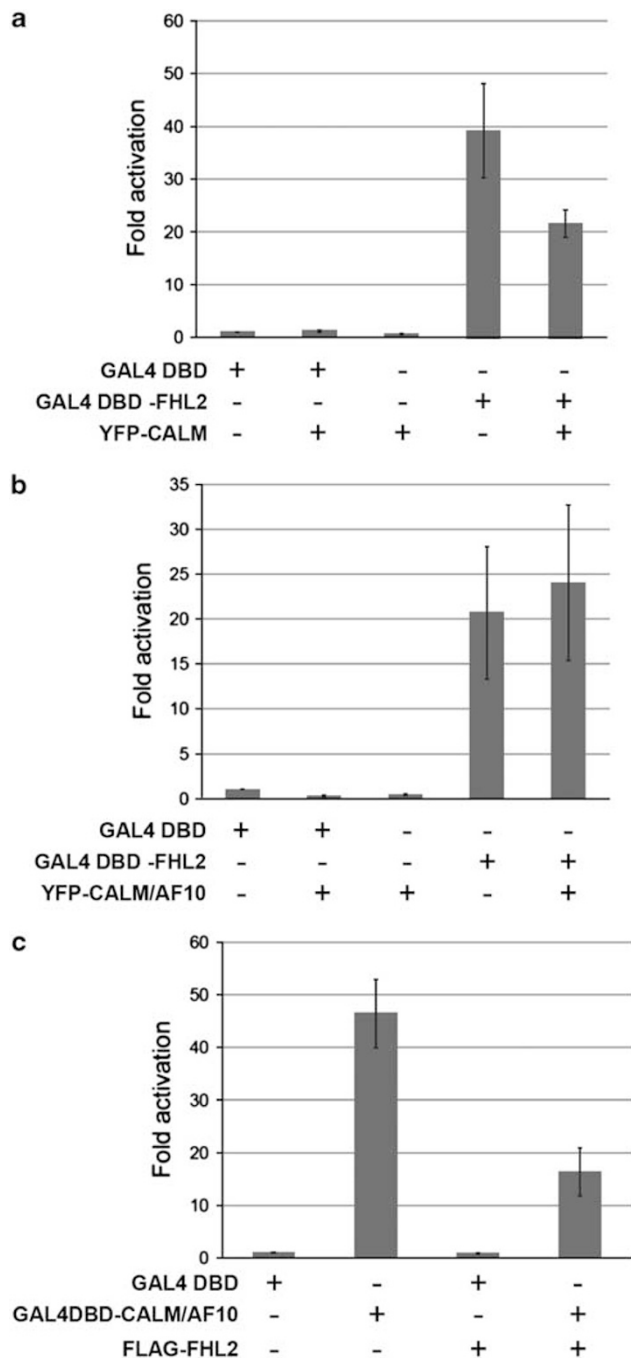

Figure 6 (a) CALM inhibits FHL2-dependent activation of transcription. HEK293T cells were transiently transfected with plasmids expressing GAL4DBD, GAL4DBD-FHL2 and YFP-CALM, a luciferase reporter plasmid and a Renilla luciferase control plasmid as indicated. The bars indicate fold activation of luciferase activity compared to GALDBD. Four independent experiments with three measurements per sample were performed. The error bars represent standard deviations. The firefly luciferase values were normalized to the Renilla luciferase values. (b) CALM/AF10 does not inhibit FHL2-dependent activation of transcription. HEK293T cells were transiently transfected with plasmids expressing GAL4DBD, GAL4DBD-FHL2 and YFP-CALM/CAF10 and a luciferase reporter plasmid as indicated. Co-transfection of YFP-CALM/AF10 did not have a significant influence on the transcriptional activation mediated by GAL4DBD-FHL2. (c) FHL2 inhibits CALM/AF10-dependent activation of transcription. HEK293T cells were transiently transfected with plasmids expressing GAL4DBD, GAL4DBD-CALM/AF10, YFP-CALM/AF10 and a luciferase reporter plasmid as indicated. In this setting, GAL4DBD-CALM/AF10 is a strong transcriptional activator. A threefold reduction of the GAL4DBD-CALM/AF10-mediated transcriptional activation is observed when FLAG-FHL2 is co-expressed. Three independent experiments were performed and measured in duplicate.
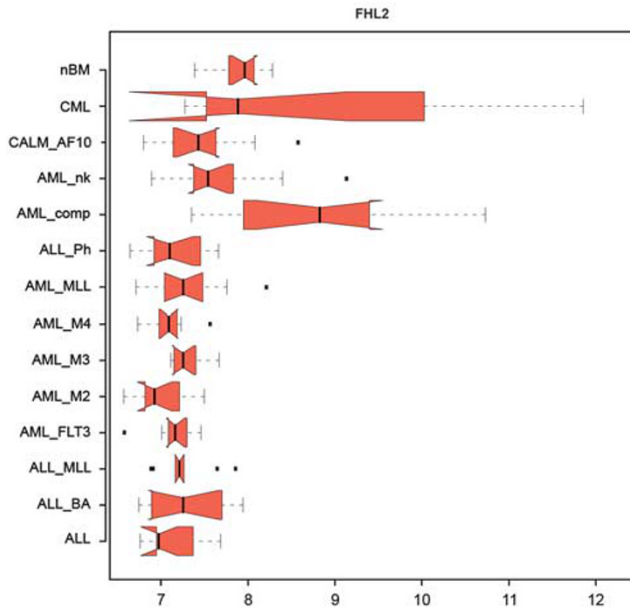
Figure 7 Expression of FHL2 in leukemia patient samples. FHL2 is expressed at higher levels in CML and AML with complex aberrant karyotype. A microarray analysis was performed on 129 patient samples and 10 normal bone marrow (nBM) samples. The boxplots depict the expression levels of FHL2 in nBM, CML, CALM/AF10-positive leukemias, in seven different AML subtypes (AML_nk: AML with normal karyotype; AML_comp: AML with complex aberrant karyotype; AML_MLL: AML with an MLL rearrangement; AML_M4: AML with CBFB/MYH11 fusion; AML_M3: acute promyelocytic leukemia with a PML/RARA fusion; AML_M2: AML with an AML1/ETO fusion; AML_FLT3: AML with normal karyotype and with an fms-like tyrosine kinase receptor-3 (FLT3) internal tandem duplication), and four ALL subtypes (ALL_Ph: ALL with BCR/ABL fusion; ALL-MLL: ALL with an MLL rearrangement; ALL_BA: c-ALL: (common ALL) and ALL: pro-B-ALL). Each group is composed of 10 patient samples, except the ALL-MLL patient group, which contains 9 samples. Expression intensities are depicted on a logarithmic scale. The median of the distribution is indicated by a bar. The box presents 50% of the expression values in that group and is called the interquartile range (IQR). An expression value is considered 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 is connected to the box via a horizontal line. If the notches of the boxes do not overlap between two groups, there is evidence that the medians are significantly different between the groups.

In reporter gene assay, FHL2 functions as a transcriptional activator when fused to the GAL4-DBD.[30] We could show that co-expression of CALM significantly disturbed the transcriptional activation induced by FHL2. Since the FHL2 interaction domain of CALM is present in the leukemogenic CALM/AF10 fusion protein, we also tested whether CALM/AF10 would influence the transcriptional activation function of GAL4-DBD-FHL2. Interestingly, this was not the case. The transcriptional activation mediated by FHL2 was inhibited by CALM, but not by CALM/AF10, suggesting that the CALM/AF10 fusion protein might interfere with a physiological negative regulatory role of CALM on the function of FHL2. In other words, CALM/AF10 might change the function of FHL2 and this could be an important mechanism in CALM/AF10-induced leukemia. In particular, the loss of FHL2 inhibition by CALM may potentiate the enhanced proliferation of myeloid progenitors that was observed upon forced expression of FHL2 in murine bone marrow cells.[56]
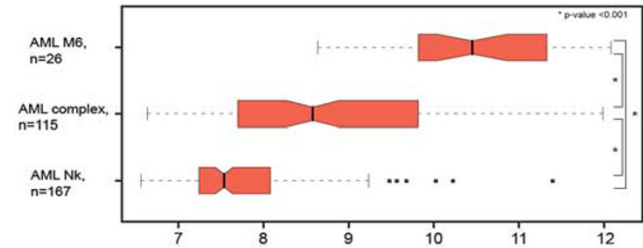
Figure 8 Expression of FHL2 in samples from patients with AML with complex aberrant karyotypes, AML M6 and AML with normal karyotypes. FHL2 microarray expression levels from 308 AML. Group 1 consists of 167 AML patients with normal karyotypes of all FAB subtypes, except M6, group 2 consists of 115 samples from patients with AML with complex aberrant karyotypes, and group 3 contains 26 samples from patients with AML M6. FHL2 is significantly higher expressed in patients with AML M6 than in AML patients with a normal karyotype or in patients with complex aberrant karyotypes ($P = 2.2 \times 10^{-16}$ and $P = 9.967 \times 10^{-10}$, respectively). A significant difference in the expression of FHL2 was found between AML patients with normal karyotype and patients with AML complex karyotype with a P-value $= 1.04 \times 10^{-12}$.
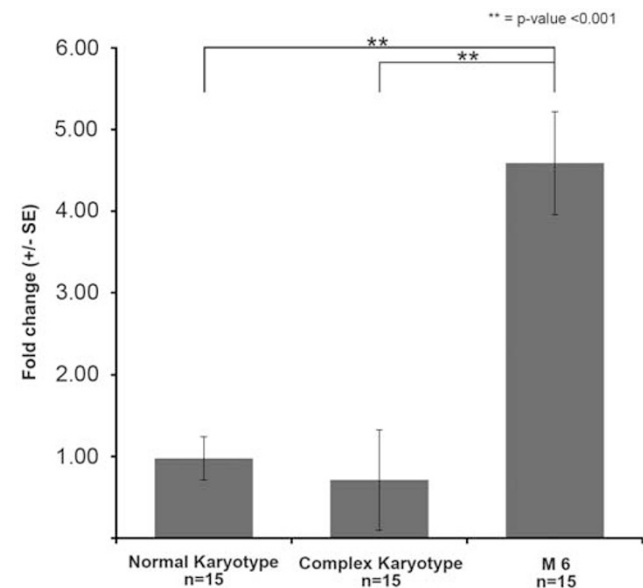
Figure 9 FHL2 is highly expressed in patients with AML M6 karyotype. *FHL2* expression was determined using qRT-PCR in 45 AML patient samples. High expression of FHL2 was observed in patients with AML M6 in comparison to patients with complex or normal karyotype (P-value: 0.000). No statistical significance was discovered between AML patients with complex karyotype and patients with normal karyotype (P-value: 0.685). The data are presented as fold change in gene expression compared to the gene expression of AML patients with normal karyotype.

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.[57] Interestingly, Qian *et al.*[56] showed that therapy-related myelodysplastic syndrome and 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. Another characteristic of AML with complex aberrant karyotypes is the frequent loss of chromosome 7 that is also associated with adverse prognosis.[58]

We also observed high expression levels of FHL2 in patients with complex aberrant karyotype and with CML. Although analyzing the expression levels of FHL2 in patients with monosomy 5 or del(5)(q), five patients with very high *FHL2* expression levels were noted (data not shown). Three of these outliers had erythroleukemia (AML M6) and two had AML M4. When we performed a new analysis using the *FHL2* expression data from all available M6 patient samples, a significantly higher *FHL2* expression in patients with AML M6 was discovered. Erythroid leukemia has a poor prognosis.[59] These results are in line with reports that high expression levels of FHL2 in breast and prostate cancer have recently been shown to be associated with an adverse prognosis.[20–22] Interestingly, we could recently show that the expression levels of another member of the FHL family, namely FHL1, correlated closely with a poor prognosis in cytogenetically normal AML.[25] Early erythroid cells are characterized by an increased expression of FHL2 (GNF Expression Atlas 2 Data from U133A and GNF1H Chips; http://www.genome.ucsc.edu). Thus, the observed overexpression of FHL2 could be only a marker of the erythroid lineage. Additional experiments are required to determine the causes and/or consequences of elevated *FHL2* expression levels in erythroid leukemia.

Our results, combined with previous reports of FHL2 being associated with adverse prognosis in different cancers as well as acting on the cell cycle program, thus regulating cell proliferation[46] and cell differentiation,[60] suggest that there might be a common oncogenic pathway in the development and progression of leukemogenesis as well as in tumorigenesis. However, more studies will be required to understand the role of the CALM–FHL2 interaction in leukemogenesis and especially its role in erythroleukemia.

Conflict of interest

The authors declare no conflict of interest.

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