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The Clathrin-Binding Domain of CALM-AF10 Alters the Phenotype of Myeloid Neoplasms in Mice

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Abstract

The *PICALM (CALM)* gene, whose product is involved in clathrin-mediated endocytosis, has been identified in two recurring chromosomal translocations, involving either *MLL* or *MLLT10* (*AF10*). We developed a mouse model of CALM-AF10^{+} leukemia to examine the hypothesis that disruption of endocytosis contributes to leukemogenesis. Exclusion of the C-terminal portion of CALM from the fusion protein, which is required for optimal binding to clathrin, resulted in the development of a myeloproliferative disease, while inclusion of this domain led to the development of acute myeloid leukemia and changes in gene expression of several cancer-related genes, notably *Pim1* and *Crebbp*. Nonetheless, the development of leukemia could not be attributed directly to interference with endocytosis or consequential changes in proliferation and signaling. In leukemia cells, full-length CALM-AF10 localized to the nucleus with no consistent effect on growth factor endocytosis, and suppressed H3K79 methylation regardless of the presence of clathrin. Using FRET analysis, we show that CALM-AF10 has a propensity to homo-oligomerize, raising the possibility that the function of endocytic proteins involved in chimeric fusions may be to provide dimerization properties, a recognized mechanism for unleashing oncogenic properties of chimeric transcription factors, rather than disrupting the internalization of growth factor receptors.

Keywords

AML; MPD; endocytosis; Dot1l; H3K79 methylation; oligomerization

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Introduction

Endocytosis has long been regarded as a mechanism of signaling attenuation through the degradation of signaling receptors. However, emerging evidence suggests that endocytic proteins are involved in additional cellular processes, including mitosis, and the regulation of signaling, cell cycle, apoptosis and cell fate determination (Lanzetti and Di Fiore, 2008). Given their involvement in a variety of physiological processes, an alteration in expression of endocytic pathway proteins is predicted to play a role in cancer. In support of this hypothesis, changes in expression levels of the endocytosis regulators, *HIP1*, *RAB25* and *NUMB*, have been linked to human cancer (Bradley *et al.*, 2007; Cheng *et al.*, 2004; Colaluca *et al.*, 2008), and chromosomal translocations involving genes encoding endocytic proteins, such as the clathrin heavy chain (*CTLC*), clathrin assembly lymphoid myeloid leukemia (*PICALM/CALM), EPS15*, *HIP1*, and *CBL* genes, have been identified in human hematological malignant diseases (Bernard *et al.*, 1994; Chikatsu *et al.*, 2003; Dreyling *et al.*, 1998; Fu *et al.*, 2003; Wechsler *et al.*, 2003). It is not known, however, whether the fusion proteins resulting from these translocations disrupt endocytic processes, and whether this contributes to malignant transformation. Here, we address this issue for acute leukemias with such translocations.

The t(10;11) (p13;q14) is a recurring translocation observed in both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), and has been shown to involve the fusion of the (*MLLT10/AF10*) gene on chromosome 10, with the (*PICALM/CALM*) gene on chromosome 11 (Carlson *et al.*, 2000; Dreyling *et al.*, 1996; Dreyling *et al.*, 1998). AF10 functions as a putative transcription factor binding DNA through an AT hook motif, and interacting with the SWI/SNF chromatin remodeling complex (Debernardi *et al.*, 2002; Linder *et al.*, 2000). The CALM protein binds clathrin and regulates clathrin-mediated endocytosis through its ability to regulate the orderly progression of clathrin-coat formation (Meyerholz *et al.*, 2005; Tebar *et al.*, 1999). Recent data suggests that CALM-AF10 interacts with the histone H3 lysine 79 (H3K79)-specific methyltransferase DOT1L, through the OM-LZ domain in AF10. This interaction leads to local H3K79 hypermethylation on the *Hox* loci and upregulation of *Hoxa* cluster genes (Caudell *et al.*, 2007; Deshpande *et al.*, 2006; Okada *et al.*, 2006). Expression of CALM-AF10 can also lead to a global reduction in H3K79 methylation, by sequestering DOT1L from chromatin, and this correlates with increased chromosome instability (Lin *et al.*, 2009). These particular transformation activities can be attributed to the AF10 component of the fusion protein, whereas the contribution of the fused CALM moiety to the transformation process remains unclear.

CALM has been shown to undergo nucleocytoplasmic shuttling (Vecchi *et al.*, 2001), suggesting that its fusion to AF10 might contribute to transcriptional deregulation; however, whether CALM itself possesses transcriptional activity remains unclear (Archangelo *et al.*, 2006; Vecchi *et al.*, 2001). Additionally, the fusion of CALM to AF10 could lead to a disruption of the endocytic process and enhancement of growth factor receptor signaling. Lastly, CALM's contribution to the oncogenic potential of AF10 could be attributed to its scaffolding properties. Another endocytic protein, EPS15, has been shown to contribute to oncogenic activation of *MLL-EPS15* by promoting dimerization of MLL (So *et al.*, 2003). By analogy, CALM may promote self-association of the CALM-AF10 fusion. In fact,

oligomerization of chimeric fusion transcripts, including MLL, RUNX1/AML1 and RARA fusions, has emerged as a powerful mechanism for activating their oncogenic properties (So and Cleary, 2004).

To decipher CALM's role in CALM-AF10^{+} leukemias, we set out to determine whether defective endocytosis contributes to the development of leukemia. Notably, the clathrin-binding domain of CALM-AF10 influenced the severity of disease. Although the presence of this domain was required for CALM-AF10 to inhibit transferrin endocytosis in a transfected human cell line, internalization of growth factor receptors and their signaling was not affected in leukemia cells lines or in primary leukemia cells isolated from mice transplanted with cells transduced with CALM-AF10. Moreover, the presence of CALM-AF10 in transduced mouse cells led to a global reduction in H3K79 methylation, independent of clathrin expression. However, using FRET analysis we showed that $CALM_{2091}AF10$ has a propensity to homo-oligomerize, suggesting that the oncogenic function of the CALM domain may be to confer oligomerization properties that influence transcription, rather than through its effect on endocytosis. Our results suggest the possibility that oligomerization via the CALM domain could increase the binding affinity of AF10-interacting proteins, such as DOT1l, thereby modifying epigenetic patterns, altering transcriptional properties and promoting the development of AML.

Results

CALM-AF10 inhibits clathrin-mediated endocytosis in 293T cells

In patients with the t(10;11)(p13;q14), eight alternative *CALM-AF10* fusion transcripts have been identified, since there are two breakpoints in *CALM* and four breakpoints in *AF10* (Bohlander *et al.*, 2000; Carlson *et al.*, 2000). All fusion proteins retain the OM-LZ domain of AF10. Relevant to this study, there are two breakpoints in *CALM* at positions 1926 and 2091(GenBank accession number U45976), referred herein as $CALM_{1926}AF10$ and $CALM_{2091}AF10$ (Fig. 1A). The $CALM_{2091}AF10$ transcript encodes a protein that retains almost the entire coding region of CALM (only the last four amino acids of CALM are replaced by AF10), whereas $CALM_{1926}AF10$ is missing the C-terminal 59 amino acids of CALM. CALM associates with clathrin heavy chain and co-immunoprecipitation studies using fragments of CALM demonstrated that the C-terminal domain of CALM was required for optimal binding to clathrin heavy chain (Tebar *et al.*, 1999). To examine the interaction of truncated CALM with clathrin, we cloned CALM ending at breakpoint 1926 or breakpoint 2091, with or without exon 13, which encodes a DPF motif that binds the endocytic adaptor AP2. In transfected 293T cells, we demonstrated that $CALM_{2091}$, but not $CALM_{1926}$, was enriched in clathrin-coated vesicles (Fig. 1A), and only $CALM_{2091}$ displayed a punctate pattern of staining, typical of clathrin-coated pits (Fig. 1B). Exclusion of exon 13 didn't change the pattern, since there are additional AP2 binding sites (Meyerholz *et al.*, 2005)). Next, we engineered constructs expressing chimeric fusion proteins of $CALM_{1926}AF10$ or $CALM_{2091}AF10$ with the green fluorescent protein (GFP). An examination of the fusion proteins revealed that $CALM_{2091}AF10$-GFP localized to the cytoplasm with aggregates of variable size and number, whereas $CALM_{1926}AF10$-GFP displayed a much more diffuse pattern throughout the cytoplasm (Fig. 1C). When cells were

treated with leptomycin B, a chemical that blocks nuclear export, both CALM-AF10 fusion proteins localized to the nucleus suggesting that CALM-AF10 shuttles to and from the nucleus in 293T cells. Interestingly, $CALM_{2091}AF10$-GFP retained a punctate appearance in the nucleus, whereas $CALM_{1926}AF10$-GFP was diffusely localized within the nucleus. Clathrin (a major binding partner of CALM) did not co-localize with $CALM_{2091}AF10$ clusters within the nucleus (Supplementary Fig. S1), suggesting that $CALM_{2091}AF10$ clustering occurs independently of clathrin association. Together, these results suggest that $CALM_{2091}AF10$ retains a region of CALM that is critical for clathrin binding, but it has a tendency to form microscopic aggregates regardless of cellular localization or its association with clathrin. The presence of these aggregates can be attributed to the presence of the clathrin-binding region of CALM and not the GFP tag, as this behavior is not displayed by $CALM_{1926}AF10$-GFP.

To determine whether CALM-AF10 disrupts internalization, we first examined transferrin endocytosis, as a marker for clathrin-mediated internalization. The uptake of fluorescently-labeled transferrin was examined in GFP-positive 293T cells expressing the GFP-tagged chimeric fusion proteins (Fig. 1D). Approximately 92-96% of cells expressing either the CALM or AF10 individual moiety internalized transferrin. However, only ~40% of cells expressing $CALM_{2091}$-AF10 and ~80% of cells expressing $CALM_{1926}$-AF10 endocytosed transferrin. This suggests that expression of $CALM_{2091}$-AF10 impairs the ability of 293T cells to undergo transferrin endocytosis, consistent with the fact that $CALM_{2091}$-AF10 retains the clathrin-binding region of CALM.

The absence of the clathrin-binding region of CALM alters the phenotype of CALM-AF10^{+} myeloid neoplasms

To examine the role that the CALM moiety, and how its ability to bind to clathrin, might modulate disease phenotype, fetal liver progenitors were infected with retroviral vectors expressing CALM-AF10 and transplanted into lethally-irradiated BALB/c recipient mice. In contrast to controls (MIGR1), mice receiving progenitors expressing either $CALM_{2091}AF10$ or $CALM_{1926}AF10$ succumbed within 8 months to disease with 100% penetrance (median latency, 147 and 117 days, respectively) (Fig. 2B). Moribund $CALM_{2091}AF10$[+] mice were generally anemic (7/9= 78%), whereas, fewer $CALM_{1926}AF10$[+] mice had anemia (2/12=17%) (Table 1). $CALM_{1926}AF10$[+] mice displayed slightly higher leukocyte counts (median 164×10^6 ml^{-1}) than $CALM_{2091}AF10$[+] mice (median: 127×10^6 ml^{-1}). Analysis of blood smears revealed that the increased leukocyte counts were due to granulocytosis. In all mice succumbing to disease, spleens were markedly enlarged (3-9 fold); however, enlarged lymph nodes and thymus were observed more frequently in $CALM_{1926}AF10$[+] mice. In both, a large percentage (41-96%) of the cells in the bone marrow and spleen were GFP[+], a surrogate marker for CALM-AF10. CALM-AF10^{+} cells were Mac-1(CD11b)[hi]/ Gr-1[med-hi], indicative of granulocytes or their progenitors (Fig. 2C). Histological analysis of bone marrow and spleen revealed an enrichment of myeloid blasts adjacent to moderately differentiated granulocytic cells in $CALM_{2091}AF10$[+] mice, whereas a massive enrichment of mature granulocytes without enrichment of myeloid blasts was observed in $CALM_{1926}AF10$[+] mice (Fig. 2D). In both, non-hematopoietic tissues, such as the liver, were infiltrated with myeloid cells, and the disease was transplantable to secondary recipients

with a slightly lesser degree of myeloid maturation as compared to primary disease (Fig. 2A, Supplementary Table S1), as is commonly observed after secondary transplant (personal communication, Scott Kogan). Spectral karyotype analysis of $CALM_{2091}AF10^+$ (n=6) and $CALM_{1926}AF10^+$ (n=4) splenocytes revealed a normal karyotype in all cases (Table 2).

Thus, the $CALM_{2091}AF10^+$ mice develop AML, consistent with previous studies that also examined mouse models with a CALM-AF10 fusion with the 2091 breakpoint (Caudell *et al.*, 2007; Deshpande *et al.*, 2006). However, the $CALM_{1926}AF10^+$ mice do not fulfill the Bethesda proposal for AML based on several features (Kogan *et al.*, 2002): (1) < 20% nonlymphoid blasts were observed in the blood, marrow and spleen-median percent blasts was 4% for $CALM_{1926}AF10^+$ mice versus 51% for $CALM_{2091}AF10^+$ mice (P=0.009); (2) only some mice showed cytopenias: 2/7 (17%) $CALM_{1926}AF10^+$ mice had low RBC counts ($<6 \times 10^{12}$/L), vs. 7/9 (78%) for $CALM_{2091}AF10^+$; and (3) complete maturation of myeloid forms to segmented neutrophils were observed in the bone marrow and spleen. The $CALM_{1926}AF10^+$ mice do, however, meet several criteria for myeloid leukemia, including increased numbers of nonlymphoid hematopoietic cells in blood, bone marrow spleen and other tissues, and disease was transplantable to secondary recipients (Fig. 2), suggesting that these mice can be classified on the border between myeloproliferative disease (MPD) and MPD-like leukemia (personal communication, S. Kogan). Analysis of CALM-AF10 expression in spleen from diseased mice revealed variable expression, regardless of the specific breakpoint, confirming that differences in phenotype were not merely due to different CALM-AF10 expression levels (Supplementary Fig. S2). Moreover, Deshpande *et al.* (2006) first reported that CALM-AF10^+ AMLs have a unique Mac1^+ B220^+ phenotype. Similar to Caudell *et al.* (2007), we observed that ~50% of the AMLs were Mac1^+B220^+, whereas almost all MPDs had this population of cells, suggesting that both fusion proteins can give rise to this unique population of cells (Supplemental Table S2).

Microarray analysis identified 28 over-expressed and 87 under-expressed genes in $CALM_{2091}AF10^+$ AMLs compared to $CALM_{1926}AF10^+$ MPDs (Supplementary methods, Supplementary Table S3). Although the changes were small (median fold change of 2.3), Ingenuity Pathway analysis revealed a strong association of differentially expressed genes with cellular growth, hematopoiesis and cancer. We confirmed that *Pim1* and *Klf9 (BtebI)* were increased in AML samples, and *Crebbp* (CBP) and *Itgam* (Mac-1) were downregulated in AMLs relative to MPDs (Fig. 3A). The *Itgam* and *Klf9* genes are markers for myeloid differentiation (Lian *et al.*, 2002). The serine (Ser)/threonine (Thr) kinase Pim1 gene is a proto-oncogene that enhances the development of lymphoma and leukemia (Selten *et al.*, 1985; van Lohuizen *et al.*, 1989). The *Crebbp* gene encodes a transcriptional coactivator that has been shown to have tumor suppressor activity (Kung *et al.*, 2000). The oncogenic potential of CALM-AF10 has been linked to expression of *Hoxa* cluster genes (Caudell *et al.*, 2007; Dik *et al.*, 2005; Okada *et al.*, 2006). *Hoxa5, Hoxa7*, and *Hoxa9* were increased relative to control MIGR1 cells, regardless of which CALM-AF10 fusion was expressed (Fig. 3B). Thus, $CALM_{2091}AF10$ resulted in the development of AML, whereas $CALM_{1926}AF10$ induced an invasive MPD-like leukemia, with concomitant changes in gene expression of cancer-related genes, and myeloid differentiation markers.

To exclude the possibility that changes in gene expression were merely due to a comparison of blasts versus mature neutrophils, we examined changes in direct target genes, and noted some differences in gene expression as compared to leukemia cells (Supplementary Fig. S3). For example, $CALM_{2091}AF10$ expression did not result in high *Pim1* expression *in vitro*. As anticipated, *Hoxa* cluster genes, *Meis1*, and *Bmi1* were elevated (5-50 fold) in CALM-$AF10^+$ cells. Interestingly, expression of these genes was consistently and significantly ~2 fold higher (*P*<0.01) after $CALM_{1926}AF10$ expression compared to $CALM_{2091}AF10$ (Fig. 3C). As these genes are associated with stem cell proliferation and leukemogenesis, the elevated expression in CALM AF10$^+$ 1926 cells may account for why the mice with MPD succumb more rapidly than the mice with AML (median survival 117d vs. 147d).

Growth factor receptor internalization and proliferation is not altered in CALM-AF10$^+$ leukemia cells

$CALM_{2091}AF10^+$ is an effective inhibitor of transferrin endocytosis *in vitro* (Fig. 1D) and mediator of acute leukemia in mice (Fig. 2), raising the possibility that defective endocytosis may contribute to leukemogenesis. The KIT, CXCR4 and TF receptors internalize via a clathrin-mediated pathway (Broudy *et al*., 1999; Hopkins, 1985; Signoret *et al*., 1997). Internalization of these receptors in freshly isolated bone marrow cells was highly variable with no significant difference from controls, suggesting that expression of $CALM_{2091}$-AF10 or $CALM_{1926}$-AF10 does not consistently affect internalization in AMLs or MPDs (Fig. 4A-C). As signaling and endocytosis are tightly coupled, we examined whether expression of CALM-AF10 had an effect on the proliferation of BaF3 cells, a well-established IL-3 dependent hematopoietic cell line model commonly used to test oncogene effects on growth and proliferation. Expression of CALM-AF10 did not affect the growth of BaF3 cells in response to low and high levels of IL-3 (Fig. 4D) and did not enhance Erk signaling in response to IL-3 (Fig. 4E). A greater percentage of tetraploid metaphases were observed in CALM-AF10$^+$ BaF3 cells (Fig. 4F), suggesting that CALM-AF10 is physiologically active in these cells, since its expression is thought to contribute to genomic instability (Lin *et al*., 2009).

To understand why expression of CALM-AF10 did not have major effects on endocytosis or proliferation of hematopoietic cells, we generated three IL-3 independent cell lines from the malignant cells of $CALM_{2091}$-AF10$^+$ and $CALM_{1926}$-AF10$^+$ mice (Supplemental Table S4 and Fig. S4). All cell lines efficiently internalized the TF receptor. The KIT$^+$ line (CA2091-CL1) displayed impaired KIT internalization; however, we could not prove that CALM-AF10 was directly responsible for impaired KIT internalization, since a 95% decrease in CALM-AF10 expression by siRNA was unable to restore KIT internalization to wild type levels in CA2091-CL1 cells (Fig. 5A,B). To explore why CALM-AF10 did not have a major effect on endocytosis in leukemia cells, as we observed with transfected 293T cells, the CA2091-CL1 cell line was stained with an anti-FLAG or anti-CALM antibody to detect expression of $CALM_{2091}AF10$ (Fig. 5C). Similar to 293T cells, large punctate clusters variable in size and number were observed in interphase cells; however, they localized predominantly to the nucleus, where they did not co-localize with clathrin (Supplementary Fig. S5A, B). Although clathrin-mediated endocytosis ceases during early mitosis, it has been shown that clathrin localizes to mitotic spindles to aid congression of chromosomes

(Royle *et al*., 2005). Interestingly in CA2019CL1 metaphase cells, $CALM_{2091}AF10$ co-localizes with clathrin (Fig. 5C, Supplementary Fig S5B). The nuclear localization of CALM-AF10 in the CA2091-CL1 leukemia cell line and its apparent lack of effect on internalization or signaling downstream of growth factor receptors suggests that CALM-AF10 expression likely does not influence endocytic trafficking during leukemogenesis, but rather fusion of the CALM moiety to AF10 plays another crucial role.

CALM-AF10's interaction with clathrin does not influence global H3K79 hypomethylation

AF10 interacts with the H3K79 methyltransferase, DOT1L, and it has been hypothesized that CALM-AF10 prevents AF10 from targeting DOT1L to chromatin, leading to global H3K79 hypomethylation (Lin *et al*., 2009). Whether the interaction of other proteins with the CALM portion of CALM-AF10 influences DOT1L recruitment is unknown. In agreement with previous results, we observed a decrease in dimethylated H3K79 (H3K79-2me) after $CALM_{2091}AF10$ expression compared to GFP control, in both 293T cells and cells from leukemic mice (Fig. 6A). Although subtle, expression of $CALM_{1926}AF10$ did not appear to be as effective as $CALM_{2091}AF10$ at lowering H3K79 methylation. These subtle shifts in H3K79 methylation may have a consequential impact on gene expression. *Hoxa* cluster genes, whose transcription is influenced by local H3K79 methylation, were expressed at 2-fold higher levels in $CALM_{1926}AF10^+$ cells vs. $CALM_{2091}AF10^+$ cells (Fig. 3C).

These data also raised the possibility that clathrin's interaction with $CALM_{2091}AF10$ may influence recruitment of DOT1L to its targets and, thereby, alter H3K79-2me levels. The CA2091-CL1 cell line was transfected with siRNA pools directed against CALM-AF10 or clathrin heavy chain and global H3K79 methylation was examined by Western blot analysis of nuclear extracts. Whereas knock-down of $CALM_{2091}AF10$ restored H3K79 methylation as anticipated, knock-down of clathrin had no effect (Fig. 6B). These results suggest that the clathrin/CALM-AF10 interaction does not influence DOT1L recruitment and methylation of its targets, although it remains possible that residual clathrin expression may be sufficient to sequester CALM-AF10 with DOT1L away from chromatin-associated AF10.

$CALM_{2091}AF10$ forms homo-oligomers

The localization of $CALM_{2091}AF10$, in large punctate clusters, was reminiscent of some MLL fusions where oligomerization of the fusion proteins has been shown to play a critical role in promoting leukemogenesis. To test whether the clusters of $CALM_{2091}AF10$ were interacting through homo-oligomerization, we undertook FRET analysis using two distinct detection methods: acceptor photobleaching and donor photobleaching. In acceptor photobleaching, energy transfer from CFP (donor) to YFP (acceptor) is reduced upon acceptor bleaching, resulting in increased donor fluorescence (Kenworthy, 2001). FRET efficiencies were determined for a region of interest (ROI), drawn around each aggregate of $CALM_{2091}AF10$ (Fig. 7A). As a control, FRET was calculated in unbleached samples where energy transfer from CFP to YFP should not occur, and accordingly only FRET efficiencies above 3.5% were considered positive. For the experimental sample, 63% of the ROIs displayed FRET, with an average FRET efficiency of 13%, suggesting that $CALM_{2091}AF10$ forms homo-oligomers.

Donor photobleaching is based on the principle that processes such as FRET, which shorten the excited-state lifetime of a fluorophore, also protect it from photobleaching, making decay kinetics an efficient measure of FRET efficiency (Kenworthy, 2001). Fluorescence decay from cells expressing only CALM-AF10-CFP or CALM-AF10-CFP plus CALM-AF10-YFP was measured, and decay constants were determined by fitting to single exponential curves (Fig. 7B). Measurements from numerous aggregates from multiple cells show that the mean decay constant for cells expressing $CALM_{2091}AF10$-CFP alone (0.72 ± 0.014, n=88) was significantly greater ($p=1.6\times10^{-16}$) than that for cells co-expressing $CALM_{2091}AF10$-CFP and $CALM_{2091}AF10$-YFP (0.50 ± 0.021, n=110). These data are consistent with the occurrence of FRET between CFP and YFP fluorophores, and again suggest that homo-oligomerization of $CALM_{2091}AF10$ occurs within large aggregates. No difference in decay constant was observed when a similar experiment was performed with $CALM_{1926}AF10$, suggesting that this fusion protein does not homo-oligomerize as readily as $CALM_{2091}AF10$.

Discussion

Several endocytic proteins have been identified as fusion partners of transcription factors or tyrosine kinases in hematopoietic malignancies, and it has been proposed that disruption of the endocytic process may contribute to disease progression (Lanzetti and Di Fiore, 2008). To explore this hypothesis further, we examined the functional contribution of the CALM moiety in CALM-AF10-mediated transformation. $CALM_{2091}AF10^+$ and $CALM_{1926}AF10^+$ mice developed disease with a similar latency (P=0.1), yet $CALM_{2091}AF10$ affects the severity of the disease, suggesting a potential role for clathrin. However, we did not identify deficits in endocytosis, proliferation or signaling in CALM-AF10+ leukemia or hematopoietic cell line models. In contrast, inclusion of the clathrin-binding domain ($CALM_{2091}AF10$) was required to inhibit transferrin endocytosis in 293T cells efficiently. As many studies of endocytic function rely heavily on the use of epithelial cell lines, our studies emphasize caution in interpreting these results in a larger biological context. For example, CALM-AF10 localized predominantly in the cytoplasm of 293T cells (where it may have transiently interacted with clathrin-coated vesicles), but was found predominantly in the nucleus of the CA2091CL1 leukemia cell line, providing a potential explanation for the different effects on endocytosis in the two cell types.

Experimental results suggest that DOT1L and H3K79me2 are regulated during progression of the cell cycle, in which H3K79me2 levels increase during M phase (Feng *et al.*, 2002). During interphase, clathrin plays a key role in membrane trafficking; however, when the cell enters mitosis, membrane trafficking ceases and a portion of clathrin is targeted to the mitotic spindle where it stabilizes kinetochore fibers (Royle *et al.*, 2005). In the $CALM_{2091}AF10^+$ leukemia cell line, CALM-AF10 and clathrin generally segregated to either the nucleus or cytoplasm, respectively, during interphase; however, they co-localized during metaphase (Fig. 5, Supplementary Fig. S5). Therefore, we examined whether the DOT1L-sequestering effect of CALM-AF10 was influenced by interaction of the CALM moiety with clathrin. However, knock-down of clathrin had no effect on global H3K79me2 levels in a CALM AF10+ 2091 cell line, leaving open the question of how CALM-AF10 may act as a dominant negative competitor of AF10 to regulate global H3K79

hypomethylation. The genomic instability observed in CALM-AF10$^+$ cells is speculated to be associated with global H3K79 hypomethylation (Lin *et al*., 2009). Given that disruption of clathrin has also been associated with genomic instability (Lemmon *et al*., 1990; Royle *et al*., 2005), and that CALM-AF10 colocalizes with clathrin during metaphase in the leukemia cell line tested, our data raises the intriguing possibility that clathrin/CALM-AF10 interaction may also contribute to increased genomic instability.

It has been proposed that oligomerization of chimeric transcription factors may act as a universal oncogenic amplifier that enhances the transcriptional properties by augmenting and broadening the binding affinity to interacting proteins and target DNA sequences. The nature of the endocytic machinery, characterized by their self-assembly properties and ability to form higher-order complexes, may lend itself to promoting dimerization or oligomerization of transcription factors. Moreover, previous work suggests that the homo-oligomerization of AF10 may itself enhance AF10-mediated transcription (Forissier *et al*., 2007). In our mouse model, we observed a slightly more aggressive disease with a form of CALM-AF10 (CALM$_{2091}$AF10) that has a tendency to form macroscopic aggregates, regardless of cellular location, and could be shown to oligomerize by FRET analysis. The homo-oligomerization of CALM-AF10 could be envisaged to compete with endogenous AF10 for the recruitment of DOT1L, a known player in CALM-AF10-mediated transformation. Additionally, AF10 binds to GAS41 (Harborth *et al*., 2000), a protein previously found to interact with the SWI/SNF complex, which in turn binds to the nuclear mitotic apparatus protein, NuMA (Bhattacharya *et al*., 2002), which can be phosphorylated by Pim1. Alteration of the binding affinity of these interactions through oligomerization, could influence chromatin organization and transcriptional regulation, thereby, contributing to malignant transformation. Artificial oligomerization of AF10, as has been done for MLL (So *et al*., 2003), could be used to address this hypothesis and rule out other oncogenic contributions by CALM.

In patients with CALM-AF10$^+$ T-ALL there appears to be a correlation between the AF10 fusion and leukemic phenotype (Asnafi *et al*., 2003). To date, there is no known association between CALM or AF10 fusion breakpoint and leukemic phenotype in AML patients; however, varying degrees of maturation and leukemia phenotype (AML M0-M5) have been noted (Carlson *et al*., 2000). Also, CALM-AF10 fusions are found in <1% of AML cases, and only a small number of cases have been well-characterized. Nonetheless, mouse models have proven to be a powerful biological tool in deciphering the underlying mechanisms of disease. In this study, we exploited the alternate CALM fusions to decipher the functional contribution of the CALM protein in CALM-AF10-mediated transformation. Our data suggest that increased proliferation through perturbed endocytosis is not a major contributing factor to oncogenic transformation, as originally hypothesized. Instead, our findings point to an alternative mechanism involving homo-oligomerization of CALM-AF10 that could increase its binding affinity for interacting proteins, such as DOT1L or other co-activators, that influence chromatin organization and transcriptional regulation, promoting the development of overt leukemia.

Methods

Cloning of CALM and CALM-AF10 expression constructs

cDNAs encoding truncated human CALM and AF10 were obtained by reverse transcriptase-PCR. Recombinatorial PCR was used to clone human CALM-AF10 fusion transcripts representing fusions isolated from patients with breakpoints at nucleotide 1926 or 2091 of CALM, and nucleotide 588 of AF10 (Carlson *et al*., 2000; Dreyling *et al*., 1996). For 293T studies, CALM, AF10 or CALM-AF10 cDNAs were cloned upstream and in-frame with the enhanced green fluorescent protein (GFP) using the pEGFP-N1 vector (BD Biosciences Clontech), and transiently-transfected using the Effectene transfection reagent (Qiagen). For retroviral expression, $CALM_{2091}AF10$ and $CALM_{1926}AF10$ cDNAs were cloned upstream of the internal ribosomal entry site (IRES) of the MIGR1 (MSCV IRES GFP) vector. Retrovirus was produced by transfection of 293T cells with the pCL ecotropic packaging plasmid and the appropriate retroviral vector. BaF3 cells or E14.5 fetal liver cells were infected by two rounds of spinoculation.

Characterization of Leukemias

Lethally-irradiated BALB/c mice (6-12 weeks old) were infected by retro-orbital injection of 3×10^6 unsorted E14.5 fetal liver cells transduced with CALM-AF10 or control vectors. Transplanted mice were monitored for the onset of disease by performing complete blood counts (Hemavet 850, CDC Technologies) every three weeks or when mice became moribund. All organs were recovered, fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 4 to 5 μm and stained with hematoxylin and eosin (H&E) for histologic examination by a pathologist (J.A.). Peripheral blood, bone marrow aspirates and spleen touch preparations were stained with Wright-Giemsa. Images were obtained using an Olympus microscope (Model BX45) equipped with an Olympus DP12 digital camera. Single cell suspensions of bone marrow and spleen were stained with phycoerythrin (PE)-conjugated antibodies specific for CD71 or Gr-1 and allophycocyanin (APC)-conjugated antibodies specific for Ter119 and Mac-1 (CD11b) (BD BioSciences). Flow cytometry was performed on a FACSCanto (BD Biosciences), and data were analyzed with FlowJo software (Treestar Software). Short-term (24-h) cultures of splenocytes from CALM-AF10^{+} mice, and spectral karyotyping were performed as described (Le Beau *et al*., 2002). At least 10 metaphase cells were analyzed per case.

Real-Time PCR Analysis

RNA was isolated using Stat-60 and cDNA was made following standard protocols. Real-time PCR was performed using a Step One Plus Thermocycler (Applied Biosystems). A list of the primers used is shown in supplemental data (Table S5). The relative amounts of each mRNA amplification products were calculated in reference to standard curves, and then normalized to the relative amounts of *Gapdh* transcripts.

Immunofluorescence and FRET Analysis

Twenty-four hours after 293T cells were transfected with CALM fused to GFP or CALM-AF10 fused to GFP, CFP or YFP, cells were plated on coverslips and grown overnight. An

IL-3-dependent cell line was generated by culturing the malignant cells of a $CALM_{2091}AF10^+$ mouse (2616b), and was named CA-2091CL1. These cells were allowed to settle on poly-L-lysine coated coverslips for 30 mins at room temperature. Cells were fixed in 4% paraformaldehyde, quenched with 50 mM NH_4Cl, permeabilized with 0.2% Triton X-100, washed, and blocked with PBS plus 5% donkey serum (Jackson ImmunoResearch) and 1% fish gelatin (Sigma) for 30 minutes. For CA2091-CL1 cells, CALM-AF10 was stained with a polyclonal anti-FLAG antibody (Sigma) or anti-CALM antibody (C18, Santa Cruz Biotechnology) followed by a Rhodamine Red-conjugated donkey anti-rabbit IgG or anti-goat IgG, respectively. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). For standard immunofluorescence, cells were viewed with a Zeiss Axioplan epi-fluorescence microscope, and images were processed using Adobe Photoshop software. For FRET analysis, methods were adapted from Cho *et al.* (Cho and Kehrl, 2007) and Muntau *et al.* (Muntau et al., 2003), and a detailed description is provided in the supplemental materials.

Preparation of clathrin-coated vesicles

Clathrin-coated vesicles were isolated by a differential centrifugation method adapted from Chakrabarti *et al.* (Chakrabarti *et al.*, 1993). A detailed description is provided in the supplemental materials.

Transfection of small interfering RNA (siRNA)

All siRNA sequences are provided in the supplementary methods. CA2091-CL1 cells were transfected with 50nM of each hAF10 siRNA, 100 mM of the CLTC pools or 100 nM of the non-targeting siRNA using the Amaxa nucleofection technology.

Western blot analysis

Western blot analysis was performed using standard protocols. Primary antibodies included: FLAG (M2) (Sigma) to detect CALM-AF10, β-tubulin (AA2, Millipore), phospho p44/42 MAPK (Erk1/2) and p44/42 MAPK (Cell Signaling Technologies), H3K79-2me and H3 (Abcam), and clathrin (TD.1, Santa Cruz Biotechnology). To quantitate levels of H3K79-2me, the intensity of the bands was measured using Adobe Photoshop and normalized to H3 loading controls. Clathrin and CALM-A10 levels were normalized to tubulin.

Internalization Assays

CALM-AF10-positive cells or E14.5 fetal liver cells (BALB/c mice) were incubated 15 min on ice with 100 ng/ml SCF or SDF1α (CXCL12) (R&D Systems), then at 37°C for 0-30 minutes (internalization) and washed in ice-cold PBS to remove ligand. Cells were stained with APC-conjugated anti-KIT receptor and PE-conjugated anti-CXCR4 antibodies; receptors remaining on the cell surface were measured by flow cytometric analysis. Internalization was calculated as $(MFI_{time0}-MFI_{timeX})/MFI_{time0} \times 100\%$, where MFI is the mean fluorescent intensity of KIT or CXCR4 receptors. For cells treated with siRNA and controls, the GFP$^+$ cells were gated for analysis. Transferrin uptake assays are described in the supplementary materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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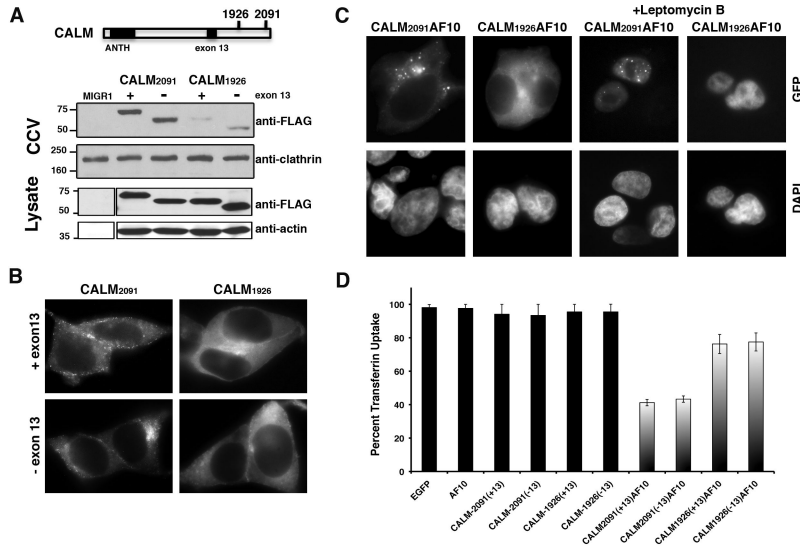
Fig. 1. CALM-AF10 inhibits clathrin-mediated endocytosis in 293T cells
(A) Schematic diagram of the CALM protein indicating nucleotide positions where fusion to AF10 occurs. The large black box refers to the ANTH domain (PIP_2-binding) and the small box refers to the location of exon 13, which is alternatively spliced and contains a DPF peptide motif involved in, but not essential for, AP2 binding. Clathrin-coated vesicle fractions (CCV) or total cell lysates, isolated from NIH3T3 cells infected with retroviral vectors expressing FLAG-tagged CALM constructs ending at breakpoint 1926 or 2091, with or without exon 13, were resolved by electrophoresis and blotted with FLAG, clathrin and actin-specific antibodies. **(B, C)** 293T cells were transfected with chimeric transcripts of $CALM_{2091}$, $CALM_{1926}$, $CALM_{2091}AF10$ or $CALM_{1926}AF10$ fused to GFP. Expressed proteins were detected by GFP fluorescence and nuclei were visualized by DAPI staining. To block nuclear export, transfected cells were treated with 20 nM leptomycin B, where indicated. Original magnification × 787.5 for all panels. **(D)** Forty-eight hours after transfection with GFP-fused constructs, a total of 150 GFP⁺ 293T cells were scored for uptake of fluorescent transferrin, from three independent experiments (50 cells per experiment). There was a significant difference in transferrin uptake between control (EGFP-N1) and $CALM_{1926}AF10$ ($P<0.02$) and control and $CALM_{2091}AF10$ ($P<0.0001$), with and without exon 13.
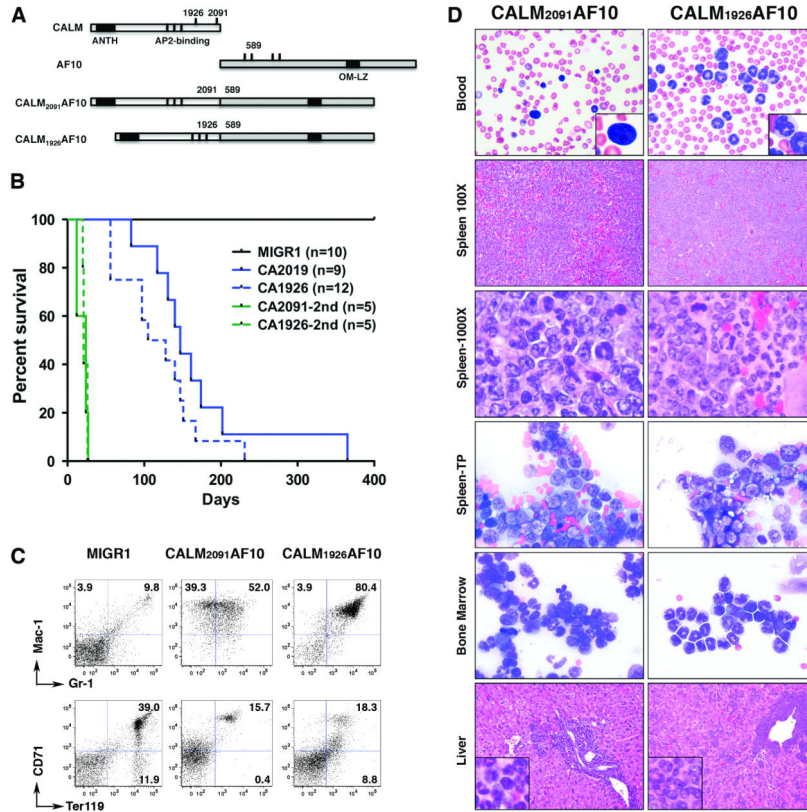
Fig. 2. The absence of the clathrin-binding region of CALM alters the phenotype of CALM-AF10+ myeloid neoplasms

(A) Schematic representation of CALM, AF10 and CALM-AF10 fusion proteins. The two breakpoints in CALM, and one of the four breakpoints in AF10 are indicated by black lines and the base pair position. The two CALM-AF10 fusion proteins used in this study are shown. (B) Survival curves of mice injected with progenitor cells transduced with $CALM_{2091}AF10$, $CALM_{1926}AF10$ or MIGR1 (control vector). CA= CALM-AF10; 2ND = secondary recipients of primary MPD or AML cells. (C) Fluorescence-activated cell sorter analysis of cells isolated from spleen of CALM-AF10 mice. Analysis of GFP-gated cells reveals an enrichment of myeloid lineage-specific markers (CD11b+Gr-1med-hi) and decrease in erythroid cells (CD71+Ter119+) in $CALM_{2091}AF10$ and $CALM_{1926}AF10$ mice compared to control (MIGR1) mice. (D) Peripheral blood, bone marrow aspirates and spleen touch preparations (TP) were stained with Wright-Giemsa. Paraffin-embedded liver and spleen sections were stained with hematoxylin and eosin. Original magnification ×100 for spleen and liver; ×500 for blood, spleen-TP and bone marrow; ×1000 for spleen and insets.
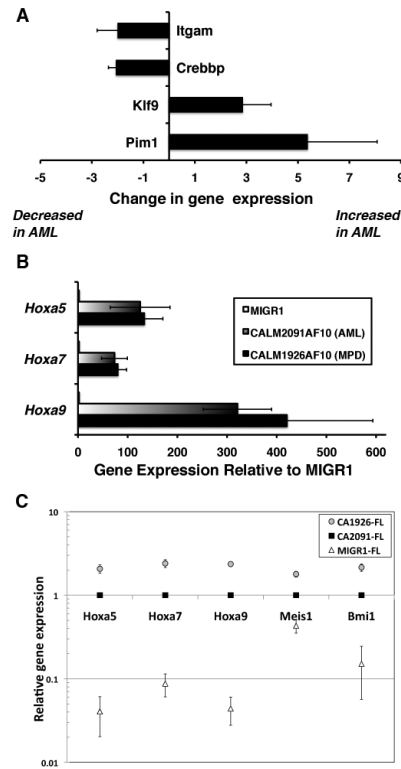
Fig. 3. Differential gene expression in CALM$_{2091}$AF10 AMLs and CALM$_{1926}$AF10 MPDs
(A) RNA expression levels were quantified by real-time RT-PCR. Fold change in RNA expression in spleen cells from mice with AML relative to MPD is shown. A positive value indicates the gene is up-regulated in AML, and a negative value indicates it is down-regulated in AML compared to MPD samples. **(B)** *Hoxa* cluster gene expression relative to MIGR1 control (normalized to 1). Whereas *Hoxa* genes were significantly up-regulated compared to controls, there was no difference between AML and MPD samples. Results are expressed as the mean ± standard error from three independent experiments. **(C)** Fetal liver (FL) progenitors, infected with retroviral vectors expressing either CALM$_{2091}$AF10 or CALM$_{1926}$AF10 were grown *in vitro* for 10-12 days; changes in gene expression relative to the MIGR1 control were examined by real-time PCR (four independent experiments). To assess differences between CALM-AF10 constructs, relative gene expression was set as '1' for CALM$_{2091}$AF10$^+$ cells. *Hoxa5, Hoxa7, Hoxa9, Meis1,* and *Bmi1* gene expression was consistently increased ~2 fold in CALM$_{1926}$AF10$^+$ cells vs. CALM$_{2091}$AF10$^+$ cells ($P<0.01$).
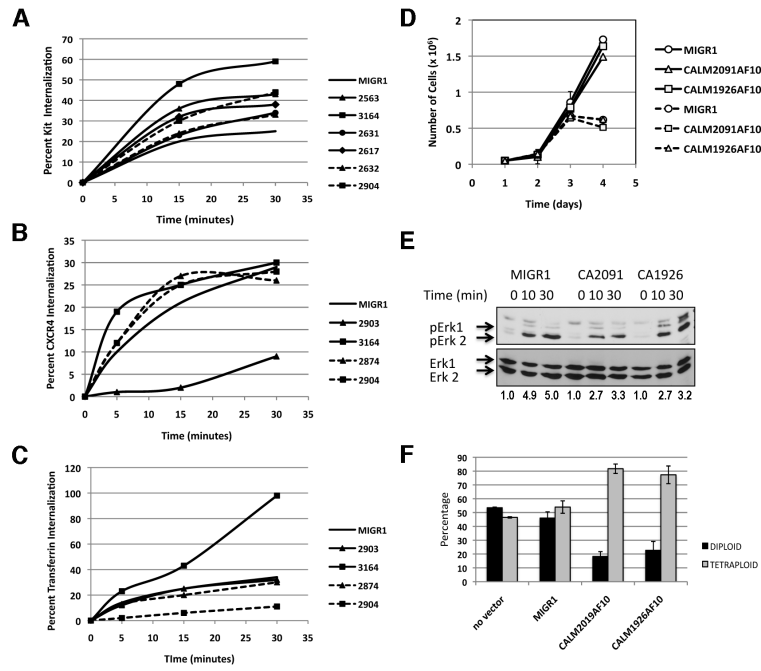
Fig. 4. Internalization and proliferation is not altered in CALM-AF10^{+} hematopoietic or leukemia cells

Internalization of KIT (**A**), CXCR4 (**B**), or TF (**C**) receptor, gated on GFP[+] bone marrow cells isolated from $CALM_{2091}AF10^+$ AML mice (solid lines) or $CALM_{1926}AF10^+$ MPD mice (dashed lines), compared to control MIGR1 mice. (**D**) Growth curve of trypan blue-excluded viable BaF3 cells expressing CALM-AF10 or control MIGR1 vector grown in low (0.01 ng/ml, dashed line) or high (1ng/ml, solid line) levels of IL-3,. (**E**) BaF3 cells expressing CALM-AF10 or control MIGR1 vector were serum starved, treated with IL-3 for times indicated, and lysates were immunoblotted with a phospho-Erk 1/2 antibody. No increase in Erk phosphorylation, normalized to Erk 2 levels, was observed in CALM-AF10 expressing cells. (**F**) To estimate the frequency of tetraploid metaphase cells, BaF3 cells were treated with Colcemid™, and metaphase cells were prepared with standard cytogenetic techniques. A higher percent of tetraploid metaphase cells were found in BaF3 cells expressing CALM-AF10 compared to MIGR1 controls. An average of three independent experiments is shown. In each experiment, 30-50 metaphases were scored for each line.
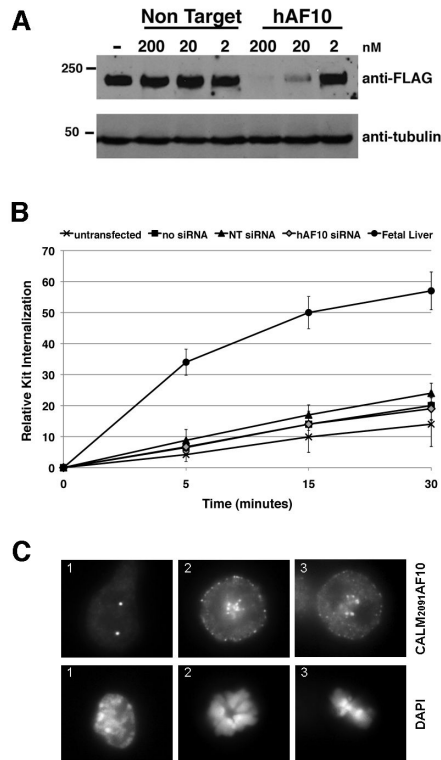
Figure 5. CALM$_{2091}$AF10 localizes to the nucleus and does not influence Kit internalization in a myeloid line derived from CALM$_{2091}$AF10$^+$ AML cells

(**A**) Protein expression of CALM$_{2091}$AF10, detected using an anti-FLAG antibody, 48 hours after nucleofection with siRNA specific for human AF10 (hAF10) or non-targeting (NT) control siRNA in mouse CA2091-CL1 cells, an IL-3 dependent line derived from CALM-AF10$^+$ AML mouse cells. (**B**) CA2091-CL1 cells were transfected with siRNAs: NT-non-targeting; hAF10-specific for CALM-AF10. Cells were treated with 200ng/ml SCF for times indicated, and KIT receptor remaining on the cell surface was measured. Fetal liver cells are shown as a positive control for KIT internalization. Knock-down of CALM-AF10 expression (hAF10 siRNA) does not increase KIT internalization. (**C**) Localization of CALM$_{2091}$AF10, expressed in CA2091-CL1 cells, during interphase (1) and metaphase (2,3). Original magnification, 787.5X.
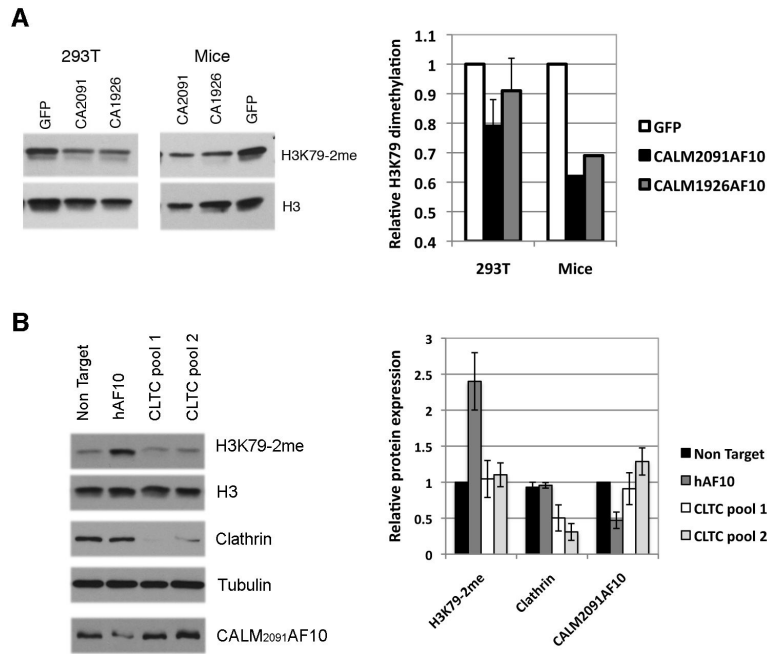
A

B

Fig. 6. CALM-AF10 interaction with clathrin does not influence global H3K79 methylation
(A) Nuclear extracts, prepared from 293T cells transfected with CALM-AF10 constructs or leukemia cells from individual transplanted CALM-AF10^{+} mice, were immunoblotted for H3K79-2me and histone H3. Quantification of H3K79-2me Western blot immunostaining, normalized to H3, confirms a decrease in H3K79 methylation upon $CALM_{2091}AF10$ expression compared to GFP control in 293T and mouse cells. Global hypomethylation after $CALM_{1926}AF10$ expression is less pronounced. **(B)** Nuclear extracts were prepared from the $CALM_{2091}AF10^+$ CA2091-CL1 cell line, transfected with non-targeting (NT), CALM-AF10 (hAF10) or clathrin specific siRNA pools (CLTC-pool1, pool2). A representative blot indicates H3K79 methylation is increased after knock-down of CALM-AF10, but not clathrin (~92% knockdown for CLTC-pool1). Quantification of the average H3K79-2me, clathrin and $CALM_{2091}AF10$ protein levels (Right panel, 3 independent experiments).
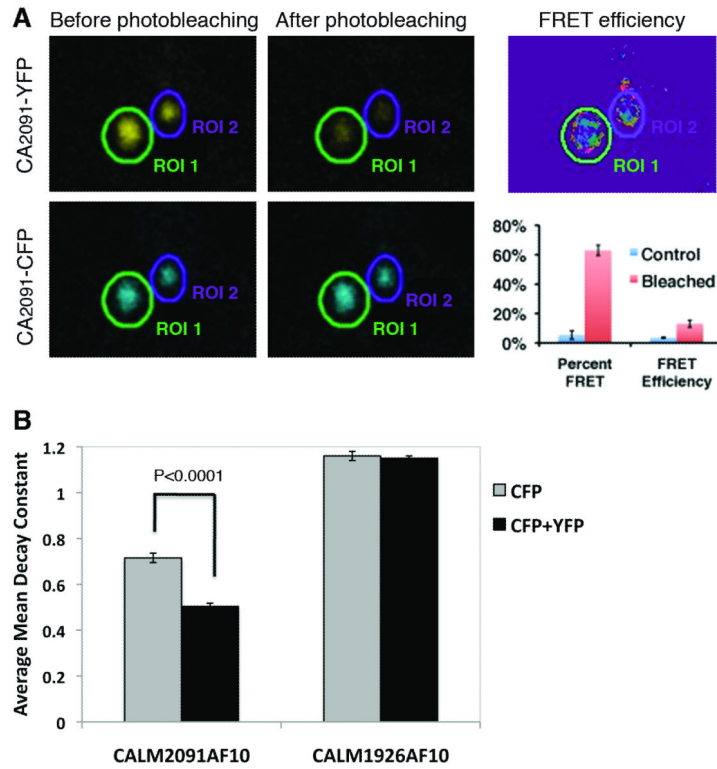
Fig. 7. Self-association properties of CALM$_{2091}$AF10 visualized by acceptor and donor photobleaching FRET

(A) 293T cells were transfected with chimeric transcripts of CALM$_{2091}$AF10 fused to YFP and CFP (CA$_{2091}$-YFP + CA$_{2091}$-CFP). A representative image of YFP and CFP fluorescence before and after photobleaching and corresponding FRET efficiency is shown. The percent of CALM$_{2091}$AF10 aggregates displaying FRET and the average FRET efficiency for bleached regions (n=147) and control (non-bleached) regions (n= 97) ± the standard error of the mean, for three individual experiments are shown. **(B)** 293T cells were transfected with CA$_{2091}$-CFP + CA$_{2091}$-YFP, CA$_{1926}$-CFP + CA$_{1926}$-YFP, CA$_{2091}$-CFP or CA$_{1926}$-CFP. Cells were bleached using the donor excitation wavelength of 458 nm, a time-lapse of images was captured, and a fluorescence decay constant was measured. Mean decay constants in cells expressing both CA$_{2091}$-CFP and CA$_{2091}$-YFP (n=110) have a slower decay constant than CA2091-CFP alone (n=88) (*P*<0.0001), suggesting the occurrence of FRET.

Table 1

Primary myeloid neoplasms in mice transplanted with CALM-AF10 expressing fetal liver cells

Mouse	Break-point	Survival (days)	WBC (10^9/L)	RBC (10^{12}/L)	HGB (g/dL)	Spleen Size (g)	% GFP$^+$ (BM/SPL)	%Mac-1$^+$Gr-1$^+$ (BM/SPL) *	% Blasts	Disease
2524	2091	83	54	**4.1**	7	0.9	96/82	80/32	52	AML
2772	2091	117	>200	8.1	15.1	0.2	96/44	81/72	34[a]	AML
2526	2091	131	102	**5.3**	**9.2**	nd	95/41	nd/88	62	AML
2617	2091	140	169	7	12.1	0.5	95/90	88/89	4	MPD
2618	2091	147	84	**5.8**	**8.1**	0.2	88/51	67/67	74	AML
2619[†]	2091	161	15	**4.6**	**10**	nd	nd	nd	nd	[†]
2616[b]	2091	174	High[‡]	**Low**[‡]	nd	0.7	51/64	9/8	20b	AML
3164	2091	202	151	**3**	**6.9**	0.4	96/80	18/24	90	AML
2903	2091	365	>200	**5.4**	**9.9**	0.7	91/80	52/47	50	AML
Median	*2091*	*147*	*127*	*5.4*	*9.6*	*0.5*	*95/72*	*67/57*	*51*	
2576	1926	56	>200	7.7	14.3	0.3	76/46	75/70	7	MPD
2577	1926	56	90	6.5	10.6	0.3	73/49	74/73	17	MPD
2578	1926	56	>200	7.5	14	0.5	80/45	82/78	8	MPD
2580	1926	97	78	**5.2**	**9.4**	0.7	90/64	88/76	4	MPD
2874	1926	97	>200	6.2	11.4	0.4	95/81	85/74	1	MPD
2662	1926	105	195	8.3	13	0.7	80/79	94/92	4	MPD
2579	1926	128	85	6.6	11.4	nd	nd	nd	1[a,c]	MPD
2963	1926	140	179	6.5	10.7	0.4	92/50	9/26	92	AML
3193	1926	147	90.1	6.8	11.5	0.7	87/64	54/37	1[a,c]	MPD
2904	1926	151	195	6.6	11.4	0.6	93/66	58/42	3	MPD
2709	1926	167	149	**4.1**	**7.4**	0.7	86/47	47/43	13[a,b]	MPD[b]
2960	1926	231	94	8	13	0.3	60/90	77/76	2	MPD
Median	*1926*	*117*	*164*	*6.6*	*11.4*	*0.5*	*86/64*	*75/73*	*4*	
MIGR1	N/A	~730	2-11	6-10	11-15	0.1	65-95	35-45 / 3-6	nd	None

WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; BM, bone marrow; SPL, spleen; nd, not determined.

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*
Analysis was performed on GFP$^+$ cells.

†
Counts obtained 5 days prior to death; tissues isolated from dead mouse suggest there are immature granulocytic elements in spleen and bone marrow.

‡
Based on blood smears, since counts not obtained Anemic mice are indicated in bold type.

a
blast count is from blood; in all other samples a differential blast count was obtained from scoring 200 bone marrow cells

b
sheets of blasts in spleen

c
sheets of segmented neutrophils in spleen and/or liver, lymph node

Table 2

Spectral karyotype analysis of CALM-AF10 myeloid neoplasms

Case No.	Mouse	Breakpoint	Recipient	Tissue	Karyotype [no. cells]
34270	2524	2091	Primary	Spleen	40,XY[19]/42,XY,+15,+17[1]
34495	2526	2091	Primary	Spleen	40,XY[11]
34582	2573	2091	Secondary	Spleen	40,XY[8]/80,XXYY[2]
34583	2574	2091	Secondary	Spleen	40,XY[10]
34752	2565	2091	Secondary	Spleen	40,XY[11]
34756	2563	2091	Secondary	Spleen	40,XY[10]
35094	2577	1926	Primary	Spleen	40,XX[10]
35095	2578	1926	Primary	Spleen	40,XY[5]/40,XX[6]
35093	2576	1926	Primary	Spleen	40,XY[10]