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The ENL YEATS epigenetic reader domain critically links MLL-ENL to leukemic stem cell frequency in t(11;19) Leukemia

Hsiangyu Hu[1,#], **Nirmalya Saha**[1,#], **Yuting Yang**[1], **Ejaz Ahmad**[1], **Lauren Lachowski**[1], **Uttar Shrestha**[1], **Vidhya Premkumar**[1], **James Ropa**[2], **Lili Chen**[1], **Blaine Teahan**[1], **Sierrah Grigsby**[1], **Rolf Marschalek**[3], **Zaneta Nikolovska-Coleska**[1], **Andrew G. Muntean**[1,*]

[1]Department of Pathology, University of Michigan Medical School, Ann Arbor, MI, USA

[2]Department of Microbiology and Immunology, Indiana University School of Medicine, 950 West Walnut Street, R2-302, Indianapolis, IN, 46202-5181, USA.

[3]Institute of Pharmaceutical Biology / Diagnostic Center of Acute Leukemia, University of Frankfurt, Frankfurt/Main, Germany.

Abstract

MLL (KMT2a) translocations are found in ~10% of acute leukemia patients, giving rise to oncogenic MLL-fusion proteins. A common MLL translocation partner is ENL and associated with a poor prognosis in t(11;19) patients. ENL contains a highly conserved N-terminal YEATS domain that binds acetylated histones and interacts with the PAF1c, an epigenetic regulator protein complex essential for MLL-fusion leukemogenesis. Recently, wild type ENL, and specifically the YEATS domain, was shown to be essential for leukemic cell growth. However, the inclusion and importance of the YEATS domain in MLL-ENL mediated leukemogenesis remains unexplored. We found the YEATS domain is retained in 84.1% of MLL-ENL patients and crucial for MLL-ENL mediated leukemogenesis in mouse models. Mechanistically, deletion of the YEATS domain impaired MLL-ENL fusion protein binding and decreased expression of pro-leukemic genes like *Eya1* and *Meis1*. Point mutations that disrupt YEATS domain binding to acetylated histones decreased stem cell frequency and increased MLL-ENL-mediated leukemia latency. Therapeutically, YEATS containing MLL-ENL leukemic cells display increased sensitivity to the YEATS inhibitor SGC-iMLLT compared to control AML cells. Our results demonstrate that the YEATS domain is important for MLL-ENL fusion protein-mediated leukemogenesis and exposes an “Achilles heel” that may be therapeutically targeted for treating t(11;19) patients.

[*]Corresponding Author: Andrew G. Muntean Ph.D., University of Michigan Medical School, 7520B Medical Science Research Building I, 1150 W. Medical Center Drive, Ann Arbor, MI 48109-5602, Fax: (734) 764-4308, andrewmu@umich.edu.

[#]These authors contributed equally

Author Contributions

H.H., N.S., Z.N.C. and A.G.M. designed the experiments. All authors participated in conducting experiments and collecting or analyzing the data. Y.Y. conducted cloning, expression and purification of recombinant proteins; E.A. conducted FP and BLI binding assays; U.S. performed synthesis of the fluorescent labeled inhibitor. R.M. provided chromosomal breakpoint data and analysis. H.H., Z.N.C. and A.G.M. wrote the manuscript. The project was overseen by A.G.M.

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Introduction:

11q23 translocations involving *MLL (KMT2a)* are found in pediatric, adult and therapy-related leukemias and about 10% of leukemias overall (1–4). Patients harboring *MLL* translocations have a poor prognosis, but this varies depending on fusion partner, leukemia subtype and age (5). The *MLL* gene codes for a large H3K4 histone methyltransferase that positively regulates *HOX* gene expression (6). Translocation events result in fusion proteins consisting of N-terminal *MLL* sequence with C-terminal sequence of one of >100 partners (7). Despite a vast number of translocation partners, most cases (>80%) involve *MLL* fusion to one of six common partners: *AF4, AF9, ENL, AF10, ELL* and *AF6*. Apart from AF6 these proteins cooperate within several similar transcriptional activation complexes including the Super Elongation Complex (SEC) (8–10). The SEC interacts and recruits positive transcriptional elongation factor b (pTEFb), which is implicated in activation of MLL target genes (8, 9, 11). pTEFb phosphorylates the C-terminus of RNA polymerase II to release it into a productive transcriptional elongation phase (12). Components of the SEC, including ENL, also interact with the DOT1L complex responsible for H3K79 methylation and necessary for MLL-fusion mediated gene activation (13–16). MLL fusion proteins localize the SEC and DOT1L to pro-leukemic target genes like *Hoxa9* and its co-factor *Meis1* to drive transcriptional elongation and MLL-fusion mediated transformation (17–19). Thus, targeting these complexes has become an attractive strategy for treatment of MLL-rearranged leukemia. Indeed, both genetic and small molecule inhibitor studies targeting DOT1L have demonstrated promise in acute leukemia mouse models, however DOT1L catalytic inhibitors displayed modest activity in clinical trials (13, 14, 20–25).

Eleven-Nineteen-Leukemia (*ENL*, also known as *MLLT1*) is the third most common *MLL* fusion partner (7) and shares high homology with another common *MLL* fusion partner AF9 (~74% gene sequence homology) (26). Recently, *ENL* was identified in a CRISPR-Cas9 screen to be essential for acute myeloid leukemia cell growth, where loss of ENL results in decreased RNA polymerase II occupancy and decreased transcriptional initiation and elongation at ENL-enriched targets (27, 28). In contrast, AF9 was found dispensable in several acute myeloid leukemia cell lines but essential for hematopoietic stem cell self-renewal and expansion (27–29). Structurally, both ENL and AF9 contain an N-terminal YEATS domain and an intrinsically disordered C-terminal Anc1-Homology Domain (AHD). The C-terminus of ENL and/or AF9 undergoes coupled binding and folding upon interaction with members of the SEC and DOT1L complexes (30–35). Indeed, the AHD is essential for MLL-ENL and MLL-AF9 mediated leukemogenesis (10, 36) and we have reported the first peptidomimetic inhibitors of the AF9/ENL AHD, demonstrating the druggability of this protein-protein interaction (22). The ENL and AF9 YEATS domains interact with histone H3 acetylated at K9, K18 or K27 and mutations disrupting the YEATS epigenetic reader function affects recruitment to target loci (27, 28, 37). Recurrent mutations in the ENL YEATS domain occur in children with Wilms tumor, which induce increased binding and spreading of ENL at target genes (38, 39). The ENL YEATS domain also directly interacts with PAF1 of the Polymerase Associated Factor 1 complex (PAF1c) (34). The PAF1c functions by recruiting epigenetic and transcription factors to influence transcriptional elongation (40). We and others have shown that the PAF1c directly interacts

with MLL and is essential for MLL-fusion mediated leukemogenesis (41–43). These studies suggest multiple functions of the ENL/AF9 YEATS domain may be critically important in regulating ENL/AF9 function in leukemic cells and several YEATS domain inhibitors have been reported (44–46). However, the importance of the YEATS domain in the context of MLL-ENL fusion proteins and leukemogenesis and its prospect as a potential therapeutic target has not been addressed.

In this study, we investigate the clinical relevance and leukemic importance of the ENL YEATS domain in MLL-ENL leukemias and our results reveal a critical role for the YEATS domain in t(11;19) patients with MLL-ENL translocations. We demonstrate the preferential inclusion of the YEATS domain in most MLL-ENL fusions (>84% of MLL-ENL patients) and exclusion of the AF9 YEATS domain in MLL-AF9 patients (>98% of MLL-AF9 patients). We report the importance of the YEATS domain on MLL-ENL localization and leukemogenesis *in vivo* and its impact on leukemic stem cell frequency that may be exploited for therapeutic intervention.

Materials and Methods

Methods are found in Supplementary Materials.

Results

The majority of t(11;19) patients retain the YEATS domain in resultant MLL-ENL fusion proteins

t(11;19) and t(9;11) translocations are common MLL translocations that fuse *MLL (KMT2a)* with *ENL (MLLT1)* or *AF9 (MLLT3)*, respectively. ENL and AF9 contain N-terminal YEATS domains, which share 88% alignment (45) that may impact MLL-fusion protein function through protein interactions (27, 28, 34, 37). To understand the functional outcomes of these protein-protein interactions, we asked whether t(11;19) patients retain the YEATS domain in resultant MLL-ENL fusion proteins. *ENL* is composed of 12 exons and located on chromosome 19p13.3 (Fig. 1A). The YEATS domain is coded from exons 2–4 (amino acids 5–140) (Fig. 1B) (47). Thus, all t(11;19) translocations with ENL breakpoints upstream of exon 2 produce MLL-ENL fusion proteins containing the YEATS domain. We mined breakpoint data from a previously described clinical cohort of 302 t(11;19) patients (7) and found that 50.7% (n=153) of all MLL-ENL patients harbor genomic breaks 5’ upstream of the *MLLT1* gene but 3’ downstream of *ACER1* (Fig. 1A). Another 33.4% (n=101) patients harbor genomic breaks within the first *MLLT1* intron (Fig. 1A). In combination, 84.1% (n=254) of t(11;19) leukemia patients retain the YEATS domain in resultant MLL-ENL fusion proteins. The remaining 15.9% (n=48) t(11;19) patients have genomic breakpoints downstream of exon 2 (Fig. 1A), leading to partial inclusion (6.0%) or exclusion of the YEATS domain (9.9%) in MLL-ENL fusion proteins (Fig. 1A, B, C). These findings align with previous studies investigating ENL breakpoints in t(11;19) patients reporting YEATS domain retention in 19 of 23 (48) and 10 of 15 (49) t(11;19) patients. Patient age group is not significantly correlated with YEATS domain retention status in MLL-ENL leukemia (Chi-sq p=0.33, df=2) (Fig. 1D). Fewer patient samples prohibited us

from comparing YEATS retention in mixed lineage leukemia, however significantly fewer AML than ALL patients retained the YEATS domain (Chi-sq p<.0001, df=1) (Fig. 1E).

Given the ENL and AF9 (*MLLT3*) YEATS domain homology, we investigated *AF9* breakpoints in t(9;11) MLL-AF9 leukemia patients (Fig. S1). In contrast to *ENL*, *AF9* genomic breaks occur almost exclusively downstream of exon 4 (98.4%, n=442) and exclude the AF9 YEATS domain from the resultant MLL-AF9 fusion proteins (Fig. S1A, S1B). Only two MLL-AF9 patients (0.5%) had breakpoints upstream of *MLLT3* exon 1, resulting in YEATS domain inclusion in the MLL-AF9 fusion product (Fig. S1B, S1C). Together, these data suggest that the YEATS domain is specifically retained in the majority of the MLL-ENL fusion proteins from t(11;19) patients but absent in the majority of the MLL-AF9 fusion proteins in t(9;11) patients.

The YEATS domain and downstream sequence is important for MLL-ENL mediated leukemogenesis

We next investigated the importance of the ENL YEATS domain in MLL-ENL mediated transformation and leukemogenesis using retroviral MLL-ENL vectors with (MLL-ENL) or without the YEATS domain (MLL-ENLΔYEATS) (Fig. 2A). The MLL-ENLΔYEATS construct includes the C-terminal AHD minimally required for MLL-ENL colony formation *ex vivo* (10). Both MLL-ENL fusion constructs displayed similar mRNA and protein levels (Fig. 2B, S2A) and were sufficient for serial colony replating as evidenced by third round colony formation (Fig. S2B), consistent with previous reports (10, 36). However, MLL-ENL cells showed significantly higher colony forming potential and proliferation rates compared to MLL-ENLΔYEATS cells (Fig. S2B, S2C). To interrogate the function of the YEATS domain in MLL-ENL leukemogenesis *in vivo*, we injected MSCV, MLL-ENL, or MLL-ENLΔYEATS transduced cells into lethally irradiated syngeneic C57Bl/6 recipient mice. Mice receiving MLL-ENL transduced cells succumbed to leukemia with a median survival of 66 days, displaying splenomegaly and leukemic infiltration in the spleen and liver (Fig. 2C, 2D, 2E). Strikingly, mice receiving MLL-ENLΔYEATS transduced cells failed to develop leukemia *in vivo* (Fig. 2C, 2D, 2E). These data suggest amino acids 5–371 of ENL, which contain the YEATS domain, are critical for MLL-ENL mediated leukemogenesis.

We next addressed specific YEATS domain functions in MLL-ENL leukemogenesis. Specifically, ENL F59A and Y78A mutants were introduced to disrupt recognition of acetylated H3 lysine marks (H3K9, K18 and K27) (Fig. 2A) (28). We also generated a deletion construct (ENL Δ21–26) that mitigates binding to PAF1 of the PAF1c (50) and a triple mutant that disrupts both (F59A, Y78A and Δ21–26) (Fig. 2A). These mutations do not impact transcript and protein expression (Fig. 2B, S2A). To assess their transformative capabilities, we performed colony and proliferation assays with MLL-ENL or MLL-ENL-mutant-YEATS transduced cells. All YEATS domain mutants displayed slower proliferation rates and a modest decrease in colony forming potential compared to MLL-ENL (Fig. S2D, S2E). To definitively assess the impact of these mutations, we performed *in vivo* leukemogenesis assays utilizing MLL-ENL fusion proteins containing YEATS mutations. All mutations significantly extended leukemic disease latency *in vivo* compared to MLL-ENL (Fig. 2F). Interestingly, combining these mutations (MLL-ENL

Triple, Fig. 2A) resulted in a significant extension of disease latency compared to MLL-ENL single YEATS mutations (Fig. 2F). All groups eventually succumbed to leukemic disease burden accompanied with splenomegaly (Fig. S2F) and leukemic infiltration in the spleen and the liver (Fig. S2G), although decreased spleen weights were observed in MLL-ENL YEATS point mutant diseased mice (Fig. S2F). These results suggest that YEATS domain protein interactions are important for MLL-ENL leukemogenesis.

Mutation of the YEATS domain alters binding to acetylated histone H3

We characterized how YEATS domain mutations impact binding to H3Kac and PAF1 using immunoprecipitation and quantitative biolayer interferometry (BLI) assays. Similar to previous reports (28), the YEATS domain binds preferably to immobilized biotin labeled acetylated H3K27ac peptide (Fig. 3A, 3B, S3A, S3B) with a binding affinity K_d of 66 μM to immobilized biotinylated H3K27ac peptide as previously reported. F59A and Y78A mutations abrogate binding to H3K27ac (Fig. 3A, 3B). Interestingly, the Δ21–26 also disrupted interaction with acetylated H3K27 peptide (Fig. 3A, 3B). This result contrasts with a previous report indicating this deletion does not alter YEATS interaction with histone H3; albeit using a different biochemical assay (50). Not surprisingly, the triple mutation (F59A, Y78A and Δ21–26) failed to bind acetylated H3K27 peptide (Fig. 3A, 3B). To further analyze the impact of YEATS mutations on H3Kac binding, we utilized the ENL/AF9 YEATS small molecule inhibitor, SGC-iMLLT (45), which binds within the H3Kac binding groove of the ENL/AF9 YEATS domain in the nanomolar range allowing quantitative analysis of the interactions with wild type and mutated YEATS domains. Based on the complex structure between SGC-iMLLT and ENL (PDB ID: 6HT1) we designed a fluorescein labeled inhibitor, Flu-SGC-iMLLT (Fig. S3C) and developed a fluorescence-polarization binding assay to determine its binding affinity to wild type and mutated ENL YEATS domain. Consistent with previous studies, Flu-SGC-iMLLT binds to the ENL YEATS domain with a K_d of 0.26 μM (Fig. 3C) (45) demonstrating the fluorescein label does not affect interaction with the YEATS domain. Importantly, we observed significantly decreased binding affinity to all YEATS mutants in the following order: Δ21–26 (K_d = 4.6 μM) > Y78A (K_d = 8.82 μM) > F59A (K_d > 8 μM) > triple mutation (K_d > 8 μM). The obtained binding affinities correlated with the binding preferences to the H3K27ac peptide, and as expected the YEATS triple mutant had the most significant impact on Flu-SGC-iMLLT binding, suggesting an additive effect (Fig. 3C, S3D).

We then addressed how YEATS domain mutations disrupt interaction with PAF1 of the PAF1c using co-immunoprecipitation experiments. Our positive control, FLAG-CDC73, a known PAF1 binding partner, efficiently co-immunoprecipitated with endogenous or exogenous PAF1 (Fig. 3D, 3E). As expected, FLAG-ENL co-immunoprecipitated endogenous PAF1 (Fig. 3D) or exogenous HA-PAF1 (Fig. 3E). Interestingly, all mutations of the YEATS domain (F59A, Y78A, Δ21–26, or Triple mutation) do not disrupt interaction with PAF1 (Fig. 3D, 3E). These results provide evidence that the H3K27ac and PAF1 binding sites are not overlapping suggesting they are not mutually exclusive. Together, these data demonstrate that the single YEATS mutations independently or additively disrupt the YEATS domain epigenetic reader function but do not alter interaction with PAF1 of the PAF1c.

Loss of YEATS domain function affects transcription of selected MLL-ENL targets

We characterized how YEATS domain mutations impair MLL-ENL leukemogenesis by investigating apoptosis, cell cycle and differentiation. We observed a modest but statistically significant increase in apoptotic populations when comparing murine MLL-ENLΔYEATS transformed cells to MLL-ENL transformed cells (Fig. S4A). A less pronounced increase in apoptotic cells is observed in MLL-ENL YEATS point mutants (Fig. S4A). Interestingly, we did not observe a significant change in expression of cell surface markers associated with myeloid differentiation (CD14 and CD11b), cell cycle or cellular morphology in MLL-ENLΔYEATS cells compared to MLL-ENL cells (Fig. S4B, S4C, S4D). Thus, loss of the YEATS domain and downstream sequence in MLL-ENL cells only modestly alters apoptosis *in vitro*. Next, we addressed how loss or mutation of the YEATS domain affects MLL-ENL-mediated transcriptional activation. We investigated the expression of confirmed MLL-ENL target genes (*Hoxa9, Meis1* and *Myc*) by qPCR. A modest expression difference was detected for *Hoxa9* and *Meis1* but not *Myc* (Fig. S4E). Given the modest change in apoptosis, we examined the expression of *Bcl2, Bclxl* and *Mcl1*, which showed mostly insignificant changes with loss or mutation of the YEATS domain (Fig. S4F). We did not detect significant increases in genes associated with myeloid differentiation (*Cd80, Id2, Itgam* and *Nab2;* (51)) consistent with our results showing loss or mutation of the YEATS domain does not affect differentiation (Fig. S4B, S4D and S4G). Finally, we tested the transcriptional activity of MLL-ENL fusion proteins with and without YEATS mutations on the *Hoxa9* promoter using a luciferase reporter. MLL-ENLΔYEATS, F59A, Y78A, Δ21–26 or triple mutant fusion proteins displayed no reduction in transcriptional activation of the *Hoxa9* promoter (Fig. S4H), suggesting transcription per se is not altered by mutation (or loss) of the YEATS domain.

Mutation of the YEATS domain alters MLL-ENL leukemic stem cell frequency

We hypothesized that the YEATS domain epigenetic reader function impacts MLL-ENL leukemic stem cell frequency. First, we investigated whether the disease latency extension following mutation of the YEATS domain (Fig. 2) was transplantable. Indeed, we observed a significant disease extension in secondary recipients following transplantation of primary MLL-ENL YEATS mutant leukemic cells (F59A or Y78A) compared to MLL-ENL leukemic cells (Figs. 4A, S5A). We then performed extreme limiting dilution analysis by transplanting primary MLL-ENL or MLL-ENL YEATS mutant (F59A or Y78A) leukemic cells into irradiated syngeneic recipients. We detected a significant decrease in leukemic stem cell frequency in MLL-ENL YEATS mutant leukemias (1/355 cells, 95% CI:1/198–1/637 cells) compared to MLL-ENL leukemia (1/108 cells, 95% CI: 1/62–1/190 cells) (Fig. 4B, C). To rule out differences in leukemic cell homing to the bone marrow we injected CD45.2 donor leukemic cells (MLL-ENL or MLL-ENL F59A) into sublethally irradiated CD45.1 syngeneic recipients and observed no difference in the percentage of donor MLL-ENL or MLL-ENL F59A leukemic cells in the bone marrow (Figs. 4D, 4E, S5B). Thus, disrupting the YEATS domain epigenetic reader function on MLL-ENL fusion proteins impacts leukemic stem cell frequency.

Loss of the YEATS domain impacts expression of select MLL-ENL target genes

To understand how the YEATS domain impacts MLL-ENL target gene expression we performed RNA-Seq analysis on RNA prepared from murine MLL-ENL and MLL-ENLΔYEATS cell lines. Differential expression analysis using a 1.5 fold cut off revealed 533 downregulated and 290 upregulated genes in MLL-ENLΔYEATS compared to MLL-ENL cells (Fig. 5A, Table S1). Gene ontology analysis revealed deregulation of several GO terms under the molecular function aspect including “DNA-binding transcription activator activity” (Fig. 5B). Next, we used Gene Set Enrichment Analysis (GSEA) to investigate gene programs misregulated in MLL-ENLΔYEATS cells (Table S1). Importantly, we found decreased expression of a HOXA9 and MEIS1 gene program, previously described in MLL-ENL cells, in MLL-ENLΔYEATS cells (Fig. 5C). A myeloid development phenotype was also more associated with MLL-ENLΔYEATS cells (Fig. 5C). Gene programs associated with hematopoietic and leukemic stem cells were enriched in MLL-ENL cells compared to MLL-ENLΔYEATS cells (Fig. 5C). Finally, direct transcriptional targets of MLL and MLL-AF9 were more enriched in MLL-ENL cells compared to MLL-ENLΔYEATS cells (Fig. 5C). Thus, we examined how many direct MLL-ENL target genes (52) were downregulated or upregulated in MLL-ENLΔYEATS cells. We identified downregulation of seven direct MLL-ENL targets: *Eya1, Ms4a3, HoxA10, Pim1, Mpo, Sox4* and *Nlp3* and upregulation of *Cdkn2c* in MLL-ENLΔYEATS cells (Fig. 5D, E). Downregulation of *Eya1, Hoxa10, Ms4a3* and *Pim1* were confirmed by qPCR from freshly prepared mRNA from MLL-ENL and MLL-ENLΔYEATS cells (Fig. 5E). These data point to differential regulation of MLL-ENL transcriptional targets depending on the presence or absence of the ENL YEATS domain.

Loss of the YEATS domain impairs MLL-ENL localization on target genes

We next performed chromatin immunoprecipitation (ChIP) assays to assess MLL fusion protein binding and changes to the epigenetic landscape on *Eya1* and *Meis1* loci. Impaired transcriptional activation of *Eya1* and *Meis1* in murine MLL-ENLΔYEATS cells observed by RNA-seq was also detected in MLL-ENL F59A, -Y78A, -Δ21–26 and -Triple mutants compared to MLL-ENL cells (Fig. 6A, 6B, S6A, S6B). We examined the promoter regions (P1 and P3) and intragenic region (P7) of *Eya1* (Fig. 6A) and the regulatory region (P1 and P2) of *Meis1* (Fig. S6A). ChIP for the N-terminal FLAG-tag revealed reduced binding of MLL-ENLΔYEATS fusion protein compared to MLL-ENL on the *Eya1* and *Meis1* loci (Fig. 6C, S6C). Interestingly, MLL-ENL F59A or -Y78A fusion proteins bound with similar affinity as MLL-ENL despite significantly lower expression levels (Fig. 6C, S6C). The binding pattern of the PAF1c subunit PAF1 mirrored the MLL-fusion proteins suggesting the PAF1c and MLL-ENL fusion proteins cooperatively assemble on these loci (Fig. 6D, S6D). Next, we interrogated histone modifications associated with transcriptional activation by MLL-ENL fusion proteins, including H3K9ac, H3K79me2 and H3K4me3, which were detected in MLL-ENL cells at the promoter region for *Eya1* and *Meis1.* Consistent with transcriptional changes observed from these loci, H3K9ac, H3K79me2 and H3K4me3 was reduced proportionally to *Eya1* and *Meis1* transcriptional output in MLL-ENLΔYEATS, -F59A and -Y78A cells (Fig. 6E, 6F, 6G, S6E, S6F, S6G). These data suggest that deletion of ENL that includes the YEATS domain impairs binding and transcriptional activation, whereas point mutations to the YEATS domain impacting H3Kac binding do not alter fusion protein localization but inhibit downstream epigenetic function.

Targeting YEATS domain binding to H3Kac impairs MLL-ENL proliferation and target gene expression

Given our *in vivo* data (Fig. 2), we hypothesized that MLL-ENL cells may be sensitive to small molecule inhibitors disrupting the YEATS-H3Kac interaction. We utilized SGC-iMLLT to test colony formation capacity of murine MLL-ENL described above (containing the YEATS domain), MLL-AF9 (without YEATS domain) and E2A-HLF AML cell lines in the presence of escalating doses of SGC-iMLLT (0.002μM to 20μM) (Fig. 7A). MLL-ENL cells showed the highest sensitivity to SGC-iMLLT (IC50=0.39μM) without a noticeable change in cell morphology after second round plating (Figs. 7A, S7A, S7B). In contrast, MLL-AF9 and E2A-HLF transformed cells showed modest sensitivity to SGC-iMLLT at the highest concentration (20μM) (Figs. 7A, S7A, S7B). Gene expression analysis revealed a dose dependent reduction of *HoxA9, Meis1* and *Eya1* expression in MLL-ENL cells treated with SGC-iMLLT without changes in myeloid differentiation genes (Fig. 7B, S7C). SGC-iMLLT exposure did not impact *Bcl2* or *Bclxl* expression in E2A-HLF cells (Fig. S7C). We next compared the sensitivity of murine cell lines generated by transduction with either MLL-ENL or MLL-ENL F59A to SGC-iMLLT in liquid proliferation assays. The F59A mutation rendered MLL-ENL cells less sensitive to SGC-iMLLT treatment (Fig. 7C), consistent with its abolished binding affinity to this mutated YEATS domain (Fig. 3C). Finally, we examined the sensitivity of human leukemic cell lines to SGC-iMLLT. We compared HB1119 cells driven by MLL-ENL fusion proteins containing the YEATS domain (3), KOPN8 cells harboring MLL-ENL fusion proteins lacking the YEATS domain (53), MOLM13 cells (MLL-AF9), MV4;11 cells (MLL-AF4) and K562 cells (BCR-ABL). All human cells were sensitive to 20 μM SGC-iMLLT, which may be cytotoxic (Fig. 7D). However, greater sensitivity was detected for HB1119 and MV4;11 cells at 2 μM consistent with a role for wild type ENL in leukemic cell survival (27). Increased sensitivity of HB1119 cells compared to KOPN8 cells is also consistent with the reliance of HB1119 cells on the YEATS domain present in the driving MLL-ENL fusion protein (Fig. 7D). Thus, leukemic cells are sensitive to targeted chemical inhibition of the YEATS domain on wild type ENL. Further, leukemic cells driven by MLL-ENL fusion proteins harboring the YEATS domain display greater sensitivity suggesting this may be an ideal target for t(11;19) patients.

Discussion

ENL and AF9 are YEATS domain proteins and *MLL* translocation partners in ~32% of MLL-rearranged leukemia (7). However, investigation of the YEATS domain inclusion in t(11;19) MLL-ENL and t(9;11) MLL-AF9 patients was lacking. We present data from t(11;19) and t(9;11) leukemia patients that shows the YEATS domain is retained in >84% of MLL-ENL fusion proteins but lost in almost all MLL-AF9 fusion proteins (Fig. 1). *In vivo* leukemogenesis assays demonstrated an important role for the YEATS domain and downstream sequence in MLL-ENL leukemias (Fig. 2). Our biochemical analysis suggests a model whereby disrupting the YEATS domain epigenetic reader function impacts MLL-ENL target activation and leukemic stem cell frequency (Fig. 8). These data identify a potential “Achilles heel” that may render MLL-ENL leukemias more susceptible to therapeutics targeting the YEATS domain.

Interestingly, our cellular characterization revealed a LSC defect following mutation of the YEATS domain in MLL-ENL fusion proteins. Transcriptomic analysis revealed differential expression of *Meis1* and *Eya1* comparing MLL-ENL and MLL-ENLΔYEATS or point mutant cells (Fig. 5, 6). *Meis1* is implicated in leukemic stem cell self-renewal, differentiation arrest, and cycling and *Eya1* can immortalize hematopoietic progenitors (18, 54). We observed lower LSC frequency following mutation of the YEATS domain affecting H3Kac binding in MLL-ENL fusion proteins (Fig. 4). It is possible that the YEATS domain directly impacts LSC frequency by affecting MLL-ENL mediated transcription of *Meis1, Eya1* and/or others.

The MLL-ENLΔYEATS construct used in this study closely models t(11;19) leukemia patients harboring ENL breakpoints between exon 6 and 7 (Fig. 1). These patients constitute ~5.3% of MLL-ENL patients (n=16/302) and fuse ENL to MLL starting at amino acid 371. In total, 15.9% of t(11;19) patients (n=48/302) express MLL-ENL fusion proteins that lack the full YEATS domain, consistent with observations from smaller cohorts (48, 49). What is the mechanism of transformation for an MLL-ENL fusion protein lacking the YEATS domain? Screening of genomic mutations in MLL-rearranged acute leukemia patients revealed secondary mutations in FLT3-ITD, KRAS/NRAS and others (1, 55). Cooperating mutations may play a more prominent role in MLL-ENL leukemias lacking the YEATS domain and downstream sequences. Further, an intrinsically disordered region (IDR; aa171–448) in ENL can initiate a liquid-liquid phase separation of pTEFb to induce transcriptional induction (56). Thus, it is noteworthy that our MLL-ENLΔYEATS constructs (aa372–559) remove the YEATS domain and part of the IDR (Fig. 2). It is possible that part of the leukemic phenotype associated with MLL-ENLΔYEATS (Fig. 2) results from phase separation defects due to partial deletion of the IDR. However, our *in vivo* experiments using single point mutations in the YEATS domain confirm that the YEATS epigenetic reader function is important for leukemogenesis. Further, our ChIP analysis suggests deletion of the YEATS domain affects fusion protein binding, whereas point mutations do not. This may point to impaired recruitment of co-activating proteins, however further experiments are needed.

Targeting the ENL YEATS domain has been established as a potential treatment for AML (27). Our data predicts targeting the ENL YEATS domain may be effective against MLL-ENL cells (Figs. 2, 7). We used the SGC-iMLLT molecule reported to bind specifically to the ENL/AF9 YEATS domain forming complementary pi-pi stacking interactions with residues F59 and Y78 (45). We observed varied sensitivity of murine and human AML cell lines to SGC-iMLLT treatment. It is noteworthy that cell lines harboring MLL-ENL fusion proteins containing the YEATS domain were amongst the most sensitive (Fig. 7). cell lines harboring MLL-ENL fusion proteins that contain the YEATS domain (HB1119 and murine MLL-ENL cells) displayed greater sensitivity compared to other AML cell lines (Fig. 7). We postulate SGC-iMLLT targets the YEATS domain of both MLL-ENL fusion proteins and endogenous ENL to inhibit cell growth. Indeed, differential transcriptional effects on MLL-ENL targets observed following genetic or pharmacological inhibition of the YEATS domain may result from inhibition of wild type ENL by SGC-iMLLT (Fig. S4, 7). A model of dual contribution of wild type ENL and MLL-ENL fusion proteins may contribute to transcription of pro-leukemic targets. Together, our study reveals the YEATS domain is

retained in the vast majority of t(11;19) MLL-ENL patients and plays a critical role during leukemogenesis that may be exploited therapeutically.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request and in the Gene Expression Omnibus (GEO) repository: GSE211523.

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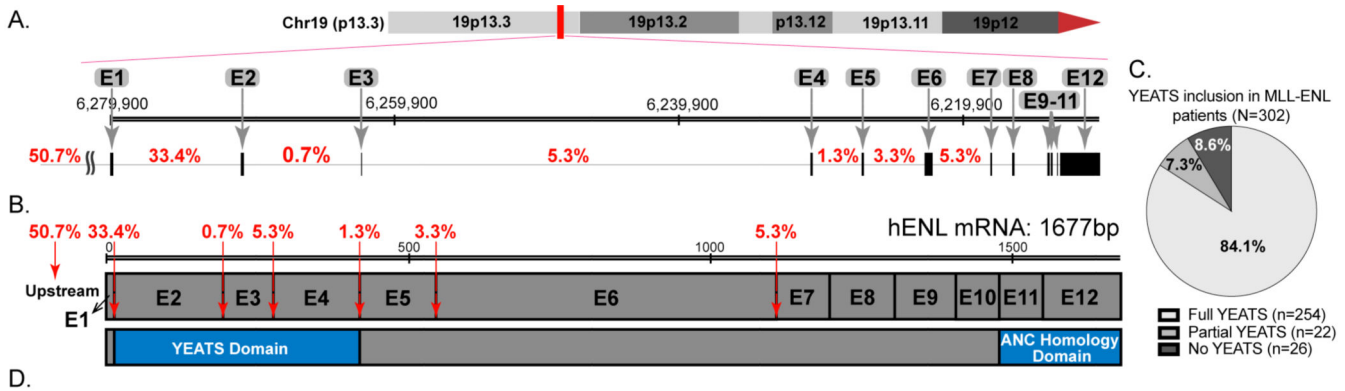

D.

	5′ Upstream	Intron 1	Intron 2	Intron 3	Intron 4	Intron 5	Intron 6	Intron 7	Intron 8	Intron 9	Intron 10	Intron 11	Total
Infant	96	44	1	9	3	3	4	0	0	0	0	0	160
Pediatric	33	30	1	4	1	4	5	0	0	0	0	0	78
Adult	24	27	0	3	0	3	1	0	0	0	0	0	58

E.

	5′ Upstream	Intron 1	Intron 2	Intron 3	Intron 4	Intron 5	Intron 6	Intron 7	Intron 8	Intron 9	Intron 10	Intron 11	Total
ALL	150	87	2	12	3	3	6	0	0	0	0	0	263
AML	1	13	0	4	1	7	10	0	0	0	0	0	36
MLL	2	1	0	0	0	0	0	0	0	0	0	0	3

Figure 1. ENL breakpoint locations in t(11;19) patients.

(A) Genomic location of the *ENL* gene and distribution of *ENL* breakpoints in t(11;19) (MLL-ENL) patients described previously (7). N=302. (B) The location of ENL breakpoints described in A is shown in relation to ENL mRNA and protein. The YEATS domain and AHD are indicated on the ENL protein schematic. (C) Pie chart summary of patient data shown in A showing percentage of t(11;19) patients harboring MLL-ENL fusion proteins containing: the full YEATS domain (n=254/302; 84.1%), partial YEATS domain inclusion (n=22/302; 7.3%), and no YEATS domain inclusion (n=26/302; 8.6%). (D and E) Summary table of ENL breakpoint data shown in A broken down by (D) infant, pediatric and adult patients or (E) leukemia subtype (AML=acute myeloid leukemia, ALL=acute lymphoid leukemia, MLL=mixed lineage leukemia).

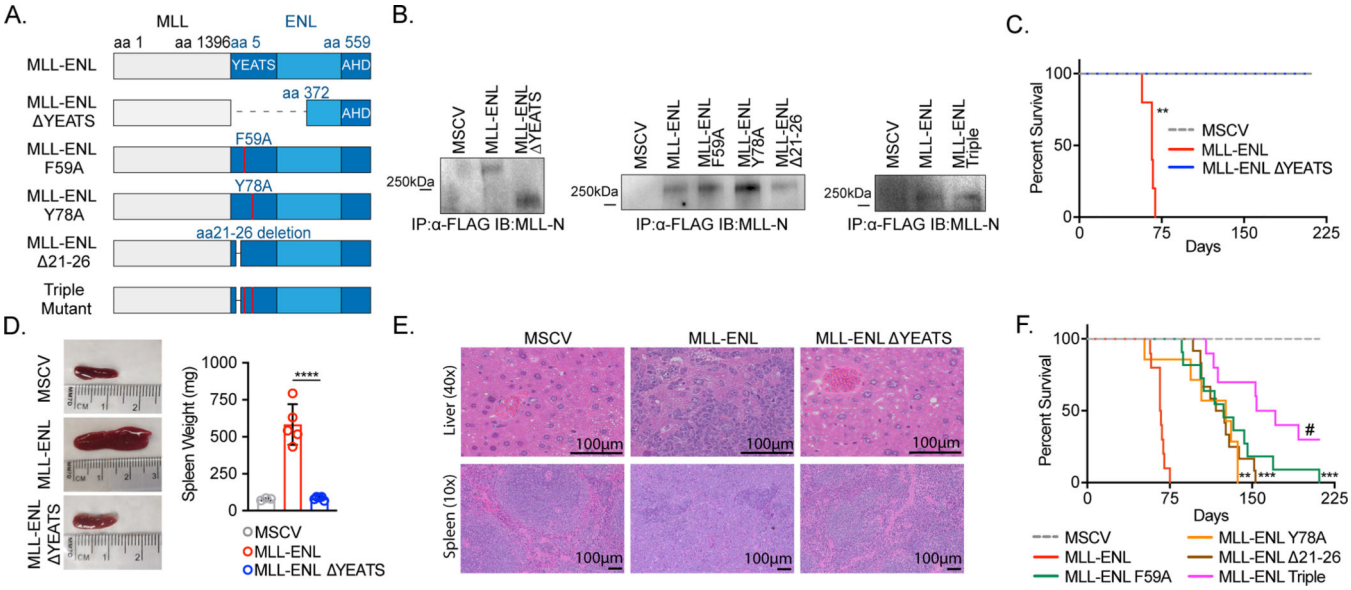

Figure 2. The ENL YEATS domain and downstream sequence is required for MLL-ENL mediated leukemogenesis.

(A) Schematic of MLL-ENL, MLL-ENLΔYEATS, and MLL-ENL YEATS mutants constructs used in this study. (B) IP-Western blots of MLL-ENL, MLL-ENLΔYEATS, MLL-ENL YEATS mutants demonstrate expression of the fusion proteins. (C) *In vivo* leukemogenesis assay performed by retroviral transduction of lin- mouse bone marrow cells from 5-FU treated C57/Bl6 mice with MSCV (n=3), MLL-ENL (n=5), or MLL-ENLΔYEATS (n=5). 80K cells were injected into lethally irradiated (950 rads) syngeneic recipients where untransduced cells served as support marrow. Statistical significance was calculated using the log-rank (Mantel-Cox) test (**: p=0.0018). (D) Spleen comparison of sacrificed animals from the MSCV (n=3), MLL-ENL (n=5) and MLL-ENLΔYEATS (n=5). (Left) Representative spleen images. (Right) Spleen weight comparison. Error bars represent SD. Statistical significance was calculated using unpaired student's t-test (****: p<0.0001). (E) Representative images of H&E stained liver and spleen of sacrificed animals from the MSCV, MLL-ENL and MLL-ENLΔYEATS groups. (F) Combination of two independent *in vivo* leukemogenesis assays performed as above with bone marrow cells retrovirally transduced with MSCV (n=6), MLL-ENL (n=10), MLL-ENLF59A (n=12), MLL-ENLY78A (n=7), MLL-ENLΔ21–26 (n=12) or MLL-ENL triple mutant (n=10). 80K cells were injected/mouse for th first experiment and 150K cells/mouse for the second experiment. Statistical test: log-rank (Mantel-Cox) (***: p<0.001; **: p<0.01). # = MLL-ENL triple vs MLL-ENLF59A: p=0.027; MLL-ENL triple vs MLL-ENLY78A: p=0.008 MLL-ENL triple vs MLL-ENLΔ21–26: p=0.002.

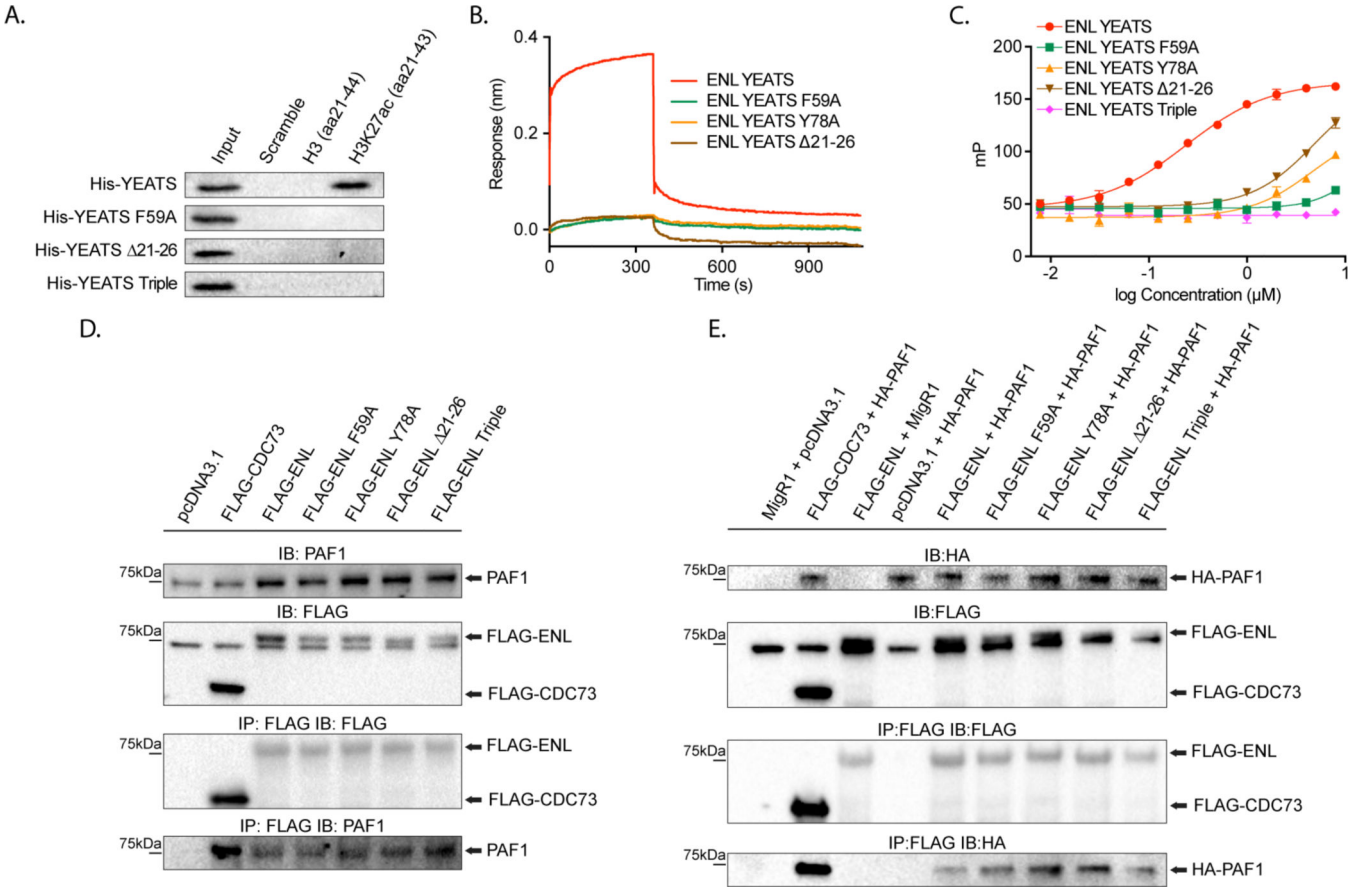

Figure 3. YEATS domain mutations impact H3Kac binding.

(A) *In vitro* binding assay of His-tagged ENL YEATS domain or mutants (F59A, Δ21–26, triple) with histone H3 peptide (aa21–44), H3K27ac peptide (aa21–43) or a scrambled peptide control. (B) Representative bio-layer interferometry (BLI) experiment of the wild type ENL YEATS domain or mutants (F59A, Y78A, and Δ21–26) with 50μM H3K27ac peptide. K_d for ENL YEATS is estimated to be 66.56 ± 7.07 μM (n=2 independent results). Binding data are obtained as the average of two or more independent experiments. (C) Fluorescence polarization experiment using fluorescently labelled SGC-iMLLT compound with the wild type ENL YEATS domain or YEATS mutants (F59A, Y78A, Δ21–26, triple). Means with SD values were plotted. Estimated K_d values: ENL YEATS: 0.26 ± 0.02 μM; ENL YEATS F59A: >8 μM; ENL YEATS Y78A: 8.82 ± 0.48 μM; ENL YEATS Δ21–26: 4.6 μM; ENL YEATS Triple: >8 μM. K_d values for ENL YEATS Y78A and Δ21–26 were calculated by constrained fitting using the Klotz plot (semi-log plot). (D) Representative co-immunoprecipitation experiment performed by immunoprecipitating FLAG-tagged ENL or ENL mutants from transiently transfected HEK293T cells and blotting for endogenous PAF1. (E) Representative co-immunoprecipitation experiment performed by immunoprecipitating FLAG-tagged ENL or ENL mutants from HEK293T transiently transfected with FLAG-ENL or ENL mutants and HA-PAF1. Precipitate was immunoblotted with either anti-HA or anti-FLAG.

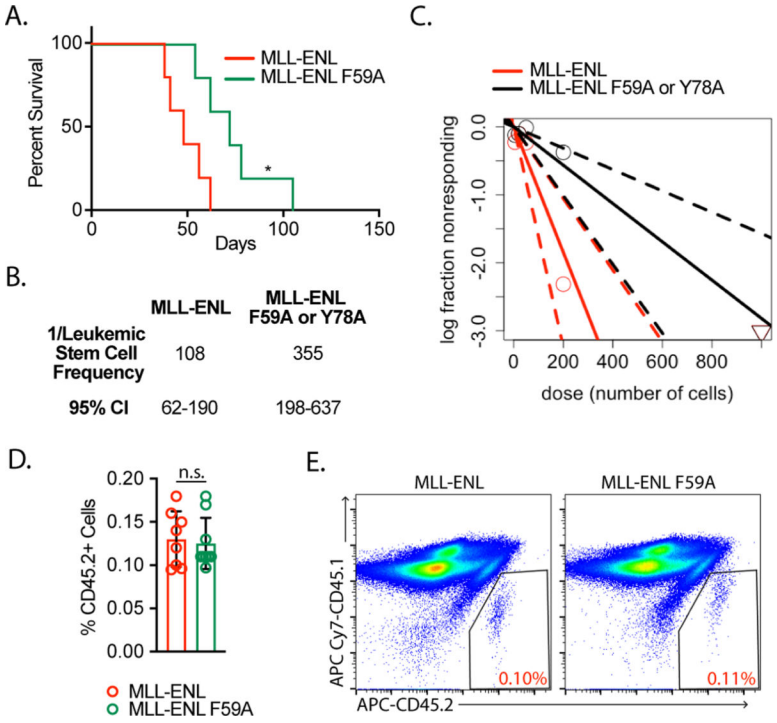

Figure 4. Disruption of the YEATS domain epigenetic reader function impacts MLL-ENL leukemic stem cell frequency.

(A) Secondary leukemogenesis assay comparing primary MLL-ENL (n=5) or MLL-ENLF59A (n=5) leukemias. 1000 primary leukemic cells were injected into sublethally (650 rads) irradiated syngeneic recipients without support bone marrow. Statistical test: log-rank (Mantel-Cox). (*: $p<0.05$) (B) Summary of the estimated leukemic stem cell frequencies with 95% confidence interval derived from extreme limiting dilution analysis of MLL-ENL leukemia compared to MLL-ENLF59A or Y78A leukemia. Analysis represents a combination of two independent experiments. In each experiment, a total of 1000, 200, 50, 20 or 5 primary mouse leukemic cells were injected into sublethally (650 rads) irradiated syngeneic recipients (n=5) without support marrow. (C) Log-fraction plot showing the leukemic stem cell frequencies in MLL-ENL and MLL-ENLF59A or Y78A leukemias according to the Extreme Limiting Dilution Analysis (ELDA). (D) Homing assay comparing the leukemic homing capacity in recipients (CD45.1+) injected with MLL-ENL (n=8) or MLL-ENLF59A (n=9) leukemias (CD45.2+). Figure shows mean with SD. Statistical test: unpaired student's t-test. n.s.: not significant. (E) Representative flow plot showing the gating for CD45.2+ leukemic cells in recipient mice injected with either MLL-ENL or MLL-ENLF59A leukemias.

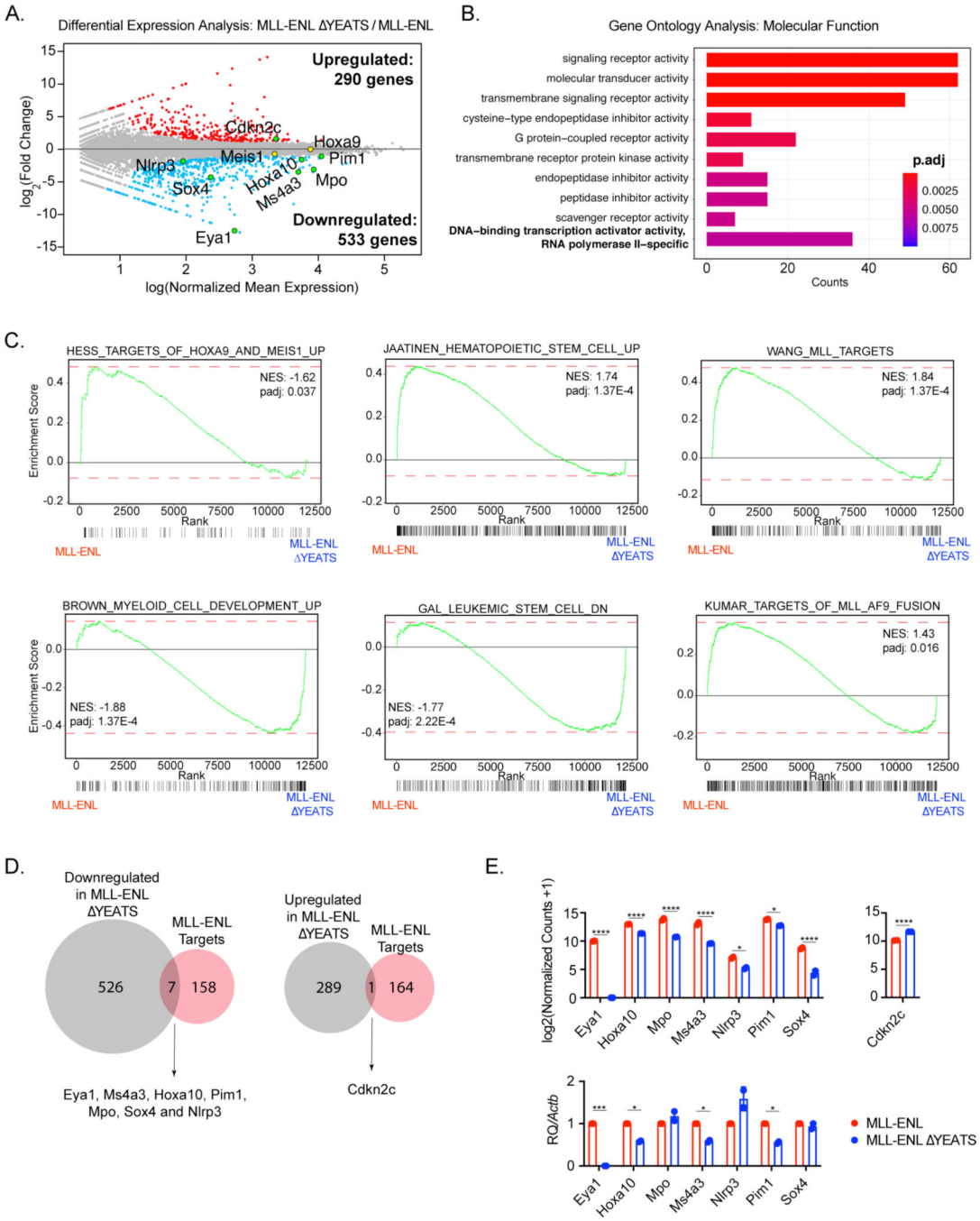

Figure 5. Transcriptomic changes associated with MLL-ENLΔYEATS cells.

(A) MA-plot showing significant differentially expressed (defined as 1.5-fold upregulated or downregulated) genes in MLL-ENLΔYEATS cells compared to MLL-ENL cells. Grey dots mark genes that are non-significant while red and light blue dots mark genes that are significantly up- or downregulated respectfully. *Hoxa9* and *Meis1* are highlighted in yellow. Green dots signify genes that are differentially expressed and targets of MLL-ENL (52). (B) Gene ontology analysis of the molecular function aspect using 1.5-fold differentially expressed genes. Figure captures top 10 GO terms with the lowest padj value (represented

with the color gradient bar). Count indicates number of differentially expressed genes mapped to the GO term. (C) Gene Set Enrichment Analysis of the C2: CGP (chemical and genomic perturbations) curation from MSigDB using differentially expressed genes. Selected pathways were highlighted in the figure shown with normalized enrichment score (NES) and adjusted p-values. (D) Venn diagram of 1.5-fold differentially expressed genes (either up- or downregulated) with targets of MLL-ENL targets (52). (E) (Top) Relative expression (presented as log2(Normalized Counts + 1)) values of differentially expressed MLL-ENL targets. Normalized Counts and statistics were obtained from DESeq2 results. Figure shows mean values with SD. ($p < 0.05$ *; $p < 0.0001$ ****). (Bottom) RT-qPCR data (shown in $\Delta\Delta$Ct) of two biological replicates probing for downregulated MLL-ENL targets identified by RNAseq. Figure shows mean values with SD. Statistics: student's unpaired T-test on ΔCt values. (*:$p < 0.05$; ***:$p < 0.001$)

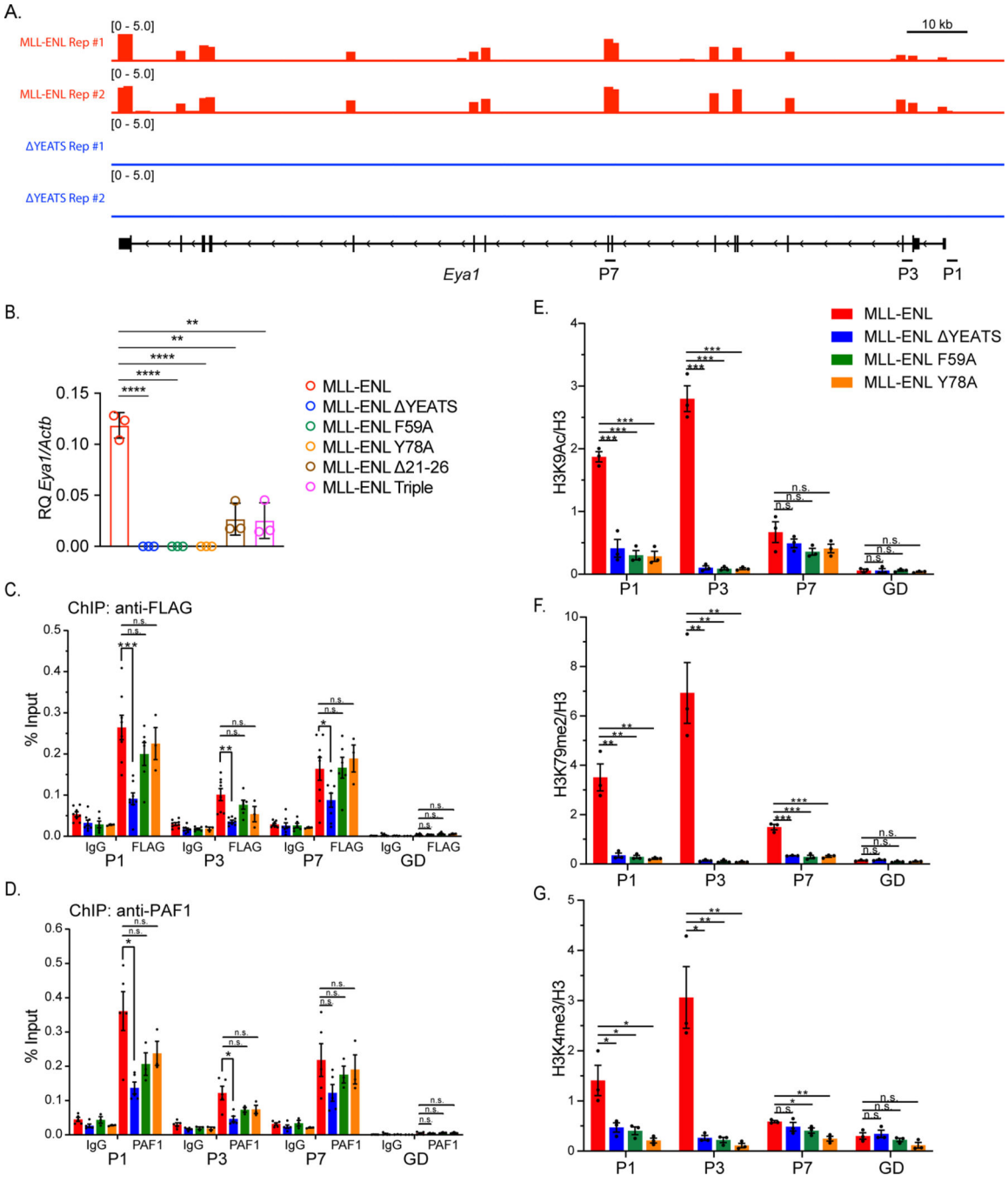

Figure 6. YEATS domain of ENL is required for epigenetic regulation of Eya1.

(A) RNA-seq genomic track showing expression of *Eya1* in MLL-ENL or MLL-ENLΔYEATS cell lines. (B) RT-qPCR analysis showing expression of *Eya1* in MLL-ENL or MLL-ENL mutant cell lines. RT-qPCR analysis is represented as mean with SD. Statistical test: unpaired student's t-test. (****: p<0.0001; **: p<0.01) (C-G) ChIP-qPCR analysis of anti-FLAG (C), anti-PAF1 (D), anti-H3K9Ac (E), anti-H3K79me2 (F) and anti-H3K4me3 (G) on *Eya1* locus in MLL-ENL, MLL-ENLΔYEATS, MLL-ENL F59A or MLL-ENL Y78A mutant cell lines. Anti-FLAG and anti-PAF1 ChIP are represented as percent input

and histone modification ChIP assays are represented as normalized value to histone H3 ChIP. qPCR amplicons are designated as P1, P3, and P7 and their locations are represented in (A) as black bars. GD represents an amplicon at a non-specific gene desert region. ChIP-qPCR analysis is represented as mean with SEM (C-G). All experiments represent at least 3 independent biological replicates. Unpaired *t-test* was performed for (E-G) and Welch's t-test was performed for (C-D). (*:$p < 0.05$; **:$p < 0.01$; ***:$p < 0.001$; n.s: not significant)

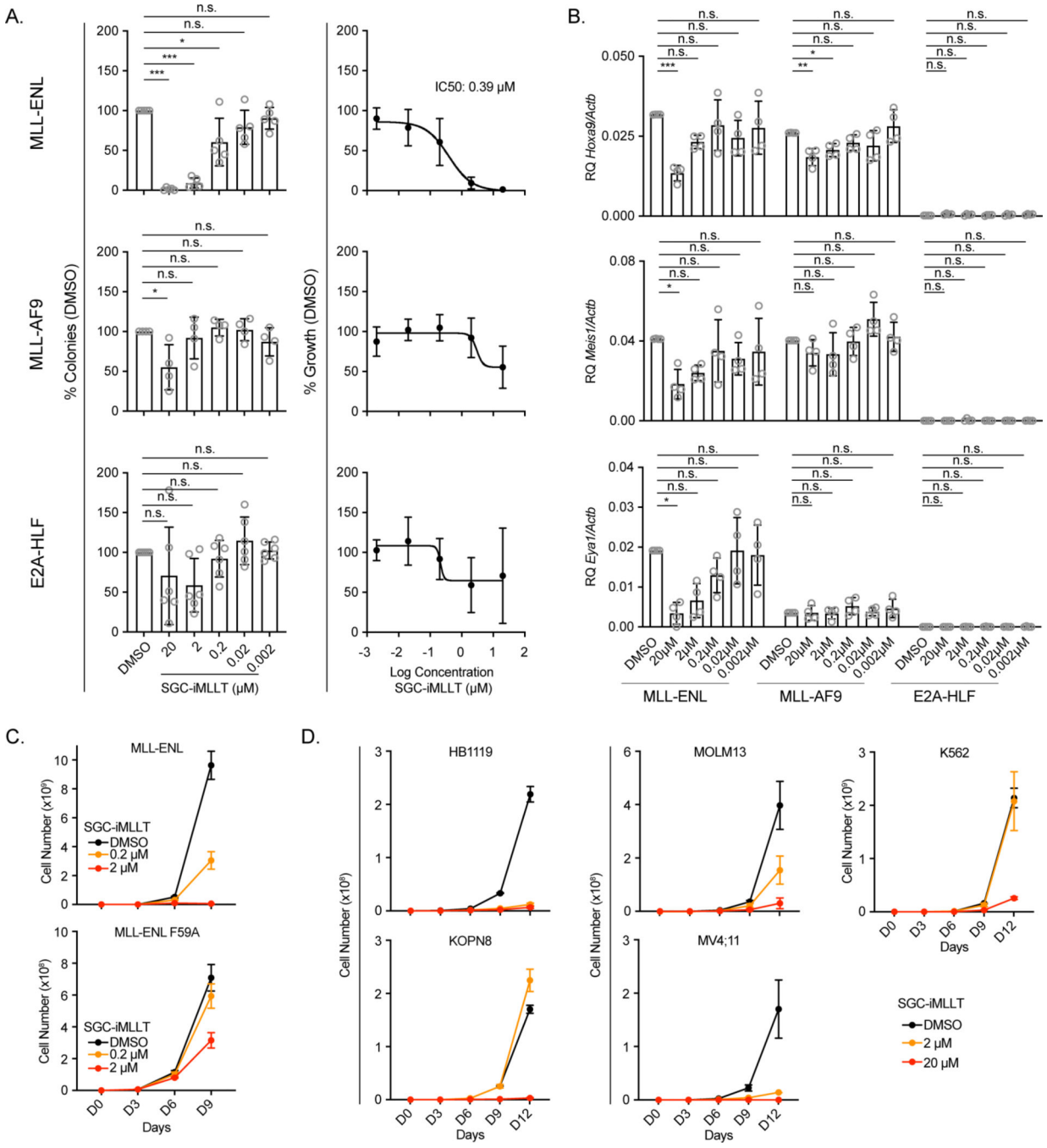

Figure 7. MLL-ENL cells display increased sensitivity to SGC-iMLLT.

(A) Left: results from second round colony formation of simultaneously generated murine MLL-ENL, MLL-AF9 and E2A-HLF cells treated with SGC-iMLLT. Results are displayed normalized to DMSO. Statistical test: one-way ANOVA test comparing treatments within each cell line (colony counts were used for significance calculation). Figure represents mean values with SD. (*: $p<0.05$; ***: $p<0.001$; n.s. not significant) Right: SGC-iMLLT dose-response curve of MLL-ENL, MLL-AF9 and E2A-HLF cell lines normalized to DMSO. Means with SD values were plotted. (B) RT-qPCR analysis of *Hoxa9*, *Meis1,* and

Eya1 expression in MLL-ENL, MLL-AF9 and E2A-HLF colonies harvested 6 days post SGC-iMLLT treatment. Figure represents mean values with SD. Data presented as ΔΔCt value (normalized to DMSO-treated samples) multiplied by a cell line-specific factor to compare expression across different cell lines. Cell line specific factors equal the average transformed ΔCt value from four biological replicates treated with DMSO. Statistical test: one way ANOVA test of ΔCt values comparing treatments within each cell line. (*: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; n.s. not significant). (C) Cell culture proliferation assay of MLL-ENL or MLL-ENLF59A cell lines treated with SGC-iMLLT. 20,000 cells were seeded on D0 and counted on D3, D6, and D9. Means with SD values were plotted. (D) Cell culture proliferation assay of HB1119, KOPN8, MOLM13, MV4;11, and K562 cell lines treated with SGC-iMLLT. 20,000 cells were seeded on D0 and counted on D3, D6, D9 and D12. Means with SD values were plotted.

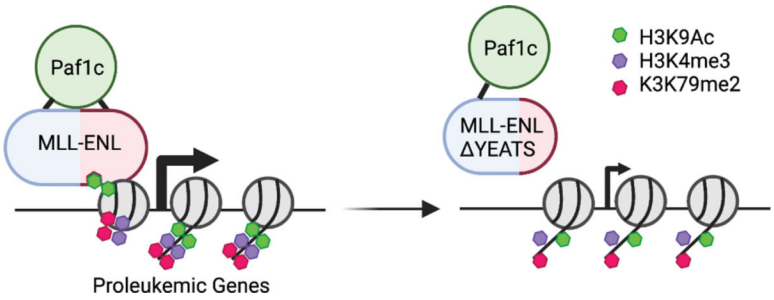

Figure 8. The YEATS domain impacts MLL-ENL fusion protein localization.

The model represents cooperative binding of MLL-ENL fusion proteins with the PAF1c at pro-leukemic targets. Loss of the YEATS domain diminishes binding of both the MLL-ENL fusion protein and the PAF1c.