The role of the PZP domain of AF10 in acute leukemia driven by AF10 translocations

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Chromosomal translocations of the AF10 (or MLLT10) gene are frequently found in acute leukemias. Here, we show that the PZP domain of AF10 (AF10PZP), which is consistently impaired or deleted in leukemogenic AF10 translocations, plays a critical role in blocking malignant transformation. Incorporation of functional AF10PZP into the leukemogenic CALM-AF10 fusion prevents the transforming activity of the fusion in bone marrow-derived hematopoietic stem and progenitor cells in vitro and in vivo and abrogates CALM-AF10-mediated leukemogenesis in vivo. Crystallographic, biochemical and mutagenesis studies reveal that AF10PZP binds to the nucleosome core particle through multivalent contacts with the histone H3 tail and DNA and associates with chromatin in cells, colocalizing with active methylation marks and discriminating against the repressive H3K27me3 mark. AF10PZP promotes nuclear localization of CALM-AF10 and is required for association with chromatin. Our data indicate that the disruption of AF10PZP function in the CALM-AF10 fusion directly leads to transformation, whereas the inclusion of AF10PZP downregulates Hoxa genes and reverses cellular transformation. Our findings highlight the molecular mechanism by which AF10 targets chromatin and suggest a model for the AF10PZP-dependent CALM-AF10-mediated leukemogenesis.
Human AF10 (or mixed-lineage leukemia translocated to 10 (MLLT10)) is essential in hematopoiesis and implicated in blood cancers. Chromosomal translocations involving the AF10 gene are frequently found in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). These aggressive forms of leukemia affect predominantly children and young adults and are characterized by poor survival rates. At least seven translocation partners of AF10 have been identified, including the most common partners clathrin assembly lymphoid myeloid leukemia (CALM) and KMT2A. The leukemia-associated AF10 translocations are shown to dysregulate downstream signaling programs since they produce aberrantly active fusion oncoproteins.

Although AF10 represents primarily a carboxy-terminal fragment in the leukemia-associated chromosomal translocations, significant heterogeneity has been reported in AF10 fusion breakpoints. Interestingly, despite this heterogeneity, all AF10 fusion chimeras contain the C-terminal octapeptide-motif leucine zipper (OM-LZ) domain of AF10 (AF10OMLZ) (Fig. 1a). AF10OMLZ is involved in the interaction with the histone methyltransferase disruptor of telomeric silencing 1-like (DOT1L), an enzyme that generates methylated H3K79 species associated with high gene expression. Furthermore, the DOT1L recruitment to target genes and the deposition of the methylated H3K79 marks require the binding of DOT1L to AF10OMLZ. This notable and strict conservation of AF10OMLZ and therefore the DOT1L-binding

Fig. 1 AF10PZP blocks transformation by the CALM-AF10 fusion. a The architecture of WT AF10 and the CALM-AF10 fusion. AF10PZP, comprised of PHD1, Zn-kn, and PHD2 (colored light green, pink, and light blue, respectively) is consistently disrupted in leukemogenic AF10 translocations. The H3K79-specific lysine methyltransferase (KMT) DOT1L binds to AF10OMLZ. b Schematic of the CFU assay, with the architecture of CALM-AF10MF and CALM-PZP-AF10MF shown at the bottom. Images of representative one-week CFU assay colonies from bone marrow-derived HSPCs transduced with each of the indicated constructs are shown at ×40 magnification. Scale bar: 100 μm. CFU assays performed in HSPCs isolated individually from 3 mice. c Different types of colonies from cells expressing each of the indicated plasmids are shown at 3 consecutive rounds of plating. CFU-G: colony-forming unit-granulocyte, CFU-M: colony-forming unit-macrophage, CFU-M: colony-forming unit-granulocyte monocyte, CFU-Blast: blast-like colonies. P values of CFU-Blast colonies (MIG vs. CALM-AF10MF) and (CALM-AF10MF vs. CALM-PZP-AF10MF) are 0.000019 for week 1, 0.00001 for week 2, and 0.000101 for week 3 (Student’s t-test).
capability in all leukemia-associated AF10 fusions suggests a likely mechanism underlyng the development of AF10-rearranged leukemias that involves the aberrant recruitment and/or stabilization of DOT1L at promoters of leukemogenic genes and constitutive activation of these genes.

The CALM-AF10 (t(10;11)(p12;q14)) translocation is particularly highly leukemogenic and is linked to aggressive acute leukemias. Wild type CALM (or PICALM) is involved in clathrin-mediated endocytosis, and an almost entire CALM protein, including its ENTH domain and the clathrin-binding clathrin-mediated endocytosis, and an almost entire CALM AF10PZP affects the transforming ability of AF10 fusions is malignant transformation, whereas the inclusion of AF10 PZP IRES-GFP (MIG) empty vector, puriﬁed transduced cells using a co-expressed ﬂuorescence marker, and tested these cells in a colony-forming unit-spleen (CFU-S) assay, in which the CALM-AF10MF fusion gene, or the CALM-PZPAF10MF fusion gene (Fig. 2b). While the injection of bone marrow-derived HSPCs transduced with CALM-AF10MF led to fully penetrant leukemias with a median of 93 days, none of the mice injected with CALM-PZPAF10MF had any replating capacity (Fig.1d). These results suggest that the incorporation of the AF10 PZP domain into the CALM-AF10 fusion abrogates the transforming ability of this chimera in vitro.

We next tested CALM-PZPAF10MF in the in vivo clonogenic colony-forming unit-spleen (CFU-S) assay, in which the CALM-AF10MF fusion was shown to confer high CFU-S capability to bone marrow-derived HSPCs. Bone marrow-derived HSPCs transduced with the CALM-AF10MF fusion formed a median of 100 colonies per 50,000 injected cells (Fig. 2a). In contrast, cells transduced with the CALM-PZPAF10MF fusion produced only a median of 20 colonies, which is at par with the MIG vector transduced cells that produced a median of 17 colonies per 50,000 injected cells. We concluded that the incorporation of AF10PZP impedes the ability of the CALM-AF10MF fusion to form a high number of CFU-S colonies in vivo.

The inclusion of AF10PZP abrogates CALM-AF10-mediated leukemogenesis in vivo. To establish whether the inclusion of AF10PZP can affect the in vivo leukemogenic activity of the CALM-AF10 translocation, we injected mice (n = 5 mice per arm) with HSPCs transduced with either the MIG empty vector control, the CALM-AF10MF fusion gene, or the CALM-PZPAF10MF fusion gene (Fig. 2b). While the injection of bone marrow-derived HSPCs transduced with CALM-AF10MF led to fully penetrant leukemias with a median of 93 days, none of the mice injected with CALM-PZPAF10MF developed disease up to 300 days post-transplantation. We next assessed whether the CALM-PZPAF10MF protein, which lacks leukemogenic activity, can also block leukemogenesis via an in trans mechanism. We used primary leukemia cells from mice with full-blown CALM-AF10MF-induced leukemia and transduced these cells with the CALM-PZPAF10MF fusion gene. Since the cells were from a primary AML, CALM-AF10MF leukemia cells produced almost exclusively blast-like colonies in CFU assays. Strikingly, retroviral transduction of the CALM-PZPAF10MF fusion in these leukemia cells almost completely abrogated their ability to form colonies (Fig. 2c). The ability of CALM-PZPAF10MF to reverse the potent transformed phenotype of the CALM-AF10MF fusion indicates that AF10PZP has a trans-dominant tumor-suppressive function over the CALM-AF10MF fusion.

Exclusion of AF10PZP is essential for Hox/Meis1 activation. The CALM-AF10 fusion is known to upregulate HOXA cluster genes and the HOX-cofactor MEIS1, which is a hallmark of this subtype of leukemia. To determine the role of AF10PZP in Hoxa gene expression, we transduced murine bone marrow-derived HSPCs with either the leukemia-associated CALM-AF10 fusion lacking the first 80 amino acids of AF10, including the first PHD ﬁnger (Fig. 1a, second schematic), or a CALM-AF10 fusion (CALM-nullAF10) which contains full-length AF10 (1–1027 amino acids), including the entire PZP domain, and measured Hoxa transcript levels by qRT-PCR. CALM-AF10 expression in murine bone marrow-derived HSPCs led to a substantial increase in Hoxa7, Hoxa9, Hoxa10, and Meis1 levels compared to the levels of these genes in CALM-nullAF10 expressing cells, indicating that the exclusion of AF10PZP may be necessary for HOX/MEIS activation by the CALM-AF10 fusion protein (Fig. 2d).

To explore whether incorporation of AF10PZP affects Hoxa gene activation by CALM-AF10 in trans, we transduced CALM-AF10MF leukemia cells with CALM-PZPAF10MF and measured Hoxa transcript levels by qRT-PCR (Fig. 2e and Suppl. Fig. 1). As
expected, CALM-AF10 cells were characterized by a high expression of CALM-AF10 target genes Hoxa7, Hoxa9, Hoxa10, and Meis1 (Fig. 2e). A considerable, ~5-10-fold downregulation of these genes observed in the cells transduced with CALM-PZPAF10 suggested that the CALM-PZPAF10 fusion can reverse Hoxa activation by the CALM-AF10 fusion oncoprotein. Together, our findings demonstrate a key role of AF10PZP in blocking leukemic transformation by CALM-AF10 through both in cis and in trans mechanisms. These results also help to explain the fact that AF10PZP is disrupted in all CALM-AF10 fusions, as analysis of the TARGET pediatric AML dataset pointed out that most of the leukemia-associated breakpoints in the AF10 gene in pediatric leukemias are located in or right after AF10PZP, and a few more breakpoints are located just upstream of AF10PZP, but importantly, in all these fusions AF10PZP is impaired or excluded (Fig. 2f).

Fig. 2 AF10PZP impairs in vivo leukemic activity of CALM-AF10. a The number of day-12 (D12) colony-forming units in the spleen (CFU-S) enumerated per 50,000 cells from mice injected with each of the indicated constructs are shown. *P value of 0.0015 and *P value of 0.04 (two tailed Student’s t-test) and data presented as mean ±/− SEM. b Kaplan Meier plot showing survival of mice injected with bone marrow-derived HSPCs transduced with MIG (vector), CALM-AF10MF, or CALM-PZPAF10MF is shown. P value of significance between CALM-AF10MF and CALM-PZPAF10MF < 0.001 (Log-rank t-test). c Number of colonies per 1000 cells with blast-like or differentiated morphology in CALM-AF10MF leukemia cells or the same cells transduced with the CALM-PZPAF10MF fusion are shown. *P value = 0.009 and *P value =0.03. data are presented as mean ±/− SEM from one representative experiment (n = 3). d q-RT PCR analysis of expression of Hoxa7, Hoxa9, Hoxa10, and Meis1 in CALM-AF10MF transduced murine bone marrow cells shown as fold change relative to expression of these genes in CALM-AF10 expressing cells. ***P value = 0.0003 (two-tailed Student’s t-test) and data are presented as mean ±/− SEM from one representative experiment (n = 3). e q-RT PCR analysis of expression of leukemia-associated Hoxa cluster genes and Meis1 in CALM-AF10MF cells expressing CALM-PZPAF10MF shown as a fold-change relative to expression of these genes in CALM-AF10MF cells. ***P value = 0.0001 (two-tailed Student’s t-test). Data are presented as mean ±/− SEM of one representative experiment, (n = 3). f Analysis of the TARGET pediatric AML dataset. Translocation breakpoints in the AF10 gene are displayed in the lolliplot. Each vertical line in the lolliplot corresponds to an individual fusion breakpoint with the height of the vertical line being proportional to the number of fusions. Different fusions are shown as concentric circles, and the orientation of the filled circle points to the position of AF10 in the fusion, i.e., right fill indicates a 3’ AF10 fusion.
histone binding activity. We generated 15N-labeled AF10PZP and tested it in 1H,15N heteronuclear single quantum coherence (HSQC) NMR experiments. The addition of increasing amounts of the H3-1-12 peptide (residues 1–12 of H3) to the AF10PZP sample resulted in large chemical shift perturbations (CSPs) in the AF10PZP spectrum. CSPs were in the intermediate exchange regime on the NMR timescale and indicated direct and tight interaction (Fig. 3a, left). Titration of the methylated H3K4me3-12 peptide into the AF10PZP sample led to an overall similar pattern of CSPs, although the magnitude of CSPs induced by H3K4me3 was smaller (Fig. 3a, right). These results suggest that the unmodified H3 peptide and H3K4me3 peptide occupy the same binding site of AF10PZP, and that AF10PZP slightly prefers an unmodified H3 tail. In agreement, binding of AF10PZP was ~3-fold tighter to the unmodified H3 peptide (dissociation constant \(K_d = 6.5 \mu M\)) than to the H3K4me3 peptide (\(K_d = 22 \mu M\)) in physiologically relevant salt concentration of 150 mM, as measured by tryptophan fluorescence (Fig. 3b, c). However, AF10PZP did not discriminate between unmodified and monomethylated, dimethylated, or trimethylated H3 peptide in low, 50 mM salt concentration, and bound equally well to all peptides with \(K_d\) of ~2–4 \(\mu M\) (Suppl. Fig. 2). No CSPs in AF10PZP were observed upon titration of the H3-1-12 peptide (residues 3–10 of H3), implying that AF10PZP does not bind to H3 lacking Ala1 and Arg2 (Fig. 3d).

Much like AF10PZP, AF10PZP was also capable of binding to the H3 tail, despite the fact that overlay of 1H,15N HSQC spectra of the proteins’ apo-states indicated differences in structures (Fig. 3e–h and Suppl. Fig. 3). Comparable \(K_d\) values, measured for the interaction of AF10PZP or AF10PHD1 with the H3-1-12 Peptide, indicated that the histone binding activity of AF10PZP is preserved in the context of AF10PHD1 (Fig. 3e). Peptide pull-down assay further showed that AF10PZP associates with the longer H3-27 but not to bind H31-21 peptide (Fig. 3f). While the pre-}

AF10PZP recognizes two regions of the H3 tail. AF10PZP has previously been shown to associate with a region of H3 spanning residues 21–27 but not to bind H31-21 peptide. While the presented here structure of H3-1-12-AF10PZP clearly demonstrates the interaction between AF10PZP and the far N-terminal part of H3, particularly residues Ala1-Thr6, in the previously reported structure of the AF10PZP-H31-36 fusion, AF10PZP associates with the middle part of H3 (Ala21-Lys27). An overlay of these structures shows that the two regions of the H3 tail occupy different binding sites of AF10PZP (Fig. 5a). While the N-terminal region of H3 (yellow) is bound by AF10PHD1, the middle region of H3 (magenta) is bound at the interface of the PHD fingers and the zinc knuckle.

To gain insight into the binding of AF10PZP to the H3 tail, we performed 1H,15N HSQC NMR titration experiments using H3 peptides of different sizes (Fig. 5b–d and Suppl. Figs. 5–7). Titration of either H3-1-12 peptide or H3-15-34 peptide to the AF10PZP NMR sample led to dissimilar patterns of CSPs, confirming that the two peptides are bound in separate binding pockets of AF10PZP (Fig. 5b, c). In both titrations, CSPs were in the intermediate exchange regime, which was in agreement with \(K_d\) of 7.5 \(\mu M\) and 2.2 \(\mu M\) measured for the interaction of AF10PZP with H3-1-12 peptide and H3-34 peptide, respectively (Fig. 5e, f). The longer H3 peptide (H3-31), however, was bound tighter by AF10PZP. Analysis of the fluorescence-derived binding curves for the AF10PZP-H3-31 interaction required a two-site binding model, and the fitting yielded two \(K_d\) values of 0.3 \(\mu M\) and 5.9 \(\mu M\), suggesting a cooperative engagement of the two regions of H3-31 (Fig. 5e, g). In support, CSPs in a slow exchange regime, indicative of a tight interaction, were observed in the AF10PZP NMR spectra upon titration with the H3-31 peptide (Fig. 5d).

An almost entire H3 tail is engaged with AF10PZP. Analyzing the crystal structures of the H3-1-12-AF10PZP and AF10PZP-H31-36 complexes (Fig. 5a), we generated AF10PZP mutants which are impaired in binding to either the Ala1-Thr6 region of H3 or the Ala21-Lys27 region of H3. Particularly, the AF10PZP E179K mutant lost its ability to bind to the H3-12 and H3-31 peptides in NMR titration experiments but retained the ability to bind to H3-12 and H3-31 peptides through the interaction with the far N-terminal part of H3 (Fig. 6a, b and Suppl. Figs. 8 and 9). Binding affinities of AF10PZP E179K for the H3-1-12 and H3-31 peptides (8.5 \(\mu M\) and 7.8 \(\mu M\)) were essentially the same as the binding affinity of WT AF10PZP for the H3-12 peptide (7.5 \(\mu M\) (Figs. 6c–e and 5e). Conversely, the AF10PZP D43K mutant was defective in binding to the H3-12 peptide but retained the ability to bind to H3-31, and H3-31 peptides through the interaction with the middle part of H3 (Figs. 6e–h and 5e and Suppl. Fig. 10). Binding affinities of AF10PZP D43K for the H3-15-34 and H3-31 peptides (2.2 \(\mu M\) and 2.9 \(\mu M\)) were similar to the binding affinity of WT AF10PZP for the H3-31 peptide (Figs. 6d and 5e). Pull-down assays using biotinylated histone peptides and the GST-AF10PZP mutants supported the conclusion derived from the NMR data and measurements of binding affinities: disruption of either binding pocket of AF10PZP, although decreases, does not abolish binding to H3. The double D43K/E179K mutation in both
Fig. 3 AF10PZP binds to the N-terminus of the H3 tail. a Overlay of \(^{1}H,^{15}N\) HSQC spectra of AF10PHD1 in the presence of the increasing amount of H3\(_{1\text{--12}}\) or H3K4me3\(_{1\text{--12}}\) peptide. Spectra are colored according to the protein:peptide molar ratio. 

b, c Binding curves used to determine \(K_d\) values by tryptophan fluorescence. \(K_d\) values are represented as mean values +/− S.D. from three independent experiments (\(n=3\)).

d Overlay of \(^{1}H,^{15}N\) HSQC spectra of AF10PHD1 in the presence of the increasing amount of H3\(_{3\text{--10}}\) peptide. Spectra are colored according to the protein:peptide molar ratio.

f Binding curves used to determine \(K_d\) by tryptophan fluorescence. \(K_d\) is represented as mean +/− S.D. from three independent experiments (\(n=3\)).

f−h Histone peptide pulldown assays of GST-AF10PZP with the indicated biotinylated peptides.
sites of AF10PZP is required to eliminate the interaction with H3 (Fig. 6i, j).

Can AF10PZP engage both the far N-terminal region and the middle region of H3 simultaneously? We addressed this question via a reverse NMR titration experiment. We produced 15N-labeled H3 tail (residues 1–44) and recorded its 1H,15N HSQC spectra while adding unlabeled AF10PZP to the sample (Fig. 6k). Synergetic resonance changes, including cross peak disappearance and shifts, were detected in all observable backbone amides between Gln5 and Ala29 of H3, suggesting that the entire Ala1-Lys27 region of the H3 tail was perturbed and therefore likely involved in the interaction. A model of the H3 1-31-AF10PZP complex generated using the simulated annealing method and both crystal structures revealed that the two regions can be bound by AF10PZP simultaneously in cis (Fig. 6l).

AF10PZP associates with both H3 and DNA within the nucleosome. To explore the histone binding mechanism of AF10PZP in the context of chromatin, we tested the interaction of AF10PZP with the nucleosome core particle (NCP) in electrophoretic mobility shift assays (EMSA) and fluorescence anisotropy assays (Fig. 7a–e). We reconstituted NCP using a 207 bp DNA (NCP207) in which 147 bp of DNA is flanked by 30 bp linker DNA on either side and internally labeled with fluorescein 27 bp in from the 5’ end. NCP207 was incubated with increasing amounts of AF10PZP, WT, and mutants, and the reaction mixtures were resolved on a 5% native polyacrylamide gel (Fig. 7a–c). A gradual increase in the amount of added WT AF10PZP resulted in a shift of the NCP207 band, indicative of the formation of the AF10PZP-NCP207 complex, but this shift was delayed when either AF10PZP D43K mutant or E179K mutant were used, implying that interaction of AF10PZP with H3 tail is important for binding to the nucleosome. However, quantitative measurement of binding affinities by fluorescence polarization revealed that the decrease in binding to NCP207 due to D43K or E179K mutation was modest. Titration of WT AF10PZP against NCP207 yielded an $S_{1/2}$ of 6 μM for the AF10PZP-NCP207 complex, whereas binding of the D43K and E179K mutants was only slightly weaker ($S_{1/2}$ = 9 μM and 14 μM, respectively) (Figs. 7d and 6e). The association of WT AF10PZP with the nucleosome reconstituted with 147 bp 601 DNA (NCP147) was also reduced ($S_{1/2}$ = 15 μM), suggesting that the extra-nucleosomal linker DNA contributes to the interaction of AF10PZP with NCP207 (Figs. 7e and 6e). This observation prompted us to investigate whether AF10PZP can also bind DNA. Indeed, a decrease in band intensity of 147 bp 601 DNA upon addition of GST-AF10PZP in EMSA and CSPs induced in AF10PZP by 147 bp 601 DNA in $^1$$H$,15N HSQC experiments indicated that AF10PZP binds to DNA
AF10PZP promotes nuclear localization of CALM-AF10MF and is required for association with chromatin. To examine the role of AF10PZP in the sub-cellular localization of CALM-AF10, we transfected Flag-tagged CALM-AF10MF and CALM-PZPAF10MF into HEK 293T cells and visualized the proteins by immunofluorescence microscopy (IF) using an anti-Flag antibody. IF analysis showed that while the CALM-AF10MF fusion protein was predominantly cytosolic, in HEK 293T cells, CALM-PZPAF10MF fusion protein, harboring D43K/E179K mutations that disrupt binding to H3 tail, lost its ability to accumulate in the nucleus and was found primarily in the cytosol, indicating the importance of functional AF10PZP for the nuclear pool of CALM-AF10 (Fig. 8a, right panels).

To assess the ability of AF10PZP to bind chromatin, we investigated the genomic occupancy of AF10PZP in the MOLM13 human leukemia cell line. We cloned AF10PZP with 2× nuclear localization signals and stably transduced MOLM13 cells. Chromatin immunoprecipitation followed by sequencing (ChIP-seq) using a custom-made antibody directed against AF10PZP showed that AF10PZP co-localizes with the transcription start sites of numerous genes (Fig. 8b). In agreement with in vitro binding data, in cells AF10PZP occupied chromatin regions enriched in H3K4me3 (as well as H3K79me2), however, did not bind to the chromatin sites enriched in H3K27me3. The inhibition of chromatin binding activity of AF10PZP by the repressive H3K27me3 methylation mark appears to be very strong as no enrichment of AF10PZP was observed at bivalent promoters associated with both H3K4me3 and H3K27me3 marks (Fig. 8c, d), which is also consistent with histone peptide pull-down results (Fig. 6i, j).

AF10PZP increases the spreading of H3K79me2. The CALM-AF10 fusion is believed to play a role in targeting DOT1L to gene loci, which results in the deposition of H3K79 methylation and transcriptional activation. We, therefore, examined whether the inclusion of AF10PZP in CALM-AF10 can lead to changes in H3K79 methylation in CALM-AF10 leukemia cells. We performed ChIP-seq experiments using in trans leukemia repression system, in which CALM-PZPAF10MF was overexpressed in CALM-AF10MF leukemia cells (Fig. 2e). ChIP-seq analysis showed that incorporation of AF10PZP by overexpressing CALM-PZPAF10MF led to the gain of H3K79me2 at a number of new genomic sites (Fig. 9a). In contrast, there were almost no sites associated with the loss of H3K79me2 upon CALM-PZPAF10MF overexpression. Furthermore, the incorporation of AF10PZP caused the spreading of H3K79me2 genome-wide beyond the H3K79me2-enriched sites in CALM-AF10MF leukemia cells (Fig. 9b). We note that most of the increase in H3K79me2 levels was found at promoter-proximal regions of genes, including those that are not CALM-AF10 targets (Figs. 9b and 10a). These results suggest that similar to overexpression of DOT1L or AF10 in leukemia cells, the inclusion of AF10PZP leads to H3K79me2 spreading and reversal of leukemogenesis.

In conclusion, our findings indicate that genomic rearrangements of AF10 in leukemia disrupt the intricate relationship between chromatin binding function of AF10PZP and chromatin...
methylation by DOT1L, leading to the establishment and/or perpetuation of oncogenic transcriptional programs. This view is supported by the observation that AF10 fusions invariably exclude the chromatin reader–AF10PZP in leukemia while always retaining AF10OMLZ and thus enabling DOT1L-mediated histone H3K79 methylation. We show that AF10PZP engages the nucleosome through multivalent contacts with histone H3 tail and DNA and binds to chromatin in cells, colocalizing with active methylation marks and discriminating against the repressive H3K27me3 mark. Our results demonstrate that CALM-PZP-AF1010MF decreases Hoxa gene expression in CALM-AF1010MF leukemia cells and that incorporation of AF10PZP in the leukemogenic fusion blocks the transforming activity in vitro and in vivo and abolishes CALM-AF10-driven leukemogenesis in vivo.

Altogether, our data suggest the molecular mechanism underlying the leukemogenic activity of the CALM-AF10 fusion (Fig. 10b). It has been shown that the nuclear export receptor CRM1 recruits CALM-AF10 to Hoxa loci via binding to the nuclear export signal of CALM26. In the absence of functional AF10PZP within the leukemogenic fusion, CALM-AF10 can trap DOT1L at the Hoxa cluster, leading to the elevated local H3K79me2 level, constitutive activation of Hoxa genes, and a decrease in global H3K79me2 level due to the inability of the fusion to spread onto chromatin regions beyond the Hoxa loci (Fig. 10b, top). Incorporation of the chromatin reader, AF10PZP in the CALM-AF10 fusion allows for spreading onto other regions of chromatin, thus disseminating DOT1L to other sites in the genome. This mechanism sheds light on the aberrant stabilization

Fig. 6 An almost entire H3 tail is engaged with AF10PZP. a, b Superimposed 1H,15N HSQC spectra of the AF10PZP E179K mutant collected upon titration with indicated H3 peptides. Spectra are color-coded according to the protein:peptide molar ratio. (c, d) Binding curves used to determine $K_b$ by tryptophan fluorescence. e Binding affinities of WT and mutated AF10PZP for the indicated ligands as measured by ($\ast$) tryptophan fluorescence and ($\ast$) fluorescence anisotropy. $K_b$s are represented as mean values $\pm$ S.D. from three independent experiments ($n = 3$). f, g Binding curves used to determine $K_b$ by tryptophan fluorescence. h Superimposed 1H,15N HSQC spectra of the AF10PZP D43K mutant collected upon titration with H31-12 peptide. Spectra are color-coded according to the protein:peptide molar ratio. i, j Histone peptide pulldown assays of WT and mutated GST-AF10PZP with the indicated biotinylated peptides. k Superimposed 1H,15N HSQC spectra of the histone H31-44 tail collected upon titration with unlabeled AF10PZP. Spectra are color-coded according to the histone:AF10PZP molar ratio. l A model for the association of AF10PZP with the histone H31-31 tail (blue) generated using Xplor 2.14.
of DOT1L at critical oncogenes and points to the CALM-AF10 fusion as a potential candidate for gene therapy aiming to eliminate the upregulation of oncogenes and reverse leukemogenesis.

**Methods**

**Plasmids and constructs.** The p-MIG-CALM-AF10 and pMIY-CALM-AF10MF constructs have been described previously. For the CALM-AF10, a PCR amplified full-length AF10 fragment (corresponding to amino acids 1–1027) was cloned downstream of the CALM part of the pMIG-CALM-AF10 construct, also amplified by PCR. For the CALM-PZPAF10MF construct, a PCR amplified fragment corresponding to amino acids 1–197 of AF10 (ENST00000377072.8) was PCR amplified and cloned into the CALM-AF10MF fusion construct using the BamHI site in between the CALM and AF10 portions. Primers used in this study are listed in the source data file.

**Mice and bone marrow transduction.** Parental strain mice were bred and maintained at the Helmholtz Centre Munich, Animal Resources at Children’s Hospital (ARCH), or the SBP animal facility. All animal experiments described in this study were approved by and adhered to the guidelines of the Sanford Burnham Prebys, Children’s Hospital Boston, or Helmholtz Center Institutional Animal Care and Use Committees under approved protocols. Lineage –ve (lin depleted) cells from murine bone marrow were isolated either by using Mouse hematopoietic progenitor cell isolation kit (STEMCELL Technologies, Canada) as per the manufacturer’s protocol or by injecting donor mice with 5-FU. Five days post 5-FU injection, bone marrow from these mice were harvested by crushing of femur and tibia and plated in bone marrow medium (Dulbecco’s modified Eagle’s medium, 15% fetal bovine serum, 1% Pen/Strep) + cytokines (100 ng/ml stem cell factor, 10 ng/ml interleukin 6 (IL6), 6 ng/ml interleukin 3 (IL3)). Forty-eight hours after prestimulation of the bone marrow cells, they were transduced with different viruses by overlaying them on virus-producing irradiated (400 cGy) GP+E86 producers in the presence of cytokines and protamine sulfate (5 μg/mL) or by spinfection with virus conditioned medium (VCM). These cells were then sorted for GFP or YFP expression using a FACSVantage (Becton Dickinson, Franklin Lakes, NJ, USA) or BD FACSAria II (BD Biosciences, US) flow sorting machine. Sorted GFP or YFP-positive cells were used for colony-forming cell (CFC) or colony-forming unit-spleen (CFU-S) assays or qRT-PCR or injected directly into recipient mice.

**Bone marrow isolation and murine transplantation assays.** CALM-AF10MF leukemia cells were transduced with the MIG empty vector or the MIG-CALM-AF10MF vector expressing viruses and sorted for GFP/YFP expression. Following sorting, 200,000 leukemia cells from these two arms were injected into 800 Gy irradiated C57BL/6J mice through tail vein injections. Hematopoietic engraftment of GFP or YFP-positive cells was assessed by flow cytometry of regularly collected peripheral blood samples. Mice were closely monitored for signs of disease manifestation and sacrificed when they showed signs of leukemic disease.

** Colony-forming unit assays.** For CFU assays, GFP or YFP sorted cells were counted and plated in 1% methylcellulose containing Iscove’s modified Dulbecco medium-based Methocult (Methocult M3434; StemCell Technologies, Vancouver, Canada) at a concentration of 1000 cells/mL.

**CFU-S assays.** Bone marrow cells from 5-fluorouracil-treated mice were isolated, transduced with retroviral supernatants from various constructs, sorted and...
injected intravenously into lethally irradiated (800 cGy of 137Cs γ-radiation) (C57BL/6J × C3H/HeJ) F1 (B6C3) mice at cell numbers adjusted to give 5 to 15 macroscopic spleen colonies. The number of macroscopic colonies was visualized after sacrificing the mice 12 days after injection, fixing the spleen in Telleyesniczky solution (absolute ethanol, glacial acetic acid, and formaldehyde mixed in a 9:1:1 ratio, respectively). For the CALM-AF10MF mutant, mice were injected with fewer cells to ensure scoring resolution (1000 GFP sorted cells per mouse).

**ChIP and ChIP-seq.** For AF10p22p, ChIP-seq, MOLM13 cells stably transduced with the retrovirally delivered AF10p22p construct were used for chromatin immunoprecipitation (ChIP) with a custom antibody generated against AF10p22p. Immunoprecipitation was performed as described earlier. Thirty million cells were fixed using 1% formaldehyde and chromatin was sheared using Diagenode Bioruptor for 15 min with 15 cycles (each 30 s on, 30 s off-cycle) setting at 4 °C. ChIP-seq for H3K79me2 was performed on 1 million CALM-AF10MF leukemia cells or the same cells transduced with the pMIG-CALM-pZPAF10MF virus and sorted for GFP 72 h after transduction and used directly for fixing and sonication as described above. The amount of each antibody used for ChIP experiments is listed in the source data file. Library preparation on eluted DNA was performed using the NEBNext Ultra II DNA library prep kit for Illumina (E7645S and E7600S) as per the manufacturer’s protocol. Library prepped DNA was subjected to sequencing by NextSeq 500 (Illumina, La Jolla, CA) at the Genomics core, MSKCC (New York, NY).

**RNA isolation and qRT-PCR.** RNA was extracted using RNeasy Mini kit (Qiagen) according to the manufacturer’s recommendations and cDNA was prepared using oligo(dT) primers and the SuperScript® III First-Strand Synthesis System (Thermo Fisher, Carlsbad, CA). cDNA was quantified by NanoDrop and used for q-RT-PCR assays with Taqman probes for Hoxa genes, Meis1 and Gapdh or B-Actin genes. Taqman probe information will be provided on request. q-RT-PCR was performed on the ABI 96-well PCR system, and data were analyzed by the delta-delta Ct method.

**Immunofluorescence.** 293T cells were seeded on coverslips and transfected with 1XFLAG CALM-AF10MF, 1XFLAG CALM-pZPAF10MF, or 1XFLAG CALM-pZPmutAF10MF. Non-transfected cells were used as controls. After 48 h of transfection, cells were washed with 1× PBS once and fixed with 2% parafomaldehyde/PBS solution for 10 mins. Cells were air-dried briefly for 2–3 mins, then washed with 1× PBS for 3 mins and permeabilized in 0.1% Triton X for exactly 5 mins. After washing with 1× PBS, cells were blocked in PBS containing 3% BSA + 0.1% Tween 20. Cells were incubated in anti-FLAG M2 (Sigma F1804, 1:500, 2 µg/mL) primary antibody in blocking buffer at 4 °C overnight. The following day, cells were washed 3 times with PBS + 0.1% Tween 20 for 5 mins each and then incubated with Alexa Fluor 647 goat anti-mouse secondary antibody (Molecular Probes A-21236, 1:1000, 2 µg/mL) in blocking buffer at 4 °C overnight. The following day, cells were washed 3 times with PBS + 0.1% Tween 20 for 5 mins each and then incubated with Alexa Fluor 647 goat anti-mouse secondary antibody (Molecular Probes A-21236, 1:1000, 2 µg/mL) in blocking buffer for 1 h at room temperature in dark/protected from light. Cells were then washed and mounted onto glass slides in ProLong Diamond Antifade Mountant with DAPI (Molecular Probes). Images were acquired with Zeiss LSM 710 NLO confocal microscope at ×40 objective.
bodies were diluted in blocking buffer (4% milk in TBS-Tween20) or in 5% BSA in

... showing genomic regions with differential H3K79me2 distribution in

... genes, whereas

... three panels show input and two replicates of CALM-AF10MF for each gene. Peak density increases from blue to red. The left three panels represent the regions with statistically insigni

... buffer and reducing reagent (ThermoFisher Scientific) in either 

... TBS-Tween20) or in 5% BSA in blocking buffer. Western blotting and CALM-AF10MF samples (left) compared to CALM-AF10MF non-transfected 293T cells were used as controls. Whole-

... with genomic regions with differential H3K79me2 distribution in CALM-AF1O MF leukemia cells and in the same cells expressing CALM-ϕϕϕϕΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦΦPhi

\begin{align}
\Delta I = \Delta I_{\text{max}} \left( [I] + [P] + K_D - [I][P] - K_D - 4[P][I] + 2[I] \right) \end{align}

where [I] is the concentration of the histone peptide, [P] is the protein concentration, \( \Delta I \) is the observed change of signal intensity, and \( \Delta I_{\text{max}} \) is the difference concentration.
Fig. 10 The role of AF10PZP in CALM-AF10-mediated leukemogenesis. 

a Representative profiles of H3K79me2 peaks in CALM-AF10MF leukemias without (upper panels, blue tracks) or with co-transduction of the CALM-PZPAF10MF fusion (lower panels, green tracks). Red tracks and orange tracks represent ChIP input control for CALM-AF10MF and CALM-AF10MF + CALM-PZPAF10MF samples, respectively. Data from 2 independent ChIP replicates is shown.

b Schematic of the mechanism of AF10PZP-dependent CALM-AF10-mediated leukemogenesis.
in signal intensity of the free and bound states of the domain. The $K_d$ values were averaged over three separate experiments, with the error calculated as standard deviation between the runs.

**Peptide pull-down assay.** One microgram of biotinylated histone peptides with different modifications was incubated with 1 µg of GST–AF10$_{292}$ in binding buffer (50 mM Tris-Cl pH 7.5, 250 mM NaCl, 0.1% NP-40, and 1 mM PMSF) overnight. Streptavidin magnetic beads (Pierce) were added to the mixture, and the mixture was incubated for 1 h with rotation. The beads were then washed three times and analyzed using SDS-PAGE and western blotting.

**Nucleosome preparation.** Human H2A, H2B, H3.2, and H4 histone proteins were expressed in *Escherichia coli* BL21 DE3 pLysS cells, separated from inclusion bodies, and purified using size exclusion and ion-exchange chromatography, as described previously. Histones were then mixed together in 7 M guanidine HCl, 20 mM Tris-Cl pH 7.5, and 10 mM dithiothreitol in appropriate molar ratios and refolded into octamer by slow dialysis into 2 M NaCl, 20 mM Tris-Cl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, and 2 mM β-mercaptoethanol. The octamer was purified from tetramer and dimer by size exclusion chromatography. Octamer was then mixed with 20% excess of DNA by slow desalting into 5 mM Tris pH 8.0 and 0.5 mM EDTA pH 8.0. Finally, the NCPs were separated from free DNA via sucrose gradient purification. DNAs used were either the 147 bp 601 Widom NPS flanked with 30 bp linker DNA on either side and internally labeled with fluorescein 27 bp in from the 5' end, or the 601 Widom NPS labeled with fluorescein or Cy3 on the 5' end.

**Fluorescence polarization.** Fluorescence polarization measurements were carried out by mixing increasing amounts of AF10 WT or D43A and E179K mutants with 5 nM NCPS in 75 mM NaCl, 25 mM Tris-Cl pH 7.5, 0.00625% Tween20, and 5 mM dithiothreitol in a 30 µL reaction volume. The samples were loaded into a Corning round bottom polystyrene plate and polarization measurements were acquired on a Tecan infinite M200 Pro plate reader by exciting at 470 nm and measuring polarized emission at 519 nm with 5 nm excitation and emission bandwidths. The fluorescence polarization was calculated from the emission polarized parallel and perpendicular to the polarized excitation light as described previously. The data were then fit to a binding isotherm to determine $S_{50}$ and the $S_{50}$ values were averaged over three separate experiments with the error calculated as standard deviation between the runs.

**EMSA.** EMSAs were performed by mixing increasing amounts of AF10$_{292}$ with 0.25 pmol of 601 Widom DNA/lane in 20 mM Tris-Cl pH 7.5 buffer supplemented with 150 mM NaCl and 5 mM dithiothreitol in a 10 µL reaction volume. Reaction mixtures were incubated at 4 °C for 10 min and loaded onto a 5% native polyacrylamide gel. Electrophoresis was performed on 0.2% Tris-borate-EDTA (TBE) buffer at 1–100 V for exposure. The gels were stained with SYBR Gold (Thermo Fisher Sci) and visualized by Blue LED (UltraThin LED Illuminator-GelCompany). EMSAs with NCPS were performed by mixing increasing amounts of AF10$_{292}$ WT or D43A and E179K mutants with 5 nM NCPS in 75 mM NaCl, 25 mM Tris-Cl pH 7.5, 10% glycerol, and 0.005% Tween 20 buffer in a 12 µL reaction volume. Each sample was incubated at 4 °C for 5 min and then loaded onto a 5% native polyacrylamide gel. Electrophoresis was performed in 0.3% Tris-borate-EDTA (TBE) at 300 V for 90 min. Fluorescence images were acquired with a Typhoon Phosphor Imager.

**FRET.** FRET efficiency measurements were carried out on a Horiba Scientific Fluoromax 4. The data were collected using Fluorescence v3.5 software and processed with MATLAB R201a. Samples were excited at 510 and 610 nm and the photoluminescence spectra were measured from 530 to 750 nm and 630 to 750 nm for donor and acceptor excitation, respectively. Each wavelength was integrated for one second, and the excitation and emission slit width was set to 5 nm with 5 nM donor and acceptor excitation, respectively. Each wavelength was integrated for one second, and the excitation and emission slit width was set to 5 nm with 5 nM donor and acceptor excitation, respectively.

**H3K79me2 ChIP-seq data analysis.** Adapter remnants of sequencing reads were removed with cutadapt v2.337. Trimmed ChIP-seq sequencing reads were aligned to mouse genome version 38 (mm10) using STAR aligner version 2.738. Chromatin-binding modules were recovered by mixing increasing amounts of AF10 WT or D43A and E179K mutants with 5 nM NCPS in 75 mM NaCl, 25 mM Tris-Cl pH 7.5, 0.00625% Tween20, and 5 mM dithiothreitol in a 30 µL reaction volume. The samples were loaded into a Corning round bottom polystyrene plate and polarization measurements were acquired on a Tecan infinite M200 Pro plate reader by exciting at 470 nm and measuring polarized emission at 519 nm with 5 nm excitation and emission bandwidths. The fluorescence polarization was calculated from the emission polarized parallel and perpendicular to the polarized excitation light as described previously. The data were then fit to a binding isotherm to determine $S_{50}$ and the $S_{50}$ values were averaged over three separate experiments with the error calculated as standard deviation between the runs.

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Author contributions

B.J.K., A.D., K.L.C., F.X., K.B., S.K., Q.T., Y.Z., P.Z. and A.S. performed experiments and together with S.K.B., X.S., H.W., M.G.P., A.J.D. and T.G.K. analyzed the data. A.J.D. and T.G.K. wrote the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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