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Symplekin, a polyadenylation factor, prevents MOZ and MLL activity on HOXA9 in hematopoietic cells

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ABSTRACT

MOZ and MLL encoding a histone acetyltransferase and a histone methyltransferase, respectively, are targets for recurrent chromosomal translocations found in acute myeloblastic or lymphoblastic leukemia. We have previously shown that MOZ and MLL cooperate to activate *HOXA9* gene expression in hematopoietic stem/progenitors cells. To dissect the mechanism of action of this complex, we decided to identify new proteins interacting with MOZ. We found that the scaffold protein Symplekin that supports the assembly of polyadenylation machinery was identified by mass spectrometry. Symplekin interacts and co-localizes with both MOZ and MLL in immature hematopoietic cells. Its inhibition leads to a decrease of the HOXA9 protein level but not of *Hoxa9* mRNA and to an over-recruitment of MOZ and MLL onto the *HOXA9* promoter. Altogether, our results highlight the role of Symplekin in transcription repression involving a regulatory network between MOZ, MLL and Symplekin.

1. Introduction

Acute myeloblastic leukemias (AMLs) are characterized by acquired somatic mutations and epigenetic alterations in genes that are essential during hematopoiesis, for cell proliferation and survival pathways. Gene expression patterns especially in AML-MLL (mixed lineage leukemia) or -MOZ (Monocytic leukemia zinc finger protein) subtypes corroborate increased expression of a subset of HOX proteins including HOXA9 [1–3]. HOXA9 is necessary for the tumoral process in MLL leukemias [4]. Deciphering the mechanisms of *HOXA9* regulation is necessary to evaluate its function in normal and leukemic myelopoiesis. MOZ and MLL are recruited to target genes including *Hox* loci, as part of complexes enhancing transcription [5–10].

MOZ (also named MYST3 or KAT6A) is a MYST (MOZ/YBF2/SAS2/TIP60 homology domain) family histone acetyltransferase (HAT), which catalyzes the transfer of an acetyl group from acetyl-CoA to the ε-amino group of specific lysine residues. MOZ forms a tetrameric complex with EAF6 (Esa1-associated factor 6 ortholog), the bromodomain PHD (plant homeodomain) finger proteins BRPF1, BRPF2 or BRPF3 (bromodomain and PHD finger-containing protein) and ING5 (inhibitor of growth 5) to execute its activities [11]. This enzyme is involved in developmental processes including hematopoiesis [12] and skeletogenesis [13,14]. Reciprocal chromosomal translocations triggering the fusion of *MOZ* to various HATs [15,16] are identified in acute myeloid leukemias (AMLs). Furthermore, analysis of *Moz* knockout mice suggests a crucial role in the maintenance of hematopoietic stem cells and differentiation of myeloid cells [12,17,18]. MOZ acts as a transcriptional co-activator of several hematopoietic transcription factors, *e.g.* Spi-1/PU.1 (spleen focus forming virus proviral integration oncogene/purine-rich box-1) or RUNX1 (runt-related transcription factor 1), *via* specific interactions [19,20].

MLL (also known as KMT2A), a histone methyltransferase (HMT) [21], is also involved in hematopoiesis [22], and is one of the most frequently rearranged genes in acute leukemias [23]. While MOZ is required for normal status of acetylated lysine 9 and 14 on histone H3 (AcH3K9, AcH3K14) [9,10], MLL catalyzes H3 lysine 4 di- and tri-methylation (Me2H3K4 and Me3H3K4) [21].

Abbreviations: AMLs, acute myeloid leukemias; BRPF, bromodomain and PHD finger-containing protein; CPSF100, cleavage and polyadenylation specificity factor 100; CTD-RNA-PolII, C-terminal domain of the RNA-polymerase II; EAF6, Esa1-associated factor 6 ortholog; HAT, histone acetyltransferase; HMT, histone methyltransferase; HOX, homeobox; HSF1, heat shock factor 1; ING5, inhibitor of growth 5; MLL, mixed lineage leukemia; MOZ, monocytic leukemia zinc finger; MYST, MOZ/YBF2/SAS2/TIP60 homology domain; PHD, plant homeodomain; RUNX1, runt-related transcription factor 1; Spi-1/PU.1, spleen focus forming virus proviral integration oncogene/purine-rich box-1

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We have previously characterized a functional interaction between MOZ and MLL, leading to the transcriptional activation of the *HOXA9* transcription factor in human hematopoietic stem/progenitor cells [24].

To better understand the mechanism of action of *HOXA9* regulation, we decided to identify MOZ and MLL interacting partners. We have isolated and functionally characterized a novel MOZ- and MLL-associated protein, Symplekin. We show here that this polyadenylation factor can act as a repressor of the MOZ-MLL complex in human immature hematopoietic cells, suggesting a regulatory network between MOZ, MLL and Symplekin.

2. Material and methods

2.1. Cells and cell culture

The human myeloblastic KG1 cell line (American Type Culture Collection, Manassas, VA, USA) was grown in an RPMI 1640 Glutamax medium (BioWhittaker) supplemented with 20% fetal calf serum (FCS) (BioWhittaker), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL) (BioWhittaker). The human HEK293T cells were maintained in 10% FCS and in DMEM supplemented with 4.5 g/L glucose (BioWhittaker). Human cord blood cells were layered over Ficoll-Paque (1.77 g/L) (Eurobio, Les Ulis, France), and the interface containing mononuclear cells was harvested after centrifugation. Then, the cells were washed, and CD34+ cells were purified using a CD34+ cells magnetic isolation kit and AutoMACS™ separator according to the manufacturer's instructions (Miltenyi Biotec, Bergish Gladbach, Germany).

2.2. Immunoprecipitation and Western blotting

The HA-MOZ and the c-Myc-MOZ vectors were provided by Issai Kitabayashi (NCCRI, Tokyo, Japan) and Edward Chan (Indiana University Cancer Center, Indianapolis, IN, USA), respectively, whereas the Flag-Symplekin plasmid was obtained from James Manley (Columbia University, New York, NY, USA). Residues 30–395 of human Symplekin (cloned in pET28a vector obtained from Liang Tong (Columbia University, New York, NY, USA)) were subcloned into the Flag-pCDNA3 vector. The N-terminal FLAG-tagged full-length human MLL complementary DNA was inserted into the pCI-neo vector (James Hsieh, Washington University School of Medicine, St. Louis, MO, USA).

HEK293T (transiently transfected as indicated above or not) or KG1 cells were harvested, washed with PBS and lysed in a cold immunoprecipitation-lysis buffer (1% NP40, 150 mM NaCl, 50 mM Tris-HCl pH8, protease inhibitor cocktail). The samples were incubated on ice for 30 min. After centrifugation, 1 mg of total proteins was then precleared with protein G agarose beads (Upstate Biotechnology). MOZ (a mouse monoclonal antibody directed against residues 856–870 of MOZ (IGBMC, Illkirch, France)), Symplekin (Becton–Dickinson), MLL-C (Upstate Biotechnology), c-Myc (9E10, Santa Cruz Biotechnology), Acetyl Lysine (Cell Signaling Technology) specific antibodies associated with protein G agarose beads, were used for immunoprecipitating proteins with gentle shaking at 4 °C overnight. As controls, each extract was also immunoprecipitated with irrelevant IgG (Santa Cruz Biotechnology). For FLAG immunoprecipitations, protein extracts were immunoprecipitated with an anti-FLAG M2 affinity gel (EZview Red Anti-Flag M2 Affinity Gel, Sigma-Aldrich, St. Louis, MO, USA). Immunoprecipitation complexes were washed five times in an ice-cold immunoprecipitation-lysis buffer. Proteins were eluted by boiling in a Laemmli buffer. Eluated proteins were then separated by SDS-PAGE and electroblotted to nitrocellulose membranes. Equivalent loading between lanes was confirmed by Ponceau Red staining. Membranes were blocked in 1× PBS-T (0.1%) and fat-free dry milk or BSA (5%) (blocking buffer) for one hour at room temperature, then incubated with the primary antibodies diluted in the blocking buffer at 4 °C overnight. Membranes were washed three times in 1× PBS-T (0.1%) for 10 min each. For immunoblotting, anti-HOXA9 and anti-CPSF100 were obtained from Santa Cruz Biotechnology, and anti-c-IAP1 from R&D Technology. For biotin blotting, streptavidin conjugated with dylight800 (Thermoscientific, Waltham, MA, USA) was used. Secondary antibodies conjugated with horseradish peroxidase (FLAG Western-blotting) or with fluorochromes were added, and the membranes were incubated at room temperature for one hour. The membranes were washed three times in 1× PBS-T (0.1%) for 10 min each. Anti-HSC70 (Santa Cruz Biotechnology) was used to check loading control. For protein detection, an ECL Western blotting reagent kit (Millipore) and the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) were used.

2.3. In vitro binding assays

For direct *in vitro* binding assays, *in vitro* translation of c-Myc-MOZ, MLL, Flag-Symplekin or Flag-Nter-Symplekin was carried out in rabbit reticulocyte lysates (T7 Quick Coupled Transcription/Translation System, Promega, Madison, WI, USA). Transcend™ tRNA (Promega) was added to the reaction mixture to biotinylate translated proteins. Unbiotinylated *in vitro* translated products were precleared with neutravidin-coated agarose beads (Pierce, Rockford, IL, USA). The proteins were incubated overnight at 4 °C. Then, biotinylated proteins were collected by incubation with neutravidin-coated agarose beads. The beads were washed five times in the IP lysis buffer. Bound proteins were eluted by boiling in the SDS loading buffer, separated by SDS-PAGE, and analyzed by immunoblotting with appropriate antibodies. The labeled protein bands were visualized by scanning the gel on an Odyssey infrared imaging system (LI-COR).

2.4. Mass spectrometry

A lysate from HEK293T cells was prepared as described above. MOZ associated with protein G agarose beads was used for immunoprecipitating proteins. Eluated proteins were then separated by SDS-PAGE. Coomassie blue stained bands were excised manually from the gel and cut into 1 mm^3 pieces. The gel fragments were prepared as recently described [25]. Peptides were then analyzed by MS and MS/MS with a MALDI-TOF/TOF UltraFlex II (Bruker Daltonics, Bremen, Germany) mass spectrometer. Proteins were identified by comparison to the human MSDB (Mass Spectrometry protein sequence DataBase) databank.

2.5. Immunofluorescence microscopy

Hematopoietic KG1 or HEK293T cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and saturated in PBS 1× 4% BSA. The cells were incubated for 1 h at RT with the anti-MOZ (N19, Santa Cruz Biotechnology) (dilution of 1:50), anti-Symplekin (dilution of 1:200), anti-FLAG (Sigma-Aldrich) (dilution of 1:500) or anti-c-Myc (dilution of 1:500). Then, the cells were incubated for 1 h with antibodies specific for mouse or goat immunoglobulin subclasses conjugated to fluorochromes (anti-goat-Alexa 488 or anti-mouse-Alexa 568) (dilution of 1:1000). Nuclei were counterstained with DAPI. The cells were analyzed using a confocal laser scanning microscope (Leica TCS SP2). Images were processed with Adobe Photoshop (adjustment of brightness and contrast).

2.6. SiRNA knockdown

Ten millions of KG1 cells were transfected with siRNAs by nucleoporation, according to the manufacturer's protocol (Amaxa). SiRNAs, synthesized by Invitrogen, target human Symplekin (Sense: 5′-CGUCUGUGCUGUUUGGAGCUGACAA-3′) or MOZ (Sense: 5′-UUAA UCUGCACUUCAGAGCCUCAGG-3′). A control siRNA was used as a negative control (*Silencer*® Negative Control No. 1 siRNA Ambion).

2.7. RNA isolation, reverse transcription, Q-PCR, poly(A) tail length

Total RNA was extracted with a TRI Reagent® (Ambion) and reverse transcribed with a random primer (Promega). Real-time PCR was performed in triplicates with TaqMan probes from Applied Biosystems and analyzed in an Applied Biosystems 7500 Fast Thermocycler. Values for each PCR were normalized with 18S. The TaqMan® assays were the following: Hs00191361_m1 (Symplekin), Hs00365956_m1 (Hoxa9),

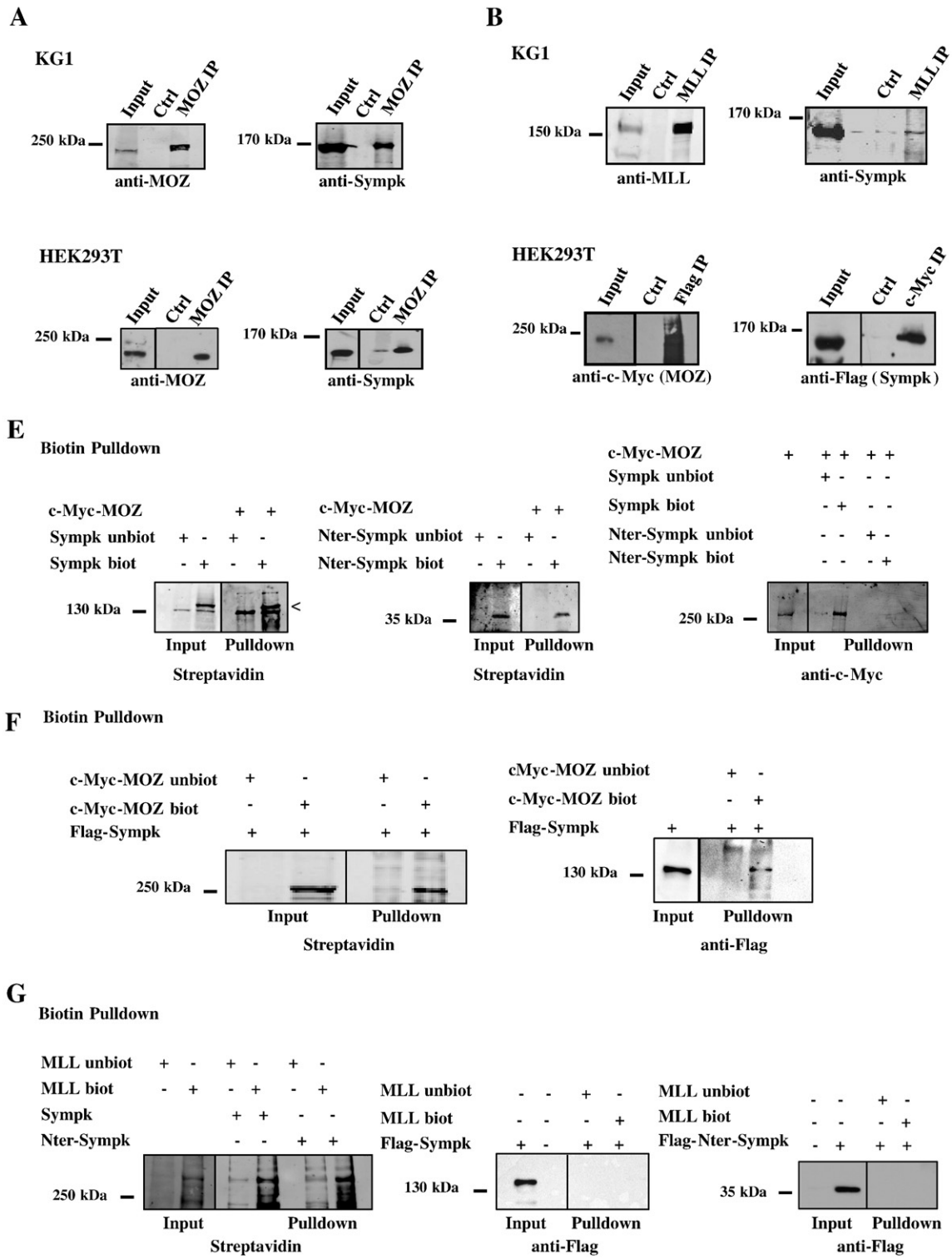

Fig. 1. Symplekin is associated with MOZ and MLL. (A) Symplekin interacts with MOZ in KG1 cells. IP was performed with anti-MOZ, then immunoprobed with anti-MOZ or anti-Sympk. (B) Symplekin interacts with MLL. IP was performed with anti-MLL, then immunoprobed with anti-MLL or anti-Sympk. (C) Symplekin interacts with MOZ in HEK293T cells. IP was performed with anti-MOZ, then immunoprobed with anti-MOZ or anti-Sympk. (D) Flag-Symplekin interacts with c-Myc-MOZ. Flag-Sympk or c-Myc-MOZ vectors were transfected into HEK293T cells. IP were performed with anti-Flag or anti-c-Myc, then immunoprobed as indicated. (E, F, G) Symplekin interacts directly with MOZ but not with MLL. As indicated, *in vitro* pulldown assays were performed with Flag-Sympk, Flag-Nter-Sympk, c-Myc-MOZ (E,F) or MLL (G), biotinylated (biot) or not (unbiot), produced by *in vitro* translation in reticulocytes lysates. Pulldown of biotinylated proteins was performed with neutravidin-coated agarose beads and protein interactions were revealed by SDS-PAGE and immunoblotted with anti-Flag, Streptavidin, anti-c-Myc.

Hs00198899_m1 (Moz), and Hs00610538_m1 (Mll). To measure poly(A) tail lengths of *Hoxa9* RNA, we employed the Poly(A) Tail-Length Assay Kit provided by Affymetrix. Two gene-specific forward and reverse primer sets were designed upstream of the polyadenylation site to correspond to controls for *Hoxa9*. The gene specific primers used were the following: site 1 forward 5′-AACTTCTGTGTACTGGGTGAT-3′; site 1 reverse CACTGGGAAATTCTTACAGCT-3′; site 2 forward 5′-TTA TACACTATGAAACCGCCATT-3′; and site 2 reverse 5′-GGCCTTGAGGT AACTATTGC-3′. The second set of primers uses the gene-specific forward primer and the universal reverse primer provided with the kit to generate a product that includes the poly(A) tails of *Hoxa9*. Human actin polyadenylation was measured as a positive control with primers from the kit. The PCR products were analyzed on polyacrylamide gels. To evaluate mRNA degradation, KG1 cells (20×10^6) were transfected with siRNAs by nucleoporation. Twenty-four hours after transfection, the cells were treated with Actinomycin D (1 μM) (Sigma-Aldrich). RNA was extracted and analyzed at a different time.

2.8. ChIP assays

Briefly, 5×10^6 KG1 cells were fixed with 1% formaldehyde to cross-link DNA with proteins, lysed, and fragmented by sonication to obtain DNA fragments of 200–1000 bp. A ChIP procedure was carried out with modifications according to the manufacturer's instructions (Upstate Biotechnology). After pre-clearing with salmon sperm DNA/protein A (antibodies specific for histone modifications) or G agarose beads, the samples underwent immunoprecipitation with antibodies specifically directed against Symplekin, MOZ, MLL-C, Me2H3K4 (Upstate Biotechnology), Me3H3K4 (Upstate Biotechnology), AcH3K9 (Upstate Biotechnology), AcH3K14 (Upstate Biotechnology), RNA polymerase II CTD repeat YSPTSPS phosphorylated Ser 5 (Abcam, Cambridge, UK), IgA (Santa Cruz Biotechnology) (Control ChIP) or IgG (Control ChIP) at 4 °C overnight. The beads were washed, protein/DNA complexes eluted, then cross-links reversed by heating at 65 °C overnight. After RNA and protein digestions, DNA was purified on a spin column (NucleoSpin Extract II: Macherey-Nagel, Düren, Germany). For sequential ChIP assays, the primary immunoprecipitation was done using a Symplekin, MLL-C or MOZ antibody. The immunoprecipitated complexes were eluted with a ChIP buffer. The eluate from Symplekin or MOZ IP was re-immunoprecipitated with the antibody corresponding to the other protein. The presence of the promoter sequences in the resulting re-ChIP immunoprecipitates were examined as described for one-step ChIP. Real-time Q-PCR analysis was performed in an Applied Biosystems 7500 Fast Real-Time PCR System. The ChIP primers used to amplify regions of the promoter locus of *HOXA9* were 5′-GGGGAGAC GGGAGAGTACAG-3′ and 5′-CGTCCAGCAGAACAATAACG-3′ (Invitrogen). For internal controls of ChIP-Q-PCR, we designed the primers to amplify an intronic region of *HOXA9* (5′-CTCCTCCCTTCAAATCCGCC-3′ and 5′-CAACTTCTGGCTCCTGGCC-3′). Input corresponding to total sonicated DNA was used as a cell number control. Delta-delta Ct values of each immunoprecipitated sample were normalized with those obtained from the amplification of their respective input and by subtracting the values obtained in the corresponding samples incubated without antibodies. Results with IgA or IgG were arbitrarily considered as 1. Error bars (standard deviation) correspond to the average of triplicates. Therefore, in Q-ChIP experiments, the relative recruitment corresponds to the ratio between the cycle threshold (Ct, obtained by Q-PCR) for each specific ChIP (MOZ, MLL, Symplekin, histone modifications or phosphorylated CTD), and the Ct obtained from the ChIP using an irrelevant antibody (IgG or IgA).

2.9. Analysis of mRNA degradation

KG1 cells (20×10^6) were transfected with siRNAs by nucleoporation. Twenty-four hours after transfection, the cells were treated with Actinomycin D (1 μM). RNA was extracted and analyzed at a different time.

2.10. Statistical analysis

A Mann–Whitney *U* test was used for statistical analysis. Differences were considered significant when *p* was less than .05. In all cases, * represents *p* less than .05; **, *p* less than .01; and ***, *p* less than .005.

3. Results

3.1. Symplekin is associated with MOZ and MLL

To isolate proteins associating with MOZ, the latter was immunoprecipitated from human embryonic kidney HEK293T cells by the anti-MOZ already described [24]. Mass spectroscopy analysis of co-eluted proteins allowed the identification of Symplekin (Supplemental Fig. 1A). Symplekin, which supports the assembly of polyadenylation complexes [26,27], has been detected among other members of the MLL supercomplex [28]. However, up to now the interaction between MLL and Symplekin has not been validated by immunoprecipitation assays.

To evaluate further the interactions that may occur between MOZ, MLL and Symplekin in human immature hematopoietic cells, we performed co-IP experiments using the KG1 AML0 cell line that expresses these proteins. We observed that Symplekin interacts with both MOZ and MLL (Fig. 1A, B). As a negative control, MOZ does not immunoprecipitate cIAP1 (Supplemental Fig. 1B). In the human cord blood CD34+ cells, Symplekin was detected after immunoprecipitation with the MOZ antibody (Supplemental Fig. 1C), indicating that the two proteins interact in normal hematopoietic stem/progenitor cells. In HEK293T cells, endogenous Symplekin and MOZ proteins interact with each other (Fig. 1C). We confirmed this interaction with exogenous epitope-tagged proteins. The Flag-tagged Symplekin co-immunoprecipitates the c-Myc-tagged MOZ from extracts of HEK293T cells co-transfected with the corresponding expression vectors (Fig. 1D).

To determine a direct interaction, we carried out *in vitro* binding assays. *In vitro* translated c-Myc-MOZ, MLL, Flag-Symplekin and Flag-

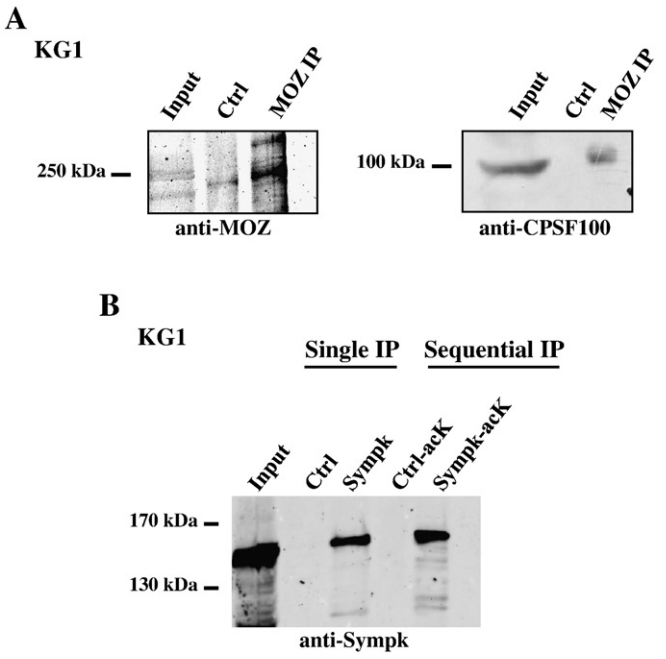

Fig. 2. (A) MOZ interacts with CPSF100 in KG1 cells. IP was performed with anti-MOZ, then immunoprobed with anti-MOZ or anti-CPSF100. (B) Symplekin is acetylated. A first IP was performed with anti-Sympk, eluated, then a second IP was performed with anti-acetylated lysine (anti-acK) and immunoprobed with anti-Sympk.

Nter-Symplekin biotinylated (biot) or not (unbiot) were used as indicated in the figure (Fig. 1E–G). Symplekin pulldown co-precipitates c-Myc-MOZ, indicating that MOZ and Symplekin interact directly (Fig. 1E, right and left panel). Otherwise, Nter-Sympk cannot interact with MOZ, suggesting that the N-terminal extremity of Symplekin is not involved in this interaction (Fig. 1E, middle and left panel). MOZ pulldown leads to the precipitation of Symplekin, confirming the direct interaction between these two proteins (Fig. 1F). We then tested if MLL interacts directly with Symplekin. Biotinylated MLL pulldown precipitates neither Symplekin nor Nter-Sympk (Fig. 1G), indicating that the interaction we detect in KG1 cells is not direct. MOZ and MLL interact, thus the interaction we detect between MLL and Symplekin may be achieved through MOZ.

In mammals, effective cleavage requires four multi-subunit complexes including CPSF (cleavage and polyadenylation specificity factor) [29]. CPSF is required for the polyadenylation step. As symplekin exists in a complex with CPSF100, we decided to examine a potential interaction between MOZ and CPSF100. CPSF100 is found to interact with MOZ (Fig. 2A) in KG1 cells, suggesting that various proteins of the polyadenylation complex could be associated with MOZ.

Since MOZ is known to acetylate not only histones, *e.g.* RUNX1 [19], we then examined whether the interaction observed between Symplekin and MOZ could lead to its acetylation. A first IP performed using anti-Symplekin followed by subsequent IP with an acetylated lysine antibody suggests its acetylation (Fig. 2B). This post-translational modification may result from its direct interaction with MOZ.

Despite its clear role in mRNA polyadenylation, Symplekin has been first identified in tight junctions and its sub-cellular localization can change depending on the cell type [30–32]. We carried out double staining of MOZ with Symplekin in KG1 cells (Fig. 3A). Symplekin, which appears to be confined in the nucleus, co-localizes with MOZ. Co-localization was confirmed in HEK293T cells, either using endogenous proteins or exogenously expressed tagged proteins, c-Myc-MOZ and Flag-Symplekin (Fig. 3B, C).

Altogether, these results demonstrate the association between Symplekin, MOZ and MLL.

3.2. Symplekin modulates HOXA9 in immature hematopoietic cells

Symplekin is an essential member of the polyadenylation machinery, supporting its assembly and its activity [27,33]. In addition, Symplekin has been described as a transcription co-regulator through its association with transcription factors such as ZONAB/DbpA and HSF1 [34,35]. Since we previously showed that MOZ and MLL cooperate to activate

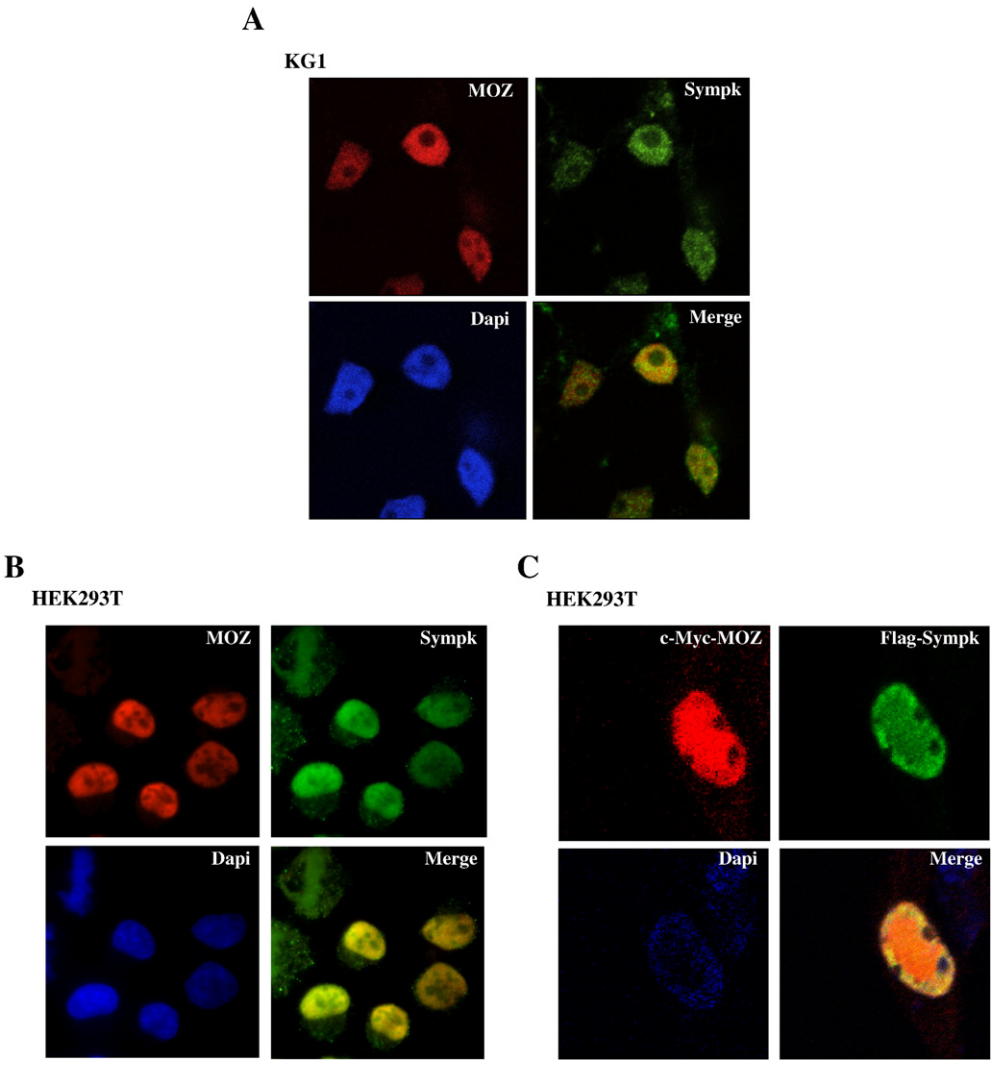

Fig. 3. Symplekin co-localizes with MOZ. (A) MOZ was stained with anti-MOZ and Symplekin with anti-Sympk in KG1 cells. Nuclei were counterstained with DAPI. The merge is the overlay of both images (scale bar: 9 μm). (B) MOZ was stained with anti-MOZ and Symplekin with anti-Sympk in HEK293T cells. Nuclei were counterstained with DAPI. The merge is the overlay of both images (scale bar: 9 μm). (C) Flag-Sympk or c-Myc-MOZ vectors were transfected into HEK293T cells. c-Myc-MOZ was stained with anti-c-Myc and Flag-Symplekin with anti-Flag. Nuclei were counterstained with DAPI. The merge is the overlay of both images (scale bar: 6 μm).

HOXA9 transcription, we thus examined whether the interaction of Symplekin with MOZ and MLL could affect *HOXA9* expression. To investigate the potential recruitment of Symplekin onto the *HOXA9* promoter, we carried out ChIP analyses in KG1 cells. After IP, the segment of the *HOXA9* promoter is amplified, indicating that Symplekin, like MOZ and MLL, is recruited onto the *HOXA9* promoter (Fig. 4A). Sequential ChIP demonstrates that Symplekin is simultaneously recruited with MOZ or MLL (Fig. 4B, C). Therefore, Symplekin, MOZ and MLL associate with the *HOXA9* locus in KG1 cells. These results may suggest that Symplekin is a regulator for *HOXA9* transcription.

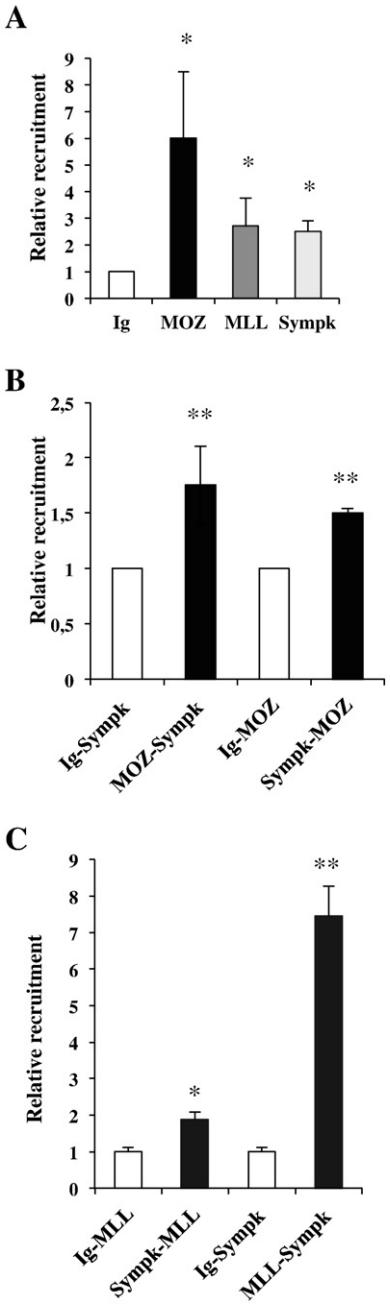

Fig. 4. Symplekin is recruited onto *HOXA9* promoter in KG1 cells. (A) The occupancy of MOZ, MLL and Sympk at the *HOXA9* promoter was measured by ChIP (n = 3). ChIP analyses examining the MOZ, MLL, and Symplekin recruitment on *HOXA9* promoter in KG1 cells were performed. The enrichment of MOZ, MLL, and Symplekin was measured by Q-PCR. The fold enrichment corresponds to the calculated ratio between the recruitment of the different proteins or modifications (values obtained by the standard curve method) and the input, normalized by the result obtained with the IgG. (B, C) MOZ or MLL are simultaneously recruited with Symplekin. For sequential ChIP experiments (n = 3), a second step of protein immunoprecipitation was carried out after the first elution.

We thus analyzed *HOXA9* expression in absence of *Symplekin* or *Moz* in KG1 cells (Supplemental Fig. 2). Surprisingly, *Hoxa9* mRNA is not affected by Symplekin down-regulation (Fig. 5A) while the HOXA9 protein level is markedly decreased (Fig. 5B). In contrast, the inhibition of *Moz* induces a down-regulation of *HOXA9* mRNA and protein level (Fig. 5A,B).

Polyadenylation, a multi-step processing consisting in the cleavage of the 3′ end of the mRNA and the synthesis of the poly(A) tail, is determinant for RNA stability or export and translation efficiency. The role of Symplekin in polyadenylation could explain why the HOXA9 protein level decreases whereas *Hoxa9* mRNA is not affected. A PCR-based assay was used to assess the effect of Symplekin on *Hoxa9* RNA 3′ poly(A) tail length. Total RNA from KG1 cells transfected with siRNA targeting *Symplekin* (si*Sympk*) or control siRNA (si*Ctrl*) (Supplemental Fig. 2) was retro-transcribed and annealed with two different primer pairs reflecting the length of the poly(A) tail of *Hoxa9* (Supplemental Fig. 3). Experiments with si*Ctrl* or si*Moz* were performed as controls. There is a marked reduction in the length of the poly(A) tail with si*Sympk* compared to that of the controls (Fig. 6A). Since polyadenylation is a determining element for translation efficacy, decreased polyadenylation by si*Sympk* could explain reduced levels of the HOXA9 protein.

Polyadenylation is crucial for RNA stability. We tested whether knockdown of Symplekin enhanced *Hoxa9* mRNA degradation. KG1 cells transfected either with the control siRNA or si*Sympk* were incubated with actinomycin D, which interferes with the process of transcription. The decay of *Hoxa9* mRNA was measured from 10 min to 4 h (Fig. 6B).

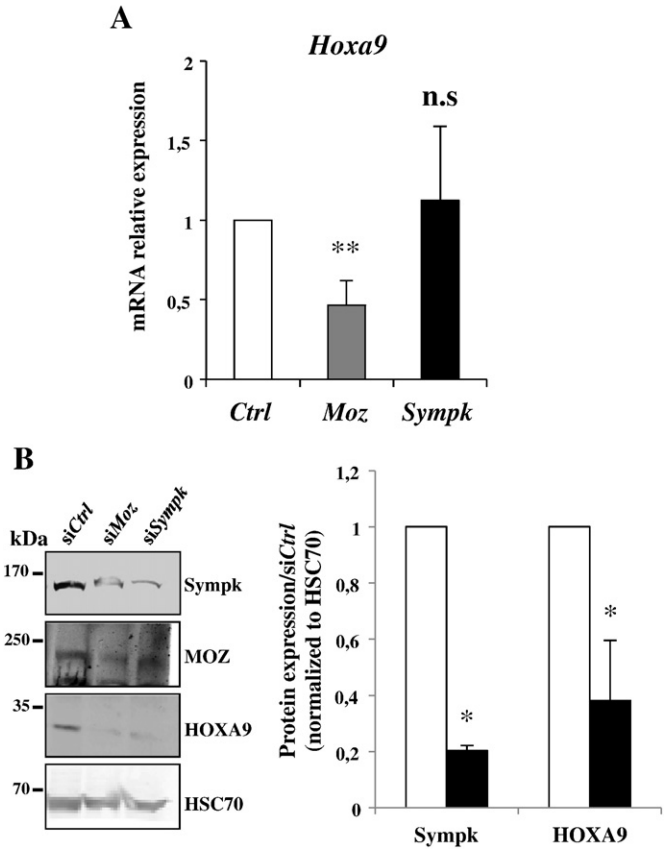

Fig. 5. Symplekin affects HOXA9 protein level in KG1 cells. (A) Symplekin impacts *HOXA9* expression. Cells were transfected with si*Ctrl*, si*MOZ* or si*Sympk*. *HOXA9* expression was measured 24 h after transfection by RQ-PCR (bar graphs: error bars represent standard deviation) (n = 5). (B) Symplekin affects HOXA9 protein level. Cells were transfected with si*Ctrl*, si*MOZ* or si*Sympk*. *HOXA9* expression was measured 24 h after transfection by immunoblotting performed with anti-Sympk, anti-MOZ, anti-HOXA9 or anti-HSC70. One independent experiment out of three is shown (left panel). Quantitation of HOXA9 and Symplekin proteins from three independent experiments are also shown (bar graphs: error bars represent standard deviation) (right panel).

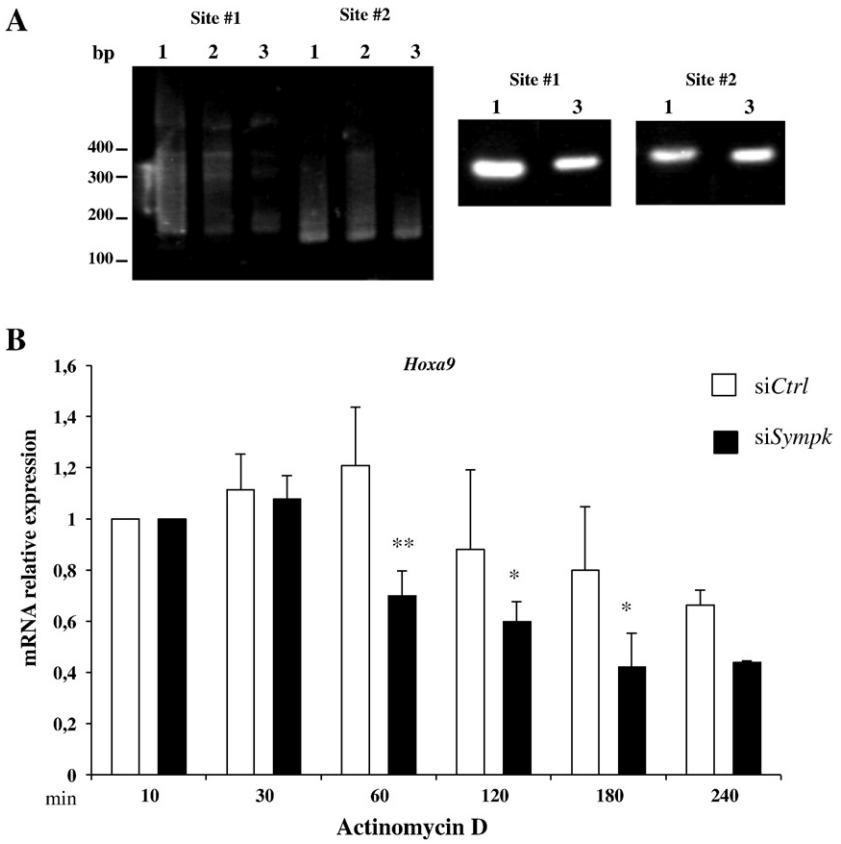

Fig. 6. Symplekin acts on polyadenylation of *Hoxa9* mRNA. (A) Impairment in *Hoxa9* polyadenylation after Symplekin knockdown. Cells were transfected with si*Ctrl* (1), si*MOZ* (2) or si*Sympk* (3). The amplified products corresponding to polyadenylated *Hoxa9* (left panel) or controls (right panels) were analyzed by PAGE. (B) Cells were transfected with si*Ctrl* or si*Sympk* and treated for 4 h with actinomycin D, 24 h after transfection. *Hoxa9* mRNA level was measured by RQ-PCR. Error bars represent standard deviation (n = 3).

After 1 h of treatment, *Hoxa9* mRNA levels were significantly lower in si*Sympk*-treated cells than in the control cells. Therefore, a lack of *Symplekin* decreases *Hoxa9* mRNA stability. This is consistent with the impairment of *Hoxa9* mRNA polyadenylation observed after *Symplekin* knockdown. From this result we could expect a decrease in global *Hoxa9* mRNA level after *Symplekin* inhibition. However, we observed no variation of *Hoxa9* mRNA level (Fig. 5A). We decided to clarify this phenomenon since a specific molecular mechanism may counteract the higher degradation of *Hoxa9* mRNA.

3.3. Symplekin affects MOZ and MLL recruitment on HOXA9 promoter

We focused our work on the transcriptional effects of Symplekin on *HOXA9*. Cells were transfected with si*Sympk*, si*Moz* or si*Ctrl*, followed by ChIP analysis of the *HOXA9* promoter. As expected from our previous report [24], binding of MOZ or MLL to the *HOXA9* promoter is decreased after *Moz* knockdown in KG1 cells, leading to a fall in histone modifications catalyzed by MOZ and MLL, *i.e.* AcH3K9, AcH3K14, Me2H3K4 or Me3H3K4 (Fig. 7A, B and Supplemental Fig. 4). Interestingly, contrary to what is observed in the intron of *HOXA9* (Supplemental Fig. 5), *Symplekin* inhibition induces an over-recruitment of MOZ or MLL onto the *HOXA9* promoter (Fig. 7A and Supplemental Fig. 4). Meanwhile, the level of the corresponding post-translational modifications is increased (Fig. 7B and Supplemental Fig. 4). To test if the accrued recruitment of MOZ and MLL is associated with an activation of *HOXA9* transcription, we assessed the phosphorylation status the C-terminal domain (CTD) of the RNA-polymerase II (CTD-RNA-PolII) by ChIP assays. Indeed, when transcription is initiated, this domain is phosphorylated, thus initiation of transcription is associated with an increase in CTD-RNA-PolII phosphorylation on the serine 5 onto target promoters. After *Symplekin* knockdown, we can observe a rise in the serine 5-phosphorylated form of the CTD-RNA-PolII onto *HOXA9* promoter (Fig. 7C). These results suggest an increase in *HOXA9* transcription after *Symplekin* inhibition through an accrued recruitment of MOZ and MLL. Thus, depletion of *Symplekin* leads to a balance between a more important *Hoxa9* mRNA degradation and a boosted transcription, making clear the constant global level of *Hoxa9* mRNA.

4. Discussion

HOXA9 is a homeobox transcription factor with pivotal functions in embryogenesis, hematopoiesis and leukemogenesis [36]. *HOXA9* is greatly expressed in mouse and human primitive blood cells, and downregulated as multipotent hematopoietic progenitors are committed into unipotent progenitors and mature cells [37–39]. High levels of *HOXA9* expression are very frequently observed in leukemic cells harboring the rearranged MLL gene [1] since *HOXA9* is a direct target gene for MLL chimeric proteins [40]. Its enhanced expression has also been demonstrated to be crucial for proliferative advantage and survival in leukemic cells [41], and it is related to poor prognosis for patients with acute myeloid leukemia [42]. We have previously reported a functional cooperation between MOZ and MLL, leading to the transcriptional activation of the *HOXA9* in human hematopoietic stem/progenitor cells [24].

In this work we identified Symplekin as a new MOZ and MLL partner, and we showed that Symplekin prevents their recruitment onto *HOXA9*.

Pta1, the yeast counterpart of Symplekin, a member of the polyadenylation machinery, is necessary for both cleavage and polyadenylation tail synthesis [43]. Pta1 is known to interact with the CTD-RNA-PolII [44] and with Ssu72, a CTD-RNA PolII phosphatase [45], linking transcription initiation to polyadenylation [46], and

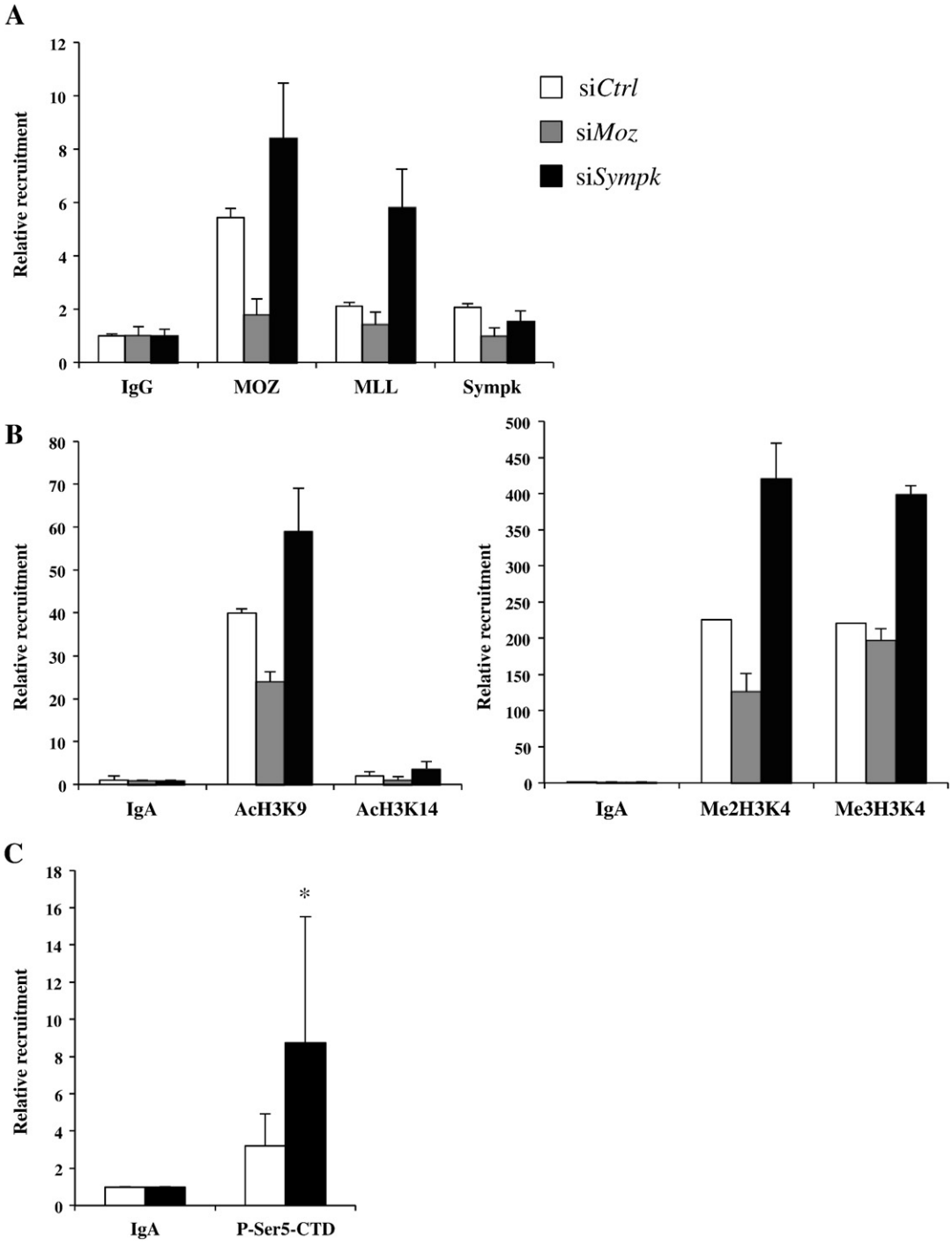

Fig. 7. Symplekin prevents MOZ and MLL recruitment on *HOXA9* promoter. ChIP experiments were carried out in cells transfected with si*Ctrl*, si*MOZ* or si*Sympk*. Cells were transfected once with 500 pmol of Ctrl siRNA, MOZ siRNA or Sympk siRNA. Then, ChIP analyses examining MOZ, MLL, Symplekin or phosphorylated CTD-RNA-PolII recruitment and the status of histone modifications on *HOXA9* promoter in KG1 cells were performed 48 h later. The enrichment of MOZ, MLL, Symplekin and posttranslational modifications of histones was measured by Q-PCR. The fold enrichment corresponds to the calculated ratio between the recruitment of the different proteins or modifications (values obtained by the standard curve method) and the input, normalized by the result obtained with the IgG. (A) This panel corresponds to ChIP measuring MOZ, MLL or Symplekin occupancy (one out of five independent experiments). (B) These panels represent post-translational modifications of histones (one out of four independent experiments). (C) This panel corresponds to ChIP measuring the occupancy of the phosphorylated CTD-RNA-PolII. Error bars represent standard deviation ($n = 3$).

improving gene-looping, a mechanism which promotes transcription [47]. *In vitro* studies performed with the N-terminal domain of human Symplekin revealed that it also interacts with human Ssu72 and the CTD of RNA PolII [48] to stimulate polyadenylation when processing is coupled with transcription.

Symplekin has also been described as a transcriptional co-regulator *via* its association with several transcription factors. In case of cell stress, Symplekin is recruited onto the *HSP70* promoter through its interaction with HSF1 (heat shock factor 1) promoting the polyadenylation linked to transcription. Thus, *Hsp70* mRNA newly transcribed is more efficiently processed, and its translation rate is improved [35]. In contrast, Symplekin is an important co-factor for ZONA/B, a repressive transcription factor, and altogether they silence *RUNX1* expression in undifferentiated intestinal cells [34]. In this case, Symplekin is crucial

for ZONA/B recruitment onto the *RUNX1* promoter. However, its role in polyadenylation was not explored. Interestingly, our results indicate that human endogenous Symplekin is bound onto a specific promoter and acts as a repressive co-regulator to prevent the recruitment of the well-known transcription co-activators MOZ and MLL (Fig. 7). Our study is in contradiction with the idea that Symplekin may be involved in the activation of transcriptional machinery. Pta1 representing ~50% of Symplekin in size and sharing only 31% of similarity [26], this could explain mechanistic differences reported by this study and other reports. Finally, a differential interaction network promoter-dependent targeted by Symplekin may also influence its transcriptional activity (co-repressor *versus* co-activator) [34,35].

Symplekin's post-translational modifications could highlight its contradictory role. Indeed, its sumoylation is essential for its activity as a polyadenylation factor [49]. Since we have shown that Symplekin is also acetylated, further studies on these modifications will be necessary to explain its complex role in regulating transcription and polyadenylation.

Altogether, these findings indicate that Symplekin exhibits a transcriptional role by controlling the function of MOZ-MLL in human immature hematopoietic cells.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.08.013.

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