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A coordinated function of lncRNA *HOTTIP* and miRNA-196b underpinning leukemogenesis by targeting FAS signaling

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Abstract

MicroRNAs (miRNAs) may modulate more than 60% of human coding genes and act as negative regulators, while long non-coding RNAs (lncRNAs) regulate gene expression on multiple levels by interacting with chromatin, functional proteins, and RNAs such as mRNAs and microRNAs. However, the crosstalk between *HOTTIP* lncRNA and miRNAs in leukemogenesis remains elusive. Using combined integrated analyses of global miRNA expression profiling and state-of-the-art genomic analyses of chromatin such as ChIRP-seq (*HOTTIP* binding in genome-wide), ChIP-seq, and ATAC-seq, we found that some miRNA genes are directly controlled by *HOTTIP*. Specifically, the HOX cluster miRNAs (miR-196a, miR-196b, miR-10a and miR-10b), located *cis* & *trans*, were most dramatically regulated and significantly decreased in *HOTTIP*^{-/-} AML cells. *HOTTIP* bound to the miR-196b promoter, and *HOTTIP* deletion reduced chromatin accessibility

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Author Contributions

Conceptualization, study design and direction, performed overall experiments, data analysis, result interpretation, writing original draft and editing of the manuscript, and project administration, A.P.S., H.L., M.M., M.E., K.H., S.H.; data curation H.L., M.M., M.E. K.H., and A.S.; manuscript review, editing, and data visualization, H.L., M.E., S.H.; project administration, S.H. All authors reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

and enrichment of active histone modifications at HOX cluster associated miRNAs in AML cells, while reactivation of *HOTTIP* restored miR gene expression and chromatin accessibility in the CTCF-boundary-attenuated AML cells. Inactivation of *HOTTIP* or miR-196b promotes apoptosis by altering the chromatin signature at the *FAS* promoter and increasing *FAS* expression. Transplantation of miR-196b knockdown MOLM13 cells in NSG mice increased overall survival of mice compared to wild-type cells transplanted into mice. Thus, *HOTTIP* remodels the chromatin architecture around miRNAs to promote their transcription, and consequently represses tumor suppressors and promotes leukemogenesis.

Keywords

Long non-coding RNA; micro-RNA; Epigenetics; FAS signaling; Leukemia

Introduction

Non-coding RNAs are emerging as important regulators of gene expression in multiple cellular processes, especially in cancer [1]. Long non-coding RNAs (lncRNAs) are involved in regulating chromatin dynamics, gene expression, cell growth, differentiation, and development [2]. Overexpression of *HOXA9* is a dominant factor driving certain subtypes of human acute myeloid leukemia (AML) [3]. Moreover, the aberrant activation of posterior HOXA gene, *HOXA9*, and its cofactor, *MEIS1*, following a variety of genetic alterations, including MLL-translocations, NUP98-fusion, CDX dysregulation, and monocytic leukemia zinc-finger fusion, was associated with poor prognosis and treatment response [4]. The regulatory mechanisms controlling expression of posterior HOXA genes remain obscure. Importantly, a treasure trove of transcribed sense and antisense noncoding RNAs exist at HOX gene locus, including *Hotair*, *Hottip*, *Hobbit*, *Halr1*, *Hotdog*, miR10, and miR196 [5]. lncRNAs regulate transcription through recruiting histone modifiers and chromatin remodeling factors that play active roles in various aspects of development and disease states [6–11]. We showed that aberrantly activated *HOTTIP* alters HOXA-driven topologically associated domains (TADs) and gene expression [12]. The lncRNA *HOTTIP*, located at the posterior end of HOXA gene cluster, acts as a scaffold to recruit the WDR5-MLL-complex to the promoters of posterior HOXA genes and positively regulates their expression in normal hematopoiesis and AML leukemogenesis [6, 12]. In contrast, loss of *HOTTIP* strongly inhibits posterior (*HOXA9-HOXA13*) compared to anterior HOXA gene expression (e.g., *HOXA1-HOXA7*) [12]. Moreover, *Hottip* aberration in mice leads to AML-like disease by altering HSC self-renewal and homeotic/hematopoietic gene-associated chromatin signatures and transcription programs [12]. Thus, *HOTTIP* functions as an oncogenic driver gene perturbing HSC function by reprogramming leukemia-associated chromatin and gene transcription.

HOTTIP provides a basis for transcriptional activation and three-dimensional (3D) chromatin organization in the 5' HOXA gene locus by acting downstream of the *CBS7/9* boundary (CTCF binding site located between *HOXA7* and *HOXA9*) [12]. Depleting *HOTTIP* reduces active histone marks (H3K4me3 and H3K79me2) and enhances repressive histone marks (H3K27me3) resulting in a switch from an active to a repressive chromatin

state in the promoter region of the 5' HOXA genes in MOLM13 cells [12]. Genome-wide binding analysis of *HOTTIP* lncRNA using chromatin isolation by RNA purification combining deep sequencing (ChIRP-seq) revealed that *HOTTIP* directly regulates its target genes through *cis* and *trans* binding at *HOXA9–13*, *RUNX1* and *MEIS1* genes. Consequently, *HOTTIP* also binds in *cis* and *trans* regulatory patterns at non-coding regions and certain annotated genes involved in chromatin organization, hematopoiesis, myeloid cell differentiation, cell-cycle progression, and JAK-STAT and WNT signaling pathways. These findings suggested that *HOTTIP* lncRNA might control gene expression by interacting with and regulating non-coding regulatory elements such as microRNAs (miRNAs) in *cis* and *trans* [12].

Growing evidence indicates that non-coding RNAs, particularly lncRNAs and miRNAs, regulate one another and cooperate to influence the levels of target mRNAs in a cell-type specific manner [13]. LncRNAs process, interact with, and regulate miRNAs at both transcriptional and post-transcriptional levels [14]. LncRNAs can function as miRNA sponges, acting as decoys to impair the functional interaction of a miRNA and its target mRNA, thereby preventing suppression of gene expression [15]. Additionally, lncRNAs can be precursors of miRNAs and regulate miRNA biogenesis at different points. Primary miRNAs (pri-miRNAs) are transcribed in the genome (i) either within the body of another gene and often their expression is linked to the expression of the parent transcript, or (ii) from independent miRNA genes, similar to mRNA, where transcription is primarily controlled by RNA polymerase II-driven promoters [16]. However, the molecular mechanisms, particularly those mediated by lncRNA *HOTTIP*, regulating miRNAs transcription remains elusive.

MicroRNAs (miRNAs) are class of 20–22 nucleotide non-coding epigenetic controllers that negatively regulate gene expression, either by repressing translation or by promoting mRNA decay, via binding at complementary seed sequences mainly located at the mRNA 3' UTR regions [17]. Growing evidence supports that miRNA might have essential roles in hematopoiesis and leukemogenesis [18]. Widespread dysregulation of certain miRNAs is associated with hematological malignancies, including acute myeloid leukemia (AML) [19], and controls leukemic and tumor suppressor gene expression [18]. Importantly, miR-10 family members (miR-10a and miR-10b) are positioned between the group 4 and 5 paralogous genes in the *HOXB* and *HOXD* clusters. This family regulates HOX genes both in *cis* and *trans*, and major signaling pathways (WNT, FGFs and NOTCH) are key non-HOX targets of the miR-10 family [20–23]. Because HOX genes also regulate these signaling pathways [24–27], the miR-10 family have feedforward regulatory inputs into HOX gene regulatory networks [5].

Three miR-196 family members, miR-196b, miR-196a-1, and miR-196a-2, are present in both the mouse and human *HOXA*, *HOXB*, and *HOXC* clusters. *HOXA9* and its neighboring non-coding RNAs miR-196b and *HOTTIP* are aberrantly activated in AML and their gene expression correlates [12, 28]. miR-196b, which is located adjacent to *HOXA9*, targets *HOXA9* and its cofactor *MEIS1* [28]. miR-196b also targets proapoptotic factor *FAS* [29], suggesting a double-edged sword function that could simultaneously repress both the oncogenic and tumor suppressor target genes. This evidence implies that integration

of miRNAs in HOX clusters provides another layer of gene regulation that plays a role in development and disease such as leukemia. Recent studies have shown the importance of miR-196b in AML [28]; however, the mechanism of its transcriptional regulation remains unknown in AML. Thus, in this study we characterize the mechanisms by which *HOTTIP* controls the expression and function of *HOXA9* and *FAS* through regulating the transcription of miR-196b in human AML cells.

Results

HOTTIP lncRNA differentially regulates microRNAs in AML

The aberrant activation and function of both *HOTTIP* and miR-196b establish their role in AML. However, key information about the functional and regulatory intervened networks of *HOTTIP*-miRNA-196b-mRNA in AML remains unknown. To identify miRNAs that are directly targeted by *HOTTIP*, small-RNA-sequencing (smRNA-seq) was performed in wild-type (WT) and *HOTTIP*^{-/-} MOLM13 AML cells (Fig. 1A). Differential expression analysis shows that a total 569 miRNAs changed more than 2-fold in *HOTTIP*^{-/-} cells compared to WT cells, including 333 down-regulated miRNAs and 236 upregulated miRNAs (Fig. 1B, C). Gene ontology (GO) analysis revealed that *HOTTIP*-regulated miRNAs were involved in many biological processes including the metabolic process, regulation of gene expression, transcription, and RNA processing (Fig. 1D). Collectively, these altered miRNAs in *HOTTIP*^{-/-} cells play vital roles in molecular and cellular processes, including hematopoiesis and leukemogenesis, suggesting that *HOTTIP* controls AML pathogenesis by regulating miRNAs.

Next, we sought to determine whether *HOTTIP*-regulated miRNAs control the expression of *HOTTIP*-regulated mRNAs. *In silico* analysis revealed that *HOTTIP*-regulated miRNAs are predicted to directly target 4704 mRNAs. Furthermore, we compared these with 1219 mRNAs that we previously demonstrated are transcriptionally regulated by *HOTTIP* [12]. Strikingly, 357 genes are co-regulated by both *HOTTIP*-associated miRNAs and *HOTTIP* itself in AML, including hematopoietic and leukemic signature genes like HOXA factors (e.g., *HOXA9*, *HOXA13*), *MYC*, *ETS1*, *VEGFA*, *ZEB2*, *TEAD4*, *WNT5a*, *WNT2* and *FAS* (Fig. 1E, F).

Depletion of *HOTTIP* inhibits the HOX clusters' miRNAs in *cis* and *trans*

Within the HOX cluster several non-coding transcripts, including miRNAs, are transcribed. Six miRNAs are transcribed within the intergenic regions of the HOX clusters (Fig. 2A). Loss of *HOTTIP* impaired posterior HOXA gene expression (e. g. *HOX13-HOXA9*) but did not affect anterior *HOXA1-7* genes, HOXB, HOXC, or HOXD genes [12]. Through analyzing the expression profile correlation of *HOTTIP*, miR-196b, HOXA genes and their co factors in available gene expression datasets of AML samples [28], we found that a strong elevation and positive correlation of expression of miR-196b with *HOTTIP* and a group of *HOXA* genes and their co factors *PBX3* and *MES11* (Fig. 2B). Furthermore, we analyzed TCGA-LAML database to examine expression pattern and overall survival. TCGA Kaplan Meier survival plots show that the expression patterns of *HOXA9*, *HOXA10*, *PBX3*, *MEIS1*, *HOTTIP*, miR-196b and miR-10a correlate and exhibit prognostic significance, and

overall survival was significantly longer in patients having AML with low expression (blue color line) than those having AML with higher expression (red color line) (Fig. 2C). Thus, *HOTTIP* and miR-196b play an important role in the pathogenesis and prognosis of AML patients, while miR-939 expression exhibits no change in overall survival (Fig. 2C) and its expression level remains unchanged upon *HOTTIP* deletion (Fig. 2F). Then we asked whether depletion of *HOTTIP* affects mRNA, and miRNAs that are transcribed in either *cis* or *trans* in the intergenic region of the HOX cluster genes. Disruption of *HOTTIP* inhibits posterior HOXA genes (Fig. 2D) and several HOX cluster-related miRNAs, including miR-196b-5p, miR-196a-5p, miR-10b-5p, and miR-10a-5p/3p (Fig. 2E, F), suggesting these miRNAs are positively regulated by *HOTTIP* in AML. Interestingly, while HOX cluster-associated miRNAs were altered, several HOX genes targeted by these miRNAs remained unaltered in *HOTTIP*^{-/-} cells (Fig. 1E). We performed correlation analysis of *HOXA9*, its cofactor *MEIS1*, miR-196b and *HOTTIP* in MOLM13 and KASUMI cells. Our data analysis shows posterior *HOXA* genes are aberrantly overexpressed in MLL-associated MOLM13 cells compared to KASUMI-1/t (8;21) cells (Fig. 2G). Similarly, pro-apoptotic genes, including *CASP3*, and *CASP9*, were also downregulated in MOLM13 (Fig. 2G). Additionally, HOX cluster miRNAs miR-196a, miR-196b, miR-10a, and miR-10b were enriched in *MLL-AF9* MOLM13 cells compared to non-MLL-rearranged leukemia cells such as KASUMI-1/t (8;21) and correlates with expression of posterior *HOXA* genes (Fig. 2G). In contrast, *MEIS1*, *FAS* and *HOTTIP* expression remained comparable in both cell lines (Fig. 2G). However, the question remains whether miR-196b targets pro-leukemic gene *HOXA9* in non-MLL-rearranged leukemia cells. The co-expression of *HOTTIP* and miR-196b and their inverse correlation with pro-apoptotic genes suggests that *HOTTIP* and miR-196b may co-target these genes in *MLL-AF9* rearranged MOLM13 AML cells. Likewise, perhaps *HOTTIP* counteracts miR196b mediated repression of *HOXA9* in AML.

***HOTTIP* established chromatin signature on HOX clusters related miRNAs loci**

To further define the mechanism by which *HOTTIP* regulates miRNA expression, we employed ChIRP-sequencing (chromatin isolation by RNA purification) to identify *HOTTIP* binding genome-wide [12]. Our *HOTTIP* ChIRP-seq data indicated that *HOTTIP* mainly binds to non-coding regions, including promoter and intergenic regions [12]. The question then arises whether *HOTTIP* binds to regulatory loci of miRNA to induce the differential expression we observed in *HOTTIP*^{-/-} compared to WT MOLM13 cells. Our data suggests that loss of *HOTTIP* greatly reduces its binding at the upstream promoter region of miR-196b compared to WT MOLM13 cells (Fig. 3A), which is correlated with attenuated expression of miR-196b upon *HOTTIP* depletion (Fig. 2E). Furthermore, transcription factor binding motif analysis revealed that the *HOTTIP* bound genomic locus at miR-196b is occupied by transcription factors, CTCF, GATA-2, *MYC* and *CEBPB*, as well as many other chromatin remodeling factors (Supplementary Fig. 1).

Next, we asked whether *HOTTIP* controls the chromatin signature at its targeted miRNA loci, using ChIP-seq (chromatin immunoprecipitation sequencing) for histone modifications (H3K4me3, H3K79me2; active; and H3K27me3; repressive) and ATAC-seq (assay for transposase-accessible chromatin using sequencing) to compare chromatin signatures between WT and *HOTTIP*^{-/-} MOLM13 cells [12]. Depletion of *HOTTIP* resulted in

marked decreases in H3K4me3 and H3K79me2 enrichment, while H3K27me3 levels were elevated at the genomic loci of all four HOX cluster miRNAs, including miR-10a, miR-10b, miR-196a-1, miR-196a-2, and miR-196b (Fig. 3B–F). The changes in histone marks (Fig. 3B–F) coincide with transcriptional changes (Fig. 2E–F) and *HOTTIP* binding pattern alteration (Fig. 3A). Concomitantly with decreased *HOTTIP* binding and active histone marks in *HOTTIP*^{-/-} cells, chromatin accessibility at regulatory loci of these four HOX cluster miRNAs was also reduced (Fig. 3G–K). Therefore, *HOTTIP* governs its target genes by regulating the chromatin signature of specific miRNAs in the *MLL1*⁺ AML.

***HOTTIP* reactivation in *CBS7/9*^{+/-} AML cells rescued the miRNA expression coupled with restored chromatin signature**

We previously demonstrated that *HOTTIP* coordinates with its upstream regulatory element the CTCF binding site located between *HOXA7* and *HOXA9* (*CBS7/9*) to activate posterior HOXA genes and other hematopoietic oncogenes [12, 30]. Reactivation of endogenous *HOTTIP* expression using sgRNA targeted dCAS9-VP160 mediated promoter activation in the *CBS7/9*^{+/-} MOLM13 cells (*CBS7/9*^{+/-}-*HOTTIP*-VP160) efficiently restored posterior HOX gene (*HOXA9*-*HOXA13*) expression. These findings led us to test whether *HOTTIP* reactivation in *CBS7/9*^{+/-} also affects miRNA expression and alters chromatin dynamics at the miRNA loci. Indeed, levels of all HOX cluster miRNAs were significantly repressed in *CBS7/9*^{+/-} MOLM13 cells and completely restored in *CBS7/9*^{+/-}-*HOTTIP*-VP160 (*CBS-HT*-VP) cells with reactivated *HOTTIP* (Fig. 4A).

Next, we carried out ATAC-seq data analysis through comparing WT, *CBS7/9*^{+/-}, and *HOTTIP*-reactivated *CBS7/9*^{+/-} MOLM13 cells. Repression of *HOTTIP* in *CBS7/9*^{+/-} cell reduced chromatin accessibility at the HOX cluster-associated miRNA loci, while accessibility was largely restored in *HOTTIP*-activated *CBS7/9*^{+/-}-HT-VP160 (*CBS-HT*-VP) MOLM13 AML cells (Fig. 4B). Hence, *HOTTIP* function is critical for regulating the chromatin accessibility and expression levels of these four HOX locus miRNAs.

miR-196b simultaneously targets oncogenes and tumor suppressors to maintain MLL-AF9 AML

miR-196b represses a subset of targets with tumor suppressor activity *in vivo* and is selectively enriched by cooperation with MLL-AF9 to promote leukemogenesis [28]. Surprisingly, miR-196b also targets *HOXA9* and *MEIS1* that play essential oncogenic roles [28]. Because *HOTTIP* positively regulates both miR-196b and its target *HOXA9* and *MEIS1* [12], we investigated the function of miR-196b in leukemogenesis. Expression of miR-196b was strongly suppressed in MOLM13 cells treated with LNA196b compared to NTC (non-targeting control) (Supplementary Fig. 2A). Although, MOLM13 cells have high levels of both miR-196b and *HOXA9* expression, we assessed the regulatory effects of miR-196b on *HOXA9* and *MEIS1* in MOLM13 cells. Both *HOXA9* and *MEIS1* were significantly elevated upon miR-196b inhibition (Fig. 5A). Reduction of miR-196b also resulted in increased expression of *HOXA13* in MOLM13 cells (Fig. 5A). This suggests that the repressive function of miR-196b might fine tune expression of *HOTTIP* activated HOX genes in acute myeloid leukemia.

To further understand the pro-oncogenic role of miR-196b, we investigated the expression of tumor suppressor *FAS*, which is a verified target of miR-196b in AML subtypes and colon cancer cells [28, 29]. Expression of *FAS* and its downstream genes *Caspase3* (*CASP3*) and *Caspase9* (*CASP9*) was significantly elevated in cells treated with LNA196b compared to NTC (Fig. 5A). However, growth competition assay shows no difference in the proliferation rate of cells treated with LNA196b compared to NTC (Supplementary Fig. 2B).

Next, we assessed whether overexpression of miR-196b simultaneously represses the expression of both oncogenes and tumor suppressors. Forced expression of miR-196b significantly represses *HOXA9*, *MEIS1*, *FAS*, *CASP3* and *CASP9* in MOLM13 cells (Fig. 5B). To reveal the mechanism underlying the oncogenic role of miR-196b, we analyzed the apoptosis rate in MOLM13 cells treated with either LNA196b or NTC. Repression of miR-196b manifested a higher rate of apoptosis compared to NTC-treated MOLM13 cells (Fig. 5C). These results indicate that miR-196b promotes leukemogenesis by down-regulating *FAS*, and its downstream genes, thus suppressing apoptosis in AML.

To further cement the oncogenic role of miR-196b, we carried out functional knockdown of miR-196b in another AML subtype with high expression of *HOTTIP*, OCI-AML3 cell. Expression level of miRs were strongly suppressed in cells introduced with LNA196 (Fig. 5D, E). Inhibition of miR-196a/b resulted significant increase in apoptotic cell death (Fig. 5F, Supplementary Fig. 2C) and enhanced expression level of proapoptotic genes (Fig. 5G, H, I). Together, these results further suggested that miR-196b functions as an oncogene to suppress proapoptotic genes in AML subtypes.

***HOTTIP* inhibits *FAS* expression to maintain MLL-AF9 leukemia**

We previously reported that exogenous expression of *HOTTIP* aberrantly elevated *HOXA9*-*HOXA13* genes, resulting in impaired hematopoietic stem cell (HSC) function and an increased leukemia stem cell (LSC) population *in vivo* [12]. Thus, we intend to define the mechanisms by which *HOTTIP* might control the LSC pool. Since *HOTTIP* regulates the chromatin signature and expression of miR-196b, which targets both oncogenes and pro-apoptotic genes (e.g., *FAS*). We investigated whether *HOTTIP* also targets *FAS* in AML. The mRNA level of *FAS* and its downstream pathway genes (e.g., *CASP3*, *CASP8*, and *CASP9*) significantly increased in *HOTTIP*^{-/-} relative to WT MOLM13 cells (Fig. 6A). Further, we found that expression of the pro-apoptotic genes was enhanced in *CBS7/9*^{+/-} cells in which *HOTTIP* expression is significantly repressed (Fig. 6B). Intriguingly, when comparing *CBS7/9*^{+/-} cells to WT cells (Fig. 6B), mRNA levels of *CASP3* and *CASP9* were significantly elevated in *CBS7/9*^{+/-} cells while *CASP8* was unaffected. We next tested whether CRISPR-mediated endogenous gene activation of *HOTTIP* in these cells represses the pro-apoptotic genes. Reactivation of *HOTTIP* in the *CBS7/9*^{+/-} cells strongly repressed *CASP3* and *CASP9* while the *CASP8* level remained unchanged in MOLM13 AML cells (Fig. 6B). Next, we carried out western blot analysis using WT, *HOTTIP*^{-/-}, *CBS7/9*^{+/-}, and the *HOTTIP*-activated *CBS7/9*^{+/-} MOLM13 cells. Cleaved CASP3 protein level was markedly increased in *HOTTIP*^{-/-} and *CBS7/9*^{+/-} compared to WT cells, whereas the change in cleaved CASP3 protein level was undetectable upon *HOTTIP* reactivation, which closely resembles WT MOLM13 cells (Fig. 6C). Then we carried out functional deletion of

HOTTIP in another AML subtype OCI-AML3 cells and examined the effects of *HOTTIP*^{-/-} on gene expression and apoptotic cell death. mRNA expression analysis in OCI-AML3 cells showed that inhibition of *HOTTIP* induced proapoptotic genes and reduced oncogenic posterior HOXA genes (Fig. 6D). Subsequently protein analysis by western blot shows the increased level of CASP8 in *HOTTIP* knockout cells compared to WT cells (Fig. 6E). Flow cytometry analysis clearly showed an increase in apoptotic cell death in *HOTTIP*^{-/-} cells compared to WT cells (Fig. 6F, Supplementary Fig. 3). Additionally, the TCGA-LAML Kaplan Meier plot shows increased expression of *CASP3* positively correlated with overall survival (Fig. 6G). Thus, *HOTTIP* directly controls AML cell survival and apoptosis by regulating the FAS-Caspase axis.

To evaluate how *HOTTIP* directly regulates *FAS* expression, we investigated *HOTTIP* binding and the chromatin signature at the promoter of *FAS* in WT, *HOTTIP*^{-/-}, *CBS7/9*^{+/-}, and *CBS7/9*^{+/-}-HT-VP160 MOLM13 cell lines. Consistently, relative enrichment of active histone modifications (H3K4me3 and H3K79me2) was increased, whereas repressive histone modification (H3K27me3) was decreased in *HOTTIP*^{-/-} compared to WT cells (Fig. 6H). Additionally, chromatin accessibility, as determined by ATAC-seq, was increased at the *HOTTIP* binding site on *FAS* in *HOTTIP*^{-/-} and *CBS7/9*^{+/-} cells, whereas reactivation of *HOTTIP* reduced accessibility, resembling WT MOLM13 cells (Fig. 6I, J). This suggests that *HOTTIP* maintains a repressive chromatin signature at the promoters of tumor suppressor genes to promote leukemogenesis.

miR-196b repression delays leukemogenesis in MOLM13 cell transplantation

miR-196b functions downstream of *HOTTIP*, and together they coordinate to repress the *FAS* tumor suppressor in MOLM13 cells. We previously showed that transplantation of *HOTTIP*^{-/-} MOLM13 cells into irradiated NSG mice delayed leukemogenesis compared to WT MOLM13 cells [12]. To further assess the function of miR-196b downstream of *HOTTIP* in leukemogenesis *in vivo*, we knocked down miR-196b in MOLM13-YFP-luciferase cells and then transplanted them into immunodeficient NOD-scid IL2r γ null (NSG) mice for luciferase-based imaging of leukemogenic burden over time and, ultimately, survival analysis. Depletion of miR-196b inhibited leukemia cell engraftment, whereas transplantation of WT MOLM13-YFP-luc cells showed remarkable expansion *in vivo* (Fig. 7A, B, C). We found that depletion of miR-196b resulted in elongated survival (19 days, OS), whereas mice receiving WT MOLM13 cells died on day 15 (overall survival, OS) (Fig. 7D). The spleen size was dramatically smaller in mice receiving miR-196b KD MOLM13-YFP-luc cells than in mice receiving WT MOLM13-YFP-luc cells (Fig. 7E, Supplementary Fig. 4). Fluorescence-activated cell sorting (FACS) analysis showed that hCD45⁺ bone marrow (BM) and spleen cells were slightly reduced in mice receiving miR-196b KD cells compared to WT cells (Fig. 7F, Supplementary Fig. 5). Thus, miR-196b functions downstream of *HOTTIP* and coordinates with the lncRNA to repress FAS signaling, and miR-196b inhibition reduces the AML leukemia burden *in vivo* (Fig. 7G).

Discussion

Long-non-coding RNAs (lncRNAs) and microRNAs (miRNAs) have emerged as biomarkers, drivers, and potential therapeutic targets for a wide array of complex disorders, including leukemia [19, 31]. A growing number of publications demonstrated that miRNAs interact with lncRNAs thereby influencing their target gene expression [32]. The role of lncRNAs in gene silencing is well established; however, less is known about their function in active genomic loci where miRNAs are transcribed [6]. *HOTTIP* (*HOXA* transcript at the distal TIP), a lncRNA, has been known to interact with WDR or WDR/MLL protein complex to epigenetically regulate the 5' *HOXA* gene by methylating histone H3K4, which is associated with active transcription. Recent studies from our laboratory combining a variety of molecular biology and bioinformatics techniques have revealed the regulatory networks activated by *HOTTIP* in malignant hematopoiesis and acute myeloid leukemia. Depletion of *HOTTIP* effectively inhibited the posterior *HOXA* genes (*HOXA9-HOXA13*), and its inhibitory effects gradually diminished on genes towards the anterior end (*HOXA1-HOXA7*) [12, 33]. However, whether miRNA genes are regulated by *HOTTIP* in a similar manner to protein coding genes remained a question relevant to hematopoiesis and leukemogenesis. LncRNA can influence miRNA function in many ways: (1) lncRNAs can serve as a source to produce mature miRNAs (2) lncRNAs act as sponge, binding miRNA to prevent them from repressing their target mRNA genes and (3) lncRNAs alter miRNA gene transcription by binding on their promoter [19]. To profile *HOTTIP* regulated miRNA in MOLM13 cells, our small RNA sequencing revealed that loss of *HOTTIP* significantly affects expression of several miRNAs when comparing WT and *HOTTIP*^{-/-} MOLM13 AML cells. Intriguingly, several ncRNAs, including lncRNAs and miRNAs, are transcribed from the intergenic regions of the HOX clusters. However, it remains unknown how *HOTTIP* may mediate ncRNA transcription, particularly of miRNAs, and how the *HOTTIP*-miRNA axis governs gene expression in malignant hematopoiesis.

HOXA9 expression is positively regulated by mixed lineage leukemia (MLL) methyltransferase, which trimethylates histone 3 lysine 4 (H3K4me3) at the *HOXA9* promoter [34]. This mechanism is directly antagonized by the sequential activity of polycomb repressive complexes PRC1 and PRC2 that trimethylated histone 3 lysine 27 (H3K27me3). Moreover, topologically associating domains (TAD) of the HOX loci within the nucleus also have an important role in coordinating expression. *HOTTIP* lncRNA interacts with the WDR5-MLL complex and localizes the complex to the 5' *HOXA* locus [12, 33]. To determine whether *HOTTIP* provides a basis for transcriptional activation and three-dimensional (3D) chromatin organization in posterior HOX gene loci, we screened all CTCF sites and lncRNAs important for *HOXA9* expression within all four HOX gene loci in *MLL-AF9* rearranged MOLM13 AML cells using a CRISPR/CAS9 lentivirus screening library [30]. The *HOTTIP* lncRNA acts downstream of the *CBS7/9* boundary to regulate expression of genes located in *cis* and *trans*, including *HOXA13-HOXA9*, *MEIS1*, and *RUNX1*, which are important for hematopoiesis, and leukemia [12]. Furthermore, ChIRP-seq analysis revealed that *HOTTIP* binds to its target genes [12] and a cohort of miRNAs. This data raises the question of whether *HOTTIP* also controls the expression of miRNAs that are involved in the management of malignant hematopoiesis.

Our sequence data analysis demonstrated that inhibition of *HOTTIP* in MOLM13 cells significantly inhibited several miRNAs, including HOX cluster miRNA, miR-196b, which targets both oncogenic *HOXA9* and the *FAS* tumor suppressor. Expression profiling of primary AML patient samples showed strong correlation among miRNA, *HOTTIP*, and their target HOX genes. Furthermore, ATAC-seq (assay for transposase-accessible chromatin using sequencing) data analysis defined chromatin signatures at differentially expressed miRNAs bound by *HOTTIP* in *cis* and *trans*, including HOX clusters miR-196b. *HOTTIP* binding on the promoter region of miRNAs that are differential expressed in *HOTTIP*^{-/-} MOLM13 cells suggests that *HOTTIP* directly controls transcriptional regulation of miRNAs.

Dynamics of chromosomal structure play important roles in gene control. Several proteins modulate chromatin dynamics by contributing to structural interactions between gene promoters and their enhancer elements. Enhancer/promoter communications for specific transcription programs are enabled by topological associated domains (TADs), which are basically structural and functional chromosomal units. Often inappropriate promoter/enhancer interactions result from altered TADs, which lead to aberrant transcription of oncogenes or tumor suppressor genes. Binding of transcription factor CTCF in chromatin boundaries plays an important role in defining TADs and chromatin signature within TADs. We previously reported that CTCF binding located in between of *HOXA7* and *HOXA9* defines posterior HOXA locus TADs and chromatin signature within the TADs. Depletion of *CBS7/9* impaired chromatin structure and altered posterior HOXA gene expression due to lacking function of *HOTTIP* lncRNA [12]. By virtue of *CBS7/9*'s role in regulating posterior HOXA genes and lncRNA *HOTTIP* expression, we show that HOX cluster miRNAs are altered in *CBS7/9*^{+/-} and that *HOTTIP* over-expression in *CBS7/9*^{+/-} cells restored HOX cluster miRNA expression and chromatin signature. Apart from the *cis* coding miR-196b, *HOTTIP* lncRNA also bound and regulated *trans* HOX and non-HOX cluster miRNAs.

To decipher the mechanisms by which *HOTTIP* exerts miRNAs to control target genes, we performed bioinformatics analysis that revealed many differentially expressed genes in *HOTTIP*^{-/-} MOLM13 cells, which are co-regulated by *HOTTIP* and miR-196b. Although *HOTTIP* positively regulates expression of their co-expressed oncogenes, several of them are negatively regulated by miR-196b. Thus, it seems that negative regulation of the co-expressed 5' HOXA genes in MOLM13 cells might be fine-tuned by miR-196b in normal hematopoiesis [28]. Notably, single miRNA (or groups of miRNAs) targets multiple genes including oncogenes and tumor suppressors simultaneously or sequentially. Partial repression of *HOXA9* and *MEIS1* by miR-196b in the human *MLL*-rearranged leukemia may not potent enough to affect their function to induce and maintain leukemia. The tumor suppressor targets (e.g., *FAS*) of miR-196b could significantly inhibit cell transformation and leukemogenesis. Indeed, miR-196b inhibition induced *FAS* expression and cleaved caspase-3. As a result, the apoptotic cell death increased upon miR-196b knockdown in MOLM13 cells. Similarly, induced expression of *FAS* and *Cas3* in *HOTTIP*^{-/-} and *CBS7/9*^{+/-} is associated with increased cell death. *HOTTIP* modulates epigenetic marks on the *FAS* promoter and thereby controls chromatin accessibility and gene expression. However, *HOTTIP* represses *FAS* expression perhaps through activation of miR-196b

that directly targets *FAS*. miR-196b-deficient MOLM13 cell transplantation in NSG mice delayed leukemogenesis. All mice transplanted with WT MOLM13 cells died within 15 days, while mice receiving miR-196b-depleted cells survived longer and exhibited fewer hCD45⁺ AML cells. Transgenic overexpression of *HOTTIP* lncRNA in mice affected HSC function and increased leukemia stem cell (LSC) pool, inducing leukemia-like disease [12]. Thus, *HOTTIP* and miR-196b deletion reduces the AML leukemic burden *in vivo*, and both coordinate to regulate *FAS* expression at the transcriptional and post-transcriptional level to promote leukemogenesis.

Taken together, we report a mechanism mediated by *HOTTIP* to regulate miR-196b expression in AML. Our study revealed a regulatory model in which *HOTTIP*-miR-196b axis represses the expression of tumor suppressor *FAS* that circumvent the negative effects of *HOXA9* repression by miR-196b in AML. The aberrant upregulation of both *HOTTIP* and miR-196b by MLL fusion results in the persistent repression of its tumor-suppressor targets (e.g., *FAS*) along with dual control (transactivation and inhibition) of their oncogenic co-targets (*HOXA9/MEIS1*). This inhibits differentiation, disrupts cell homeostasis, and promotes cell proliferation via inhibiting apoptosis, consequently maintaining leukemia stem cell pools. Apart from miR-196b, *HOTTIP* also bound and regulated a subset of non-HOX cluster miRNAs while a subset of miRNAs was upregulated in *HOTTIP*-inhibited MOLM13 cells. Future studies should aim to investigate the mechanism by which *HOTTIP* modulates expression of candidate miRNA in hematopoiesis and leukemogenesis.

Material and methods

Tissue and Cell culture

MOLM13 cells were cultured and maintained in RPMI medium supplemented with 10% FBS and 1% penicillin and streptomycin solution, and OCI-AML3 cells were cultured in alpha-MEM supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were maintained at 37°C with 5% CO₂ in a humidified incubator. All medium and supplements were purchased from Thermo Fisher Scientific.

SsecCRISPR-Cas9 Mediated *HOTTIP* lncRNA Knock-Out and Lentivirus Production

The detailed protocol of generating *HOTTIP* knockout (KO) MOLM13 leukemia cells were from our previous report [12]. In brief, CRISPR-RNA (crRNA) and tracrRNA were mixed and annealed at 95°C for 5 min and the cooled down to room temperature. Subsequently, crRNA: tracrRNA duplex and S.p. Cas9 Nuclease components were combined and then mixed with 500,000 AML cells for electroporation with Neon® System. The DNA was extracted after 24 hrs. or 96hrs. from 100ul of transfected cells using Qiagen Quick Extract kit and processed for Sanger sequencing for verification of mutation. The targeted deletion of *HOTTIP*: *HOTTIP*^{-/-} -#1 targeted region is Chr7: 27241953–27241985; *HOTTIP*^{-/-} -#2 targeted region is Chr7: 27240098–27240123.

dCas9-Mediated Overexpression of *HOTTIP* in AML Cells

HOTTIP promoter targeting guide RNA was designed using the Zhang laboratory web tool (<http://crispr.mit.edu>), and cloned into lentiviral vector the pLKO5.sgRNA.EFS.tRFP

vector (Addgene #57824). The gRNA plasmid encoding puromycin was co-transfected with a plasmid encoding dCas9-VP160 (pAC94-pmax-dCas9VP160-2A-puro, Addgene plasmid number #48226) in MOLM13 and OCI-AML3 cells. Transfected MOLM13 cells were selected with 2µg/ml puromycin for 48 hrs. Then, RFP⁺ positive cells were sorted by FACS. RNA was extracted from puromycin and RFP⁺ positive cells, and gene expression was analyzed by RT-qPCR using specific primer sets [12].

miRNA Knockdown and Over Expression

Locked Nucleic Acids (LNA) and miRNA mimics and scrambled LNA/mimic (negative control) was purchased from Qiagen, and then MOLM13 and OCI-AML3 AML cells were transfected with either LONZA nucleofector devise (program X-001) or Lipofectamine 3000 (Invitrogen), separately. 2×10^6 cells were transfected with 5ul (stock 20uM) of LNA using manufacturers protocol and LONZA nucleofector devise and transfection solution and seeded in 6-well plate. Cells were harvested on day 5 of transfection for RNA extraction and gene expression analysis.

Western Blot Analysis

Whole cell lysate extract (total protein) was prepared using RIPA buffer and quantified using Bradford method. Total protein lysates were fractionated on 4%–20% on tris-glycine polyacrylamide gradient gel and transferred onto PVDF membrane. The blot was exposed to specific antibodies to detect the endogenous protein level using chemiluminescence method and BioRad imaging system: anti-β-actin (cat#A2066–100U); Sigma at 1: 5000, anti-Cleaved Caspase-3 (cat#9661); Cell Signaling at 1:1000 dilution; anti-Cleaved Caspase-8 (cat#9746); Cell Signaling at 1:1000 dilution.

Apoptosis Analysis

MOLM13 cells transfected with either LNA against specific miRNA or scrambled non-targeting control were seeded and cultured in 6-well dishes. Cells were harvested 72hrs post transfection and washed with PBS. Apoptotic cells were detected by Accuri C6 fluorescence-activated cell sorting (FACS) using Annexin V-APC Apoptosis Detection Kit (BD) according to the manufacturer's protocol.

RNA Extraction and Real-Time qPCR

Norgen Biotek RNA purification kit was used to isolate total RNA as per manufacturer's instructions. cDNA (reverse transcription - RNA to cDNA) was made using High-Capacity RNA to cDNA kit (Life Technologies, Grand Island, NY). miRNA and mRNA qPCR were performed using TaqMan (Life Technologies) and Sybr Green assay respectively with either Taqman Universal PCR Master Mix (miRNA qPCR) or BioRad SsoAdvanced Universal Sybr Green Supermix according to the manufacturer's protocol on a Bio-Rad CFX96 Touch Real Time PCR Detection System (Bio-Rad Laboratories, Richmond, CA). PCR reactions were performed in triplicate using either U6 (miRNA qPCR), or GAPDH (human messenger RNA qPCR) as the normalizer.

Small RNA library preparation and sequencing analysis

The small RNA-seq of the MOLM13 WT and *HOTTIP*^{-/-} cells was conducted at Genome Science Facility, Pennsylvania State University College of Medicine. Small RNA-seq libraries were prepared using BioO from Perkin Elmer and Qiagen kits. Small RNAs were sequenced using a TruSeq Small RNA Sequencing Kit (Illumina, San Diego, CA, USA) according to manufacturer instructions. The quality of libraries was assessed based on size distribution and concentration using 2100 Bioanalyzer with DNA 1000 chip (Agilent Technologies). All samples were sequenced 25M reads on an Illumina NovaSeq 6000 Sequencer using the 2×50bp paired-end platform.

All of raw binary base call files from the sequencer were transformed into FASTQ format and de-multiplexing using Illumina bcl2fastq2 Conversion Software v2.20 (<https://support.illumina.com/downloads/bcl2fastq-conversion-software-v2-20.html>). Quality of the sequenced reads was evaluated using FastQC developed by Babraham Bioinformatics (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Then these fastq files were performed by adapter trimming using the FASTQ Toolkit App version 1.0 of Illumina BaseSpace (<http://basespace.illumina.com/apps/>) and sequence alignment with GRCh37 human genome assembly database using OASIS2.0 (<https://oasis.dzne.de/index.php>).

Differential expression analysis was performed using the DESeq2 algorithm [35] and the expression was normalized using de Variance Stabilizing Transformation from the DESeq2 algorithm in R. The differential expression of miRs with adjusted *p* values < 0.05, and fold change (FC) ≥ 2 representing positive log₂FC (>1.0) and negative log₂FC (<-1.0) were considered to be significantly different.

The potential targets of miRs were derived from the miRTarBase [36] in human genome (<http://mirtarbase.cuhk.edu.cn/php/index.php>). Raw sequencing data can be accessed through NCBI GEO under accession number (GSE182601).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed as the previously described [12]. Briefly, crosslinked chromatin prepared from MOLM13 cells were immunoprecipitated with antibodies against MLL1, H3K4me3, H3K9me2, H3K27me3, and H3K79me2, separately. The MLL1, H3K4me3, H3K79me2 and H3K27me3, H3K79me2 ChIP-DNA libraries were prepared using illumina's TruSeq ChIP Sample Preparation Kit according to the manufacturers' instructions (Cat # IP-202-1012). Agilent TapeStation was used to check the quality of the libraries as per manufacturer's instruction. Final libraries were submitted to paired-end sequencing of 100 bp length on an Illumina HiSeq 3000. All sequencing genomics datasets were deposited in the NCBI GEO under accession number (GSE114981).

Chromatin Isolation by RNA Immunoprecipitation (CHIRP) Assay

The detailed methods of CHIRP assay were previously described [12]. Briefly, sonicated chromatin materials diluted using hybridization buffer and hybridized with 100pmol of biotinylated tiling DNA probes targeting *HOTTIP* or *LacZ* containing 100 μ L of Streptavidin-magnetic C1 beads (Invitrogen). RNA and DNA hybrids and RNA binding

proteins were subjected to analysis by ChIRP-qPCR and ChIRP-RT-qPCR. Illumina's TruSeq ChIP Sample Preparation Kit was used according to manufacturer's instructions for preparation of CHIRP libraries and submitted to paired-end sequencing of 100 bp length on an Illumina HiSeq 2500. All sequencing genomics datasets were deposited in the NCBI GEO under accession number (GSE114981).

ChIP-seq and ChIRP-seq Data Analysis

ChIP-seq and ChIRP-seq data processing and analysis was previously described [12]. Briefly, all sequencing tracks were viewed using the Integrated Genomic Viewer [37]. Peaks annotation was carried out with the command “annotatePeaks.pl” from HOMER package [38]. For ChIRP-seq motif analysis, the *de novo* motif analysis was performed by the “findmotifsgenome.pl” from the HOMER motif discovery algorithm [38]. The genes and pathways regulated by the *HOTTIP* bound promoters or intergenic regions were analyzed and annotated by the Gene Ontology analysis with the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool (<https://david.ncifcrf.gov/>, Version 6.8) [39]. Each GO term with a p value more than 1×10^{-3} is used for cutoff (threshold: 10^{-3}). All genomics datasets were deposited in the NCBI GEO under accession number (GSE114981).

Assay for Transposase-Accessible Chromatin Using Sequencing (ATAC-seq)

ATAC-seq was previously described [12] using the Nextera DNA library preparation kit. Libraries were quantified using qPCR (Kapa Library Quantification Kit for Illumina, Roche), and purified with AMPure beads (Beckman Coulter). Quality of the DNA library was examined by Agilent bio-analyzer 2100 prior to sequencing in duplicates with 2×100 bp paired-end reads on an Illumina NextSeq 500. All genomics datasets were deposited in the NCBI GEO under accession number (GSE114981).

ATAC-seq Analysis

Detailed method of ATAC-seq analysis was previously described [12]. Briefly, all sequencing tracks were viewed using the Integrated Genomic Viewer (IGV/2.4.19) [37]. Peaks annotation was carried out with the command “annotatePeaks.pl” from HOMER package (version 4.8) [38] and GREAT [40]. DEseq2 (Benjamini-Hochberg adjusted $p < 0.05$; FoldChangeR2) were also performed to find the differential binding sites between two peak files, including control and treatment groups with C+G normalized and “reads in peaks” normalized data [41]. The *de novo* motif analysis was performed by the “findmotifsgenome.pl” from the HOMER package [38]. For each genomic feature (peaks or chromVAR annotation), we calculated the chromatin accessibility median deviation z-score (for chromVAR features) or fragment counts (for peaks) in control and treatment groups with chromVAR package in R language [42]. Pearson's correlation coefficient and Pearson's c2-test were carried out to calculate overall similarity between the replicates of ATAC-seq global open chromatin signatures. All genomics datasets were deposited in the NCBI GEO under accession number (GSE114981).

Xenotransplantation of Human Leukemic Cells and Patient-Derived Xenografts (PDX)

MOLM13 cells - WT or miR-196b KD were injected into the tail vein of the NSG mice (2–3 months old). Cells were resuspended into DPBS and injected at 5×10^5 cells (in 150 to 200 μ L DPBS) per mouse. Mice were monitored daily for symptoms of disease (ruffled coat, hunched back, weakness and reduced motility) to determine the time of killing for injected mice with sign of distress. Experimentally used NSG mice were humanely killed at the time of moribund. Blood was collected into microtubes for automated process with K₂EDTA. Bone (tibias, femurs, and pelvis) and spleen were dissected. BM cells were isolated by flushing the bones. Spleens were mashed through a 70-mm mesh filter and made into single cell suspensions. Human CD45⁺ (BD) chimerism in these hematopoietic tissues was analyzed by Accuri C6 flow cytometry.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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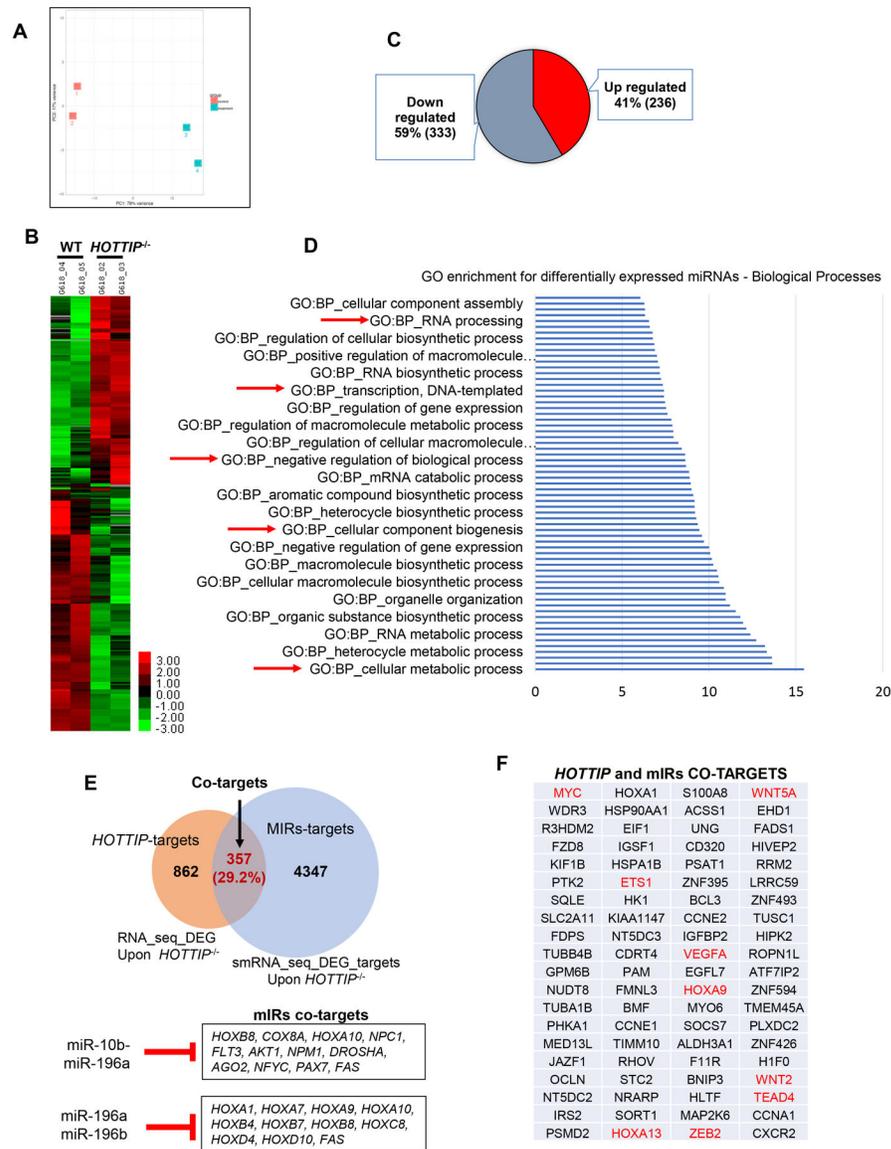


Fig. 1. LncRNA-*HOTTIP* regulates miRNAs target genes control leukemogenic program in AML cells.

(A) Principal component analysis (PCA) of the miRNAs differentially expressed in *HOTTIP*^{-/-} vs WT AML cells. (B) Heatmap of miRNAs changed >2-fold up- and down upon *HOTTIP*^{-/-} by small-RNA sequencing (smRNA-seq). (C) Pie chart shows the up- and down regulated miRNAs compared WT with *HOTTIP*^{-/-} MOLM13 cells. (D) GO (Gene Ontology) analysis of miRNAs differentially expressed in *HOTTIP*^{-/-} cells. (E) Overlapping of target genes regulated by either miRNAs or *HOTTIP*. (F) Part of overlapping targets co-regulated by both *HOTTIP*-associated miRNAs and *HOTTIP* itself in AML.

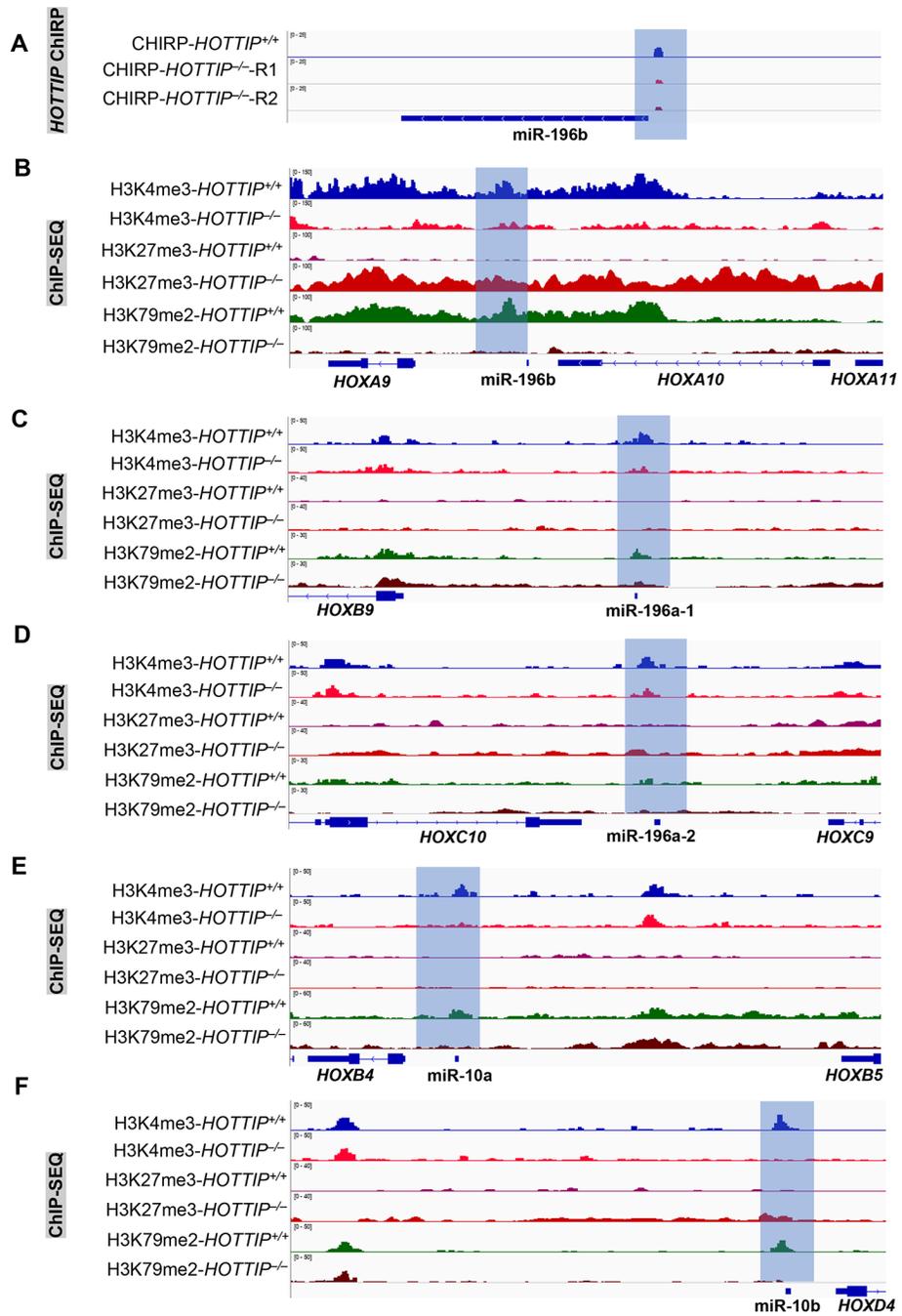
RT-qPCR analysis of the expression level of *HOTTIP*, posterior HOXA genes, and HOX cluster miRNAs in WT and *HOTTIP*^{-/-} MOLM13 cells. (F) small-RNA sequencing analysis of the expression level of indicated miRNAs compared WT and *HOTTIP*^{-/-} MOLM13 cells. (G) Relative expression level of the indicated mRNAs, miRNAs and *HOTTIP* lncRNA in the MOLM13 cells and KASUMI cells.

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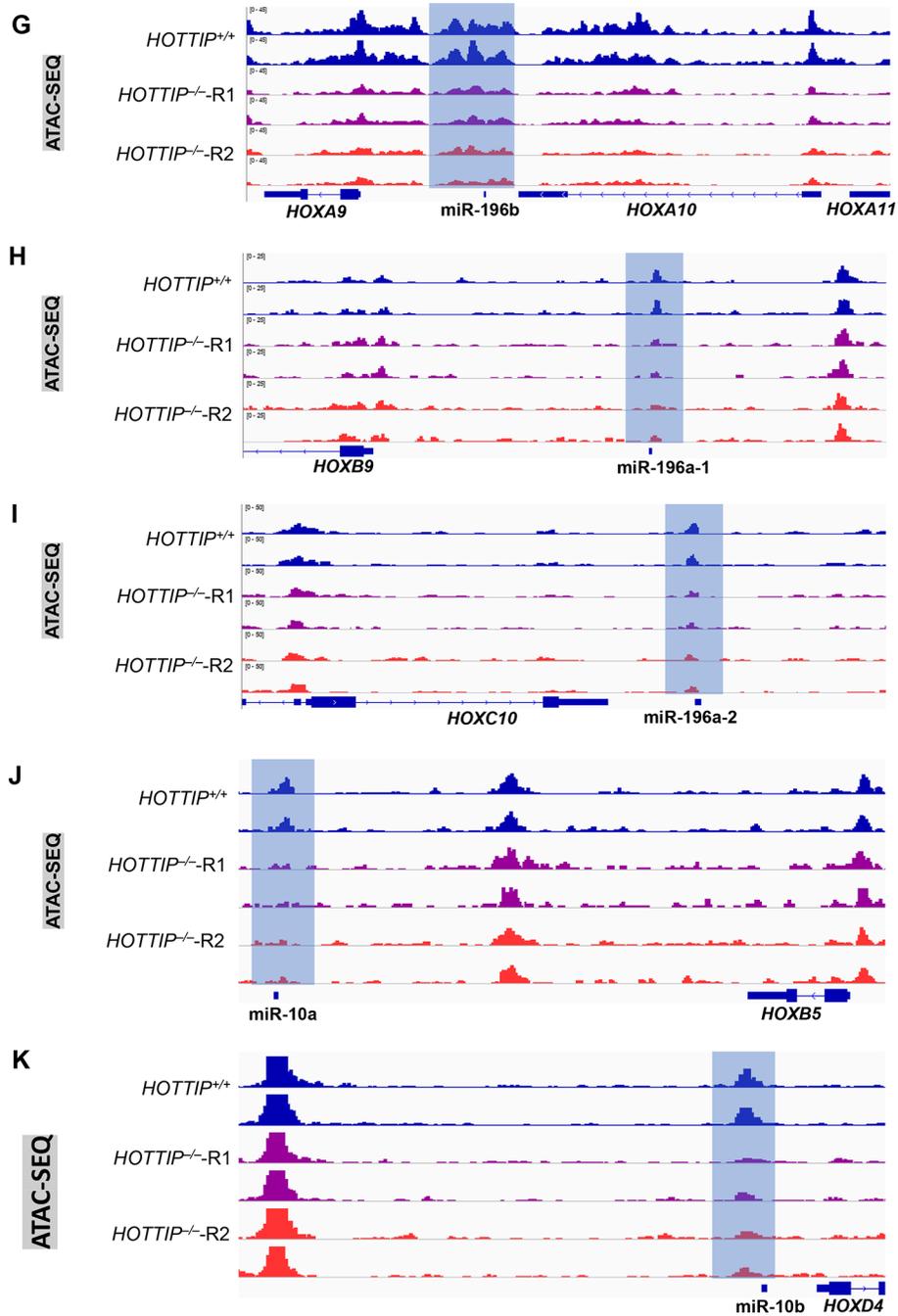


Fig. 3. *HOTTIP* controls the epigenetic modifications of HOX cluster miRNAs. (A) ChIRP-seq analysis shows *HOTTIP* binding on promoter region of miR196b in WT and *HOTTIP*^{-/-} MOLM13 cells. (B, C, D, E, F) Enrichment of the active histone marks (H3K4me3 and H3K79me2) and repressive histone mark (H3K27me3) on genomic loci of the HOX cluster miRNAs. (G, H, I, J, K) ATAC-seq analysis shows chromatin accessibility on the HOX-cluster miRNAs upon *HOTTIP*^{-/-} in MOLM13 cells, and WT cells.

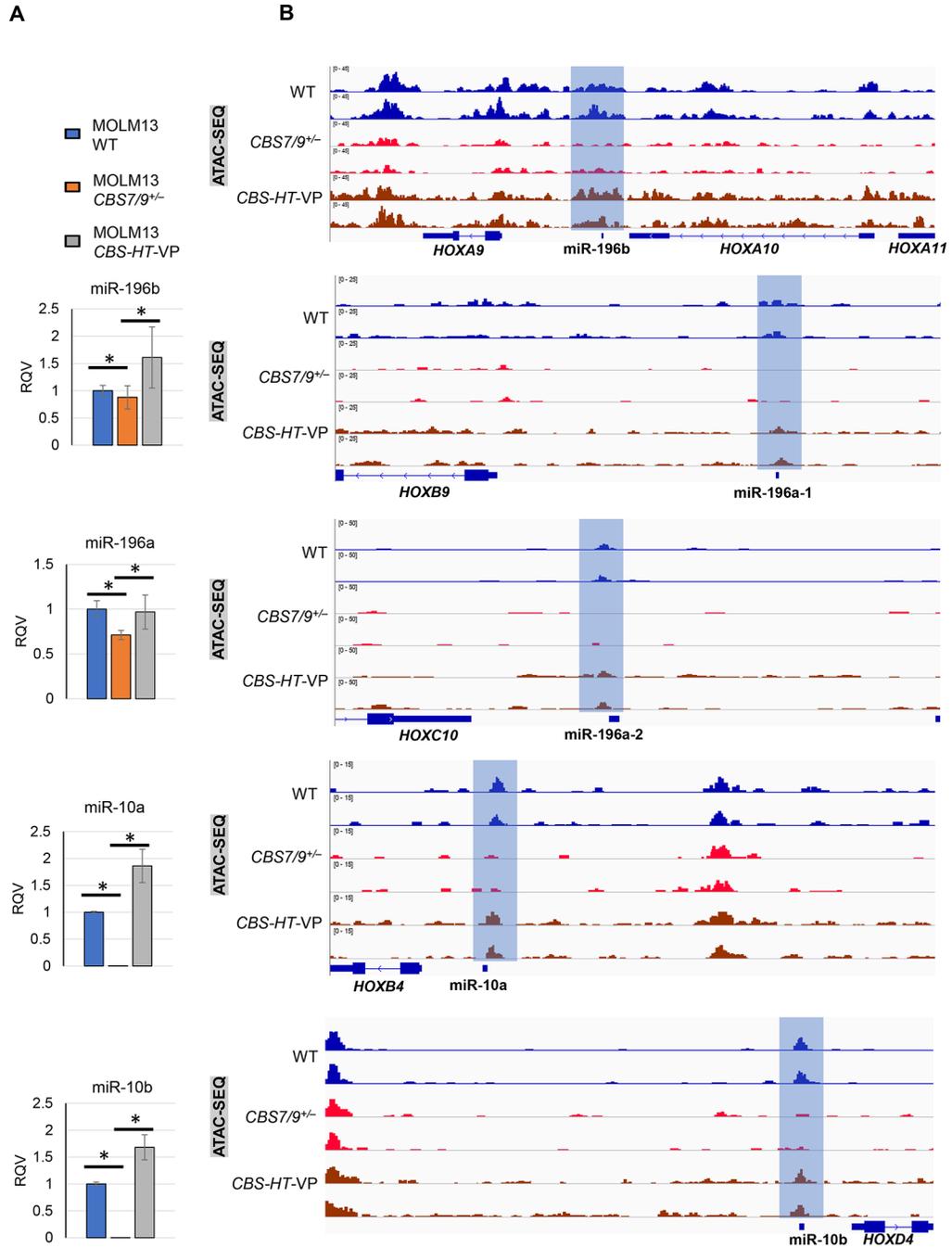


Fig. 4. CBS7/9 boundary regulates HOX cluster miRNAs chromatin neighborhood. (A) RT-qPCR analysis of HOX cluster miRNAs in WT, CBS7/9^{+/-}, and CBS7/9^{+/-} + HOTTIP-VP (CBS-HT-VP) MOLM13 cells. (B) ATAC-seq analysis shows chromatin accessibility on the loci of HOX cluster miRNAs in WT, CBS7/9^{+/-}, and dCas9-VP160-mediated HOTTIP-activated CBS7/9^{+/-} MOLM13 cells (CBS-HT-VP).

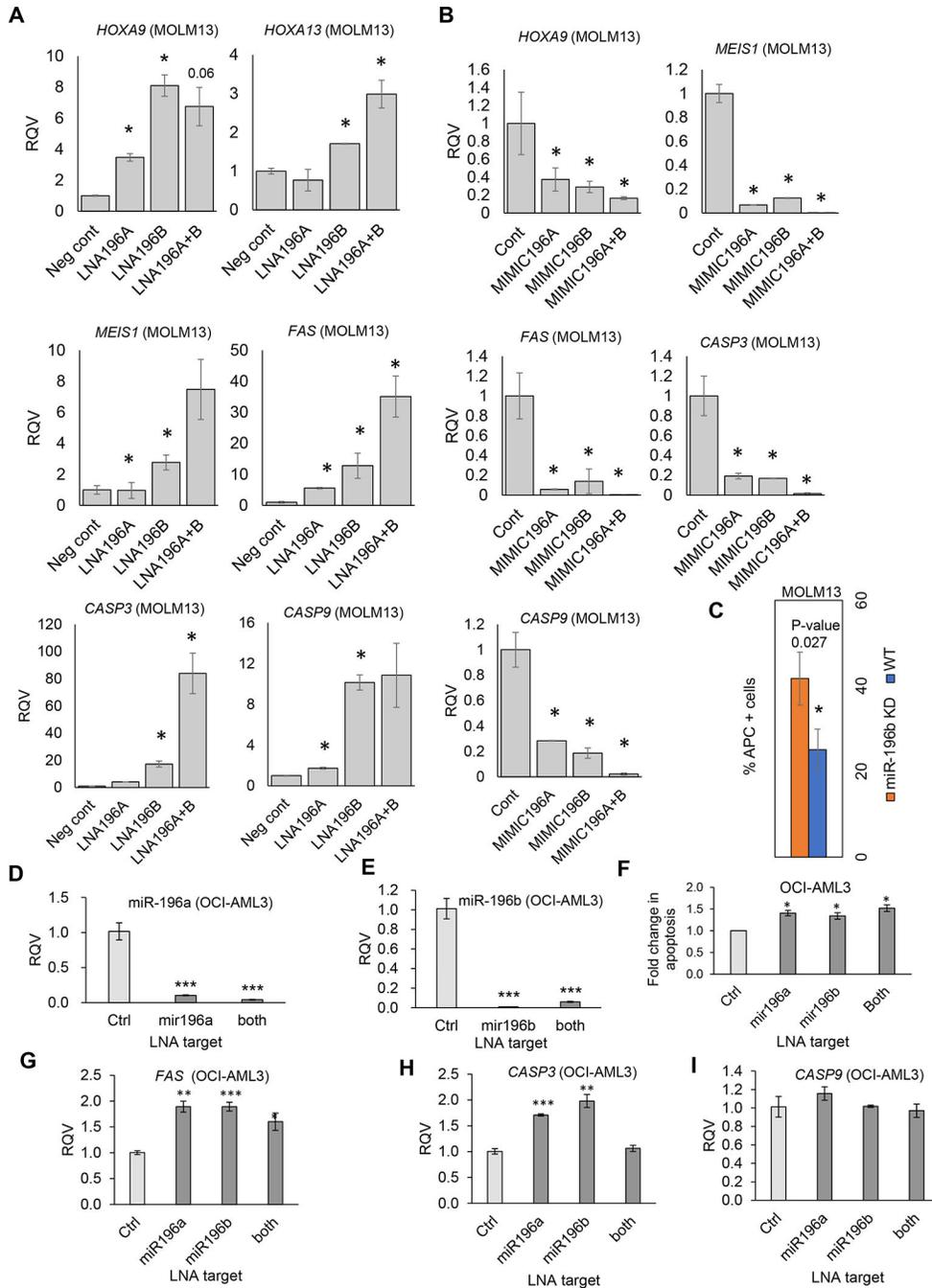


Fig. 5. Inhibition of miR-196b induced apoptosis in MOLM13 cells. (A) RT-qPCR analysis of miR-196b targets in MOLM13 cells treated with either negative control or locked nuclei acids (LNA) against miR-196b. (B) RT-qPCR analysis of miR-196b targets in MOLM13 cells treated with either mimics of specific miRNA or control. (C) Bar graph shows flow cytometry (FACS - florescence activated cell sorting) evaluated percentage of annexin V stained apoptotic population of MOLM13 cells in control and miR-196b knockdown groups. (D, E, G, H, I) RT-qPCR analysis of indicated miRNA and mRNA in OCI-AML3 cells treated with either negative control or LNA against miR-196. (F) Bar

graph shows percentage apoptotic population of OCI-AML3 cells in control and miR196 knockdown groups.

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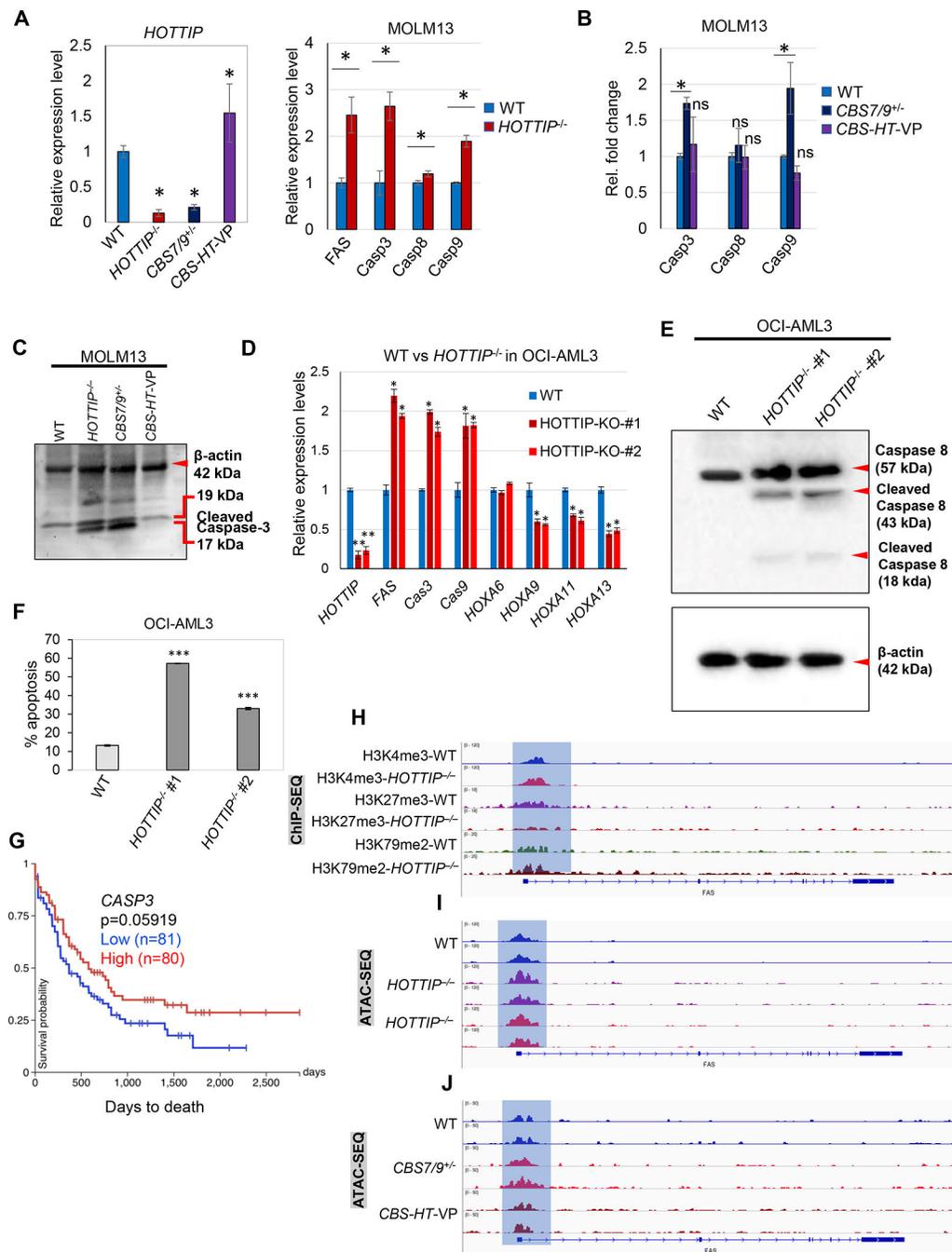


Fig. 6. *HOTTIP* targets *FAS*, and *CBS7/9* boundary play role in maintaining the *HOTTIP* targets-chromatin neighborhood.

(A) RT-qPCR analysis of *HOTTIP* expression WT, *HOTTIP*^{-/-}, *CBS7/9*^{+/-}, and *CBS7/9*^{+/-} + *HOTTIP*-VP MOLM13 (*CBS-HT-VP*) cells. (B) RT-qPCR analysis of the expression level of *Caspase* related genes in WT, *CBS7/9*^{+/-}, and dCas9-VP160-mediated *HOTTIP*-activated MOLM13 (*CBS-HT-VP*) cells, including *CASP3*, *CASP8*, and *CASP9*. (C) Western blot data shows protein level of β -actin and cleaved Caspase-3 in WT, *HOTTIP*^{-/-}, *CBS7/9*^{+/-}, and dCas9-VP160-mediated *HOTTIP*-activated MOLM13 (*CBS-HT-VP*) cells.

(D) RT-qPCR analysis shows the expression level of indicated genes in WT and *HOTTIP*^{-/-} OCI-AML3 AML cells. (E) Western blot data shows the protein level of cleaved Caspase-8 and β -actin in WT, and *HOTTIP*^{-/-} OCI-AML3 cells. (F) Bar graph shows the percentage of FACS evaluated annexin V stained apoptotic population in WT and *HOTTIP*^{-/-} OCI-AML3 cells. (G) Kaplan-Meier analysis showing overall survival of patients with LAML based on dichotomized expression (relatively high or low) of *CASP3* gene. Results were obtained from TCGA-LMAL data. (H) ChIP-seq analysis shows histone marks (H3K4me3, H3K27me3 and H3K79me2) enrichment on the *FAS* promoter in WT and *HOTTIP*^{-/-} MOLM13 cells. (I) ATAC-seq analysis of chromatin accessibility on the *FAS* promoter in WT and two *HOTTIP*^{-/-} MOLM13 clones. (J) ATAC-seq analysis of chromatin accessibility on the *FAS* promoter in WT, *CBS7/9*^{+/-}, and dCas9-VP-160-mediated *HOTTIP*-activated MOLM13 (*CBS-HT-VP*) cells.

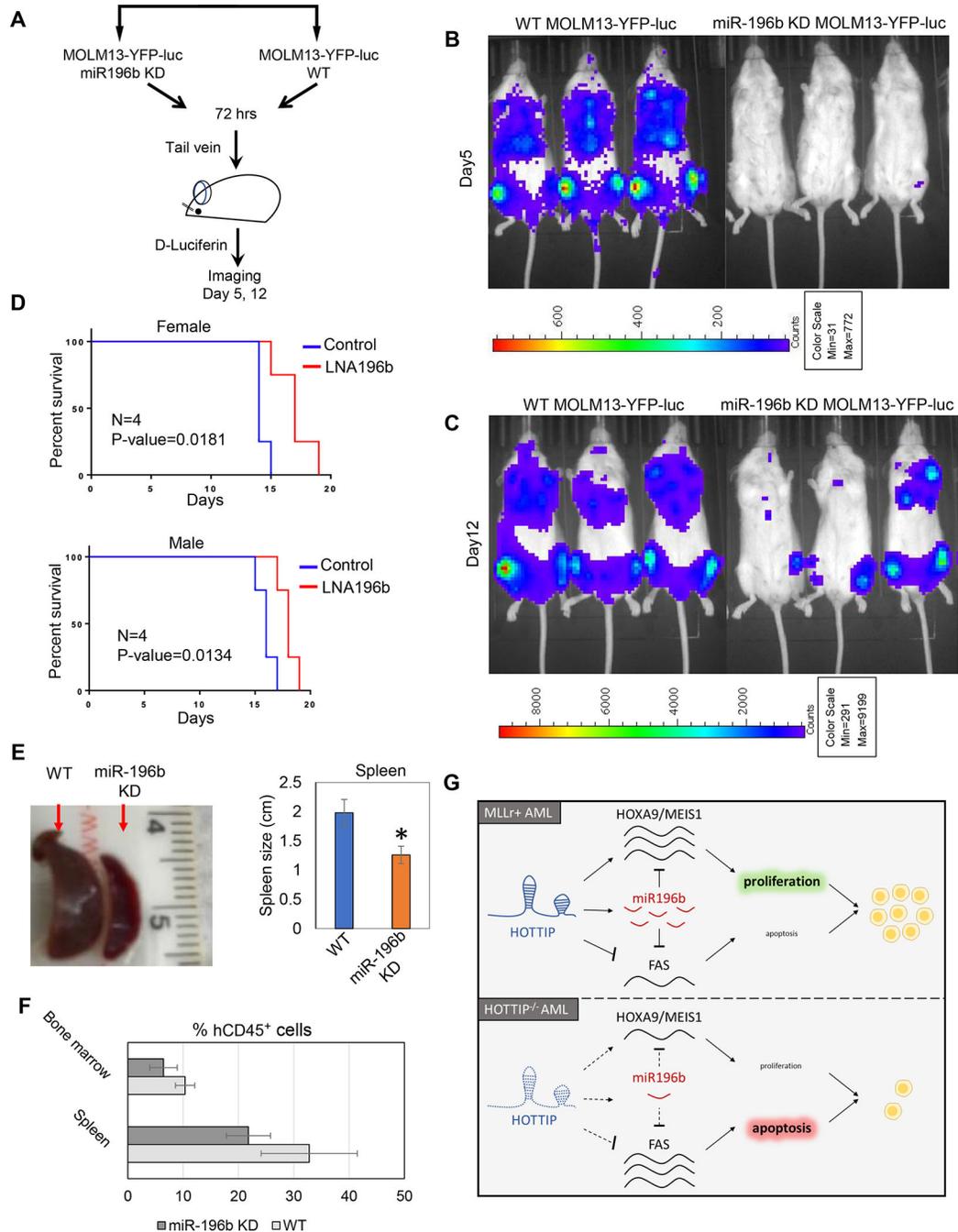


Fig. 7. Inhibition of miR-196b inhibits *in vivo* leukemogenesis.

(A) Schematic representation of the MOLM13 transplantation in NSG mice and imaging. Luciferase expressing MOLM13 cells either WT or miR196b KD were injected into NSG mice through tail vein, followed by *in vivo* imaging to assess the knockdown effects of miR-196b in leukemogenesis (B, C) Representative images of leukemogenesis in NSG mice transplanted with MOLM13 cells treated with either LNA196b or negative control. (D) Overall survival (OS) of female or male NSG mice xenografted with MOLM13 cells compared LNA196b and negative control. (E) Representative spleens images of NSG mice

after 12 days transplantation with MOLM13 cells treated with either LNA196B or negative control. Right bar graph shows relative spleen size in mice received MOLM13 cells treated with either LNA196B or control. (F) The percentage ratio of human CD45⁺ cells from bone marrow (BM) and spleen after 12 days transplantation was analyzed by FACS. (G) Model of *HOTTIP*/miR196b regulation in MLLr⁺ and NPM1^{c+} AML. *HOTTIP* activation in MLLr⁺ AML activates expression of *HOXA9*, *MEIS1*, and miR196b and suppresses *FAS*. miR196b, in turn, represses *HOTTIP* target genes to maintain a proliferative state and drive leukemogenesis. In the absence of *HOTTIP*, these pathways are inactive, therefore, *HOXA9* and *MEIS1* levels decrease, and *FAS* levels increase, resulting in leukemic cells undergoing apoptosis.