Molecules and Cells

AURKA Suppresses Leukemic THP-1 Cell Differentiation through Inhibition of the KDM6B Pathway

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Aberrations in histone modifications are being studied in mixed-lineage leukemia (MLL)-AF9-driven acute myeloid leukemia (AML). In this study, we focused on the regulation of the differentiation of the MLL-AF9 type AML cell line THP-1. We observed that, upon phorbol 12-myristate 13-acetate (PMA) treatment, THP-1 cells differentiated into monocytes by down-regulating Aurora kinase A (AURKA), resulting in a reduction in H3S10 phosphorylation. We revealed that the AURKA inhibitor alisertib accelerates the expression of the H3K27 demethylase KDM6B, thereby dissociating AURKA and YY1 from the *KDM6B* promoter region. Using Flow cytometry, we found that alisertib induces THP-1 differentiation into monocytes. Furthermore, we found that treatment with the KDM6B inhibitor GSK-J4 perturbed the PMA-mediated differentiation of THP-1 cells. Thus, we discovered the mechanism of AURKA-KDM6B signaling that controls the differentiation of THP-1 cells, which has implications for biotherapy for leukemia.

Keywords: AURKA, differentiation, KDM6B, MLL-AF9 AML, THP-1

INTRODUCTION

The mixed-lineage leukemia (MLL) protein fuses with more than 60 partners, ranging from transcription factors to cytoplasmic structural proteins. AF9 is the most common fusion partner, found in 30% of MLL-rearranged acute myeloid leukemia (AML) (Meyer et al., 2009; 2013). MLL-AF9 leukemia, which accounts for 10% of acute leukemia cases, is generally associated with a poor prognosis (Krivtsov and Armstrong, 2007). THP-1 cells are widely used for research on MLL-AF9-driven AML. THP-1 is a well-known acute monocytic leukemia cell line that exhibits similarities to primary human monocytes and differentiates into macrophages upon treatment with external stimuli, such as phorbol esters or vitamin D (Auwerx, 1991). Notably, prevalent MLL fusion proteins, including MLL-AF4, MLL-AF9, MLL-ENL, and MLL-ELL, cause leukemia through the dysregulation of MLL target gene expression, implying that inhibitors of MLL fusion oncoproteins, including DOT1L and BRD4 inhibitors, could be potential therapeutic agents (Daigle et al., 2013; Gilan et al., 2016). In a recent study, ATRA induced the granulocytic differentiation of acute promyelocytic leukemia (Nowak et al., 2009). Differentiation therapy forces malignant cells to undergo terminal differentiation, and then the differentiated cells can be treated. However, little is known about the differentiation mechanisms in MLL fusion protein-driven leukemic THP-1 cells.

Aurora kinase (AURK) A is a serine/threonine protein kinase that is amplified in many malignancies, including AML (Gu et al., 2007; Huang et al., 2008; Sakakura et al., 2001). The AURK family mediates mitotic phosphorylation of histone

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H3 serine10 (H3S10) in budding yeasts and nematodes. In particular, AURKA shows outstanding kinase activity for H3S10 (Crosio et al., 2002; Hsu et al., 2000). Remarkably, H3S10 phosphorylation is associated with mitotic chromatin condensation in early G2 phase, as well as with cell cycle progression (Hendzel et al., 1997; Van Hooser et al., 1998). A recent study suggested that AURKA can regulate the transcriptional levels of target genes through its catalytic activity (Kim et al., 2016). Alisertib (also known as MLN8237) is a novel, selective inhibitor of AURKA, which has shown remarkable anticancer effects against solid tumors and hematologic malignancies (Gorgun et al., 2010; Sehdev et al., 2012; Venkataraman et al., 2012).

Lysine (K) demethylase 6 (KDM6) family proteins possess histone H3 lysine 27 (H3K27) demethylase activity (Hong et al., 2007). Both KDM6A and KDM6B have tumor suppressive properties, such as the regulation of HOX genes, which are well-known oncogenic markers of MLL-rearranged leukemia (Agger et al., 2007). Previous studies have indicated that KDM6A is easily targeted in several types of MLL fusion leukemia cell lines with somatic mutations, and that KDM6B up-regulates the cell cycle inhibitor p21, resulting in cell cycle arrest (van Haaften et al., 2009; Zhao et al., 2013).

In this study, we investigated the role of AURKA-mediated leukemia cell differentiation via the modulation of KDM6B expression in MLL-AF9 AML cells. We demonstrated that AURKA represses KDM6B expression by interacting with YY1 and through KDM6B-dependent p21 activation. Using chromatin immunoprecipitation (ChIP) assays, we showed that AURKA and YY1 are dissociated to the *KMD6B* promoter in PMA-treated THP-1 cells. Furthermore, we found that alisertib induced leukemic THP-1 cell differentiation and that GSK-J4 repressed leukemia cell differentiation. The combined results of this study provide the evidence that AURKA plays a role in leukemogenesis via the repression of KDM6B expression.

MATERIALS AND METHODS

Cell culture

THP-1 cells were grown in RPMI-1640 and HEK 293T cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum and 0.05% penicillin-streptomycin at 37℃ in a 5% CO_2 atmosphere. For differentiation, THP-1 cells (2×10^7) were seeded in 100-mm plates and treated with 100 ng/ml PMA (Sigma-Aldrich) or DMSO (Duksan). After incubation for 48 h, the cells were harvested for experiments. For the inhibition of AURKA or KDM6B, THP-1 cells (4×10^6) were seeded in 60-mm plates and treated with 0.3 μM alisertib (LKT Laboratories) or 5 μM GSK-J4 (Cayman Chemical). After incubation for 24 or 48 h, the cells were collected and used in experiments.

Plasmid constructs

The plasmids pCMV3-Flag-GATA1 and -YY1 (Han et al., 2015; Son et al., 2012), pGFP-AURKA (Kim et al., 2016a), and pGL3-p21 have been described previously (Oh et al., 2014). The *KDM6B* promoter region was amplified from human genomic DNA using the primer pairs listed in Supplementary Table 1, then inserted into the *Nhe*I/*Hin*dIII site of the pGL3-Basic Vector (Promega). The shRNAs against human *AURKA* and *KDM6B* were designed using siRNA sequence designer software (Clontech). Double-stranded oligonucleotides for shRNA plasmid construction were produced using 5′-to-3′ primers (Supplementary Table 1). The oligonucleotides were inserted into the *Age*I/*Eco*RI site of the pLKO.1 TRC vector.

Western blot

Total proteins were isolated from the cells using RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, 0.5% SDC, 1% NP-40, and 1 mM EDTA). Western blot analysis was conducted using the indicated antibodies.

Antibodies

Antibodies against GAPDH (CSB-PA00025A0Rb; Cus Ab); AURKA (sc-373856), YY1 (sc-7341), p21 (sc-397), and H3 (sc-8654) (Santa Cruz Biotechnology); H3K4me2 (07-030), H3K9me2 (07-441), H3K27me2 (07-452), and H3K36me2 (07-274) (Millipore); and H3S10ph (9701S) (Cell Signaling Technology) were used for western blot and ChIP analyses.

Histone purification

The cells were harvested and washed once with PBS, then the cell pellets were resuspended in 1 ml of TEB lysis buffer (0.5% Triton™ X-100, 2 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail) and incubated for 30 minutes at 4℃ to promote hypotonic swelling. The cells were then recovered by centrifugation, resuspended in 400 μl of 0.4 N H_2SO_4, and incubated on a rotator overnight at 4℃. After the centrifugation, the supernatant was transferred and 132 μl of 100% TCA was added for 2 h at 4℃. The pellet was recovered by centrifugation and washed twice with acetone. The pellet was resuspended in deionized water.

Quantitative real time-PCR

Total RNA was isolated from the cells using RNAiso Plus (TaKaRa). For complementary DNA (cDNA) synthesis, we denatured 1 μg of RNA and oligo dT primers (Invitrogen) via incubation at 70℃ for 5 min. cDNA was synthesized with M-MLV reverse transcriptase according to the manufacturer's instructions (Enzynomics). Reverse transcriptase catalytic activity was inactivated by incubating the cDNA at 95℃ for 30 s. The synthesized cDNA was subjected to mRNA expression analysis. Quantitative real time-PCR (qRT-PCR) was conducted using the SYBR® Green Supermix kit (TaKaRa). The amplification reaction was performed under the following conditions: 39 cycles of denaturation at 94℃, annealing at the temperatures specified for the primers in Supplementary Table 1, and extension at 72℃. The mean threshold cycle (Ct) and standard error values were calculated from the individual Ct values obtained from triplicate reactions per stage. The normalized mean Ct value was estimated as ΔCt by subtracting the mean Ct of *GAPDH*. The ΔΔCt value was calculated as the difference between the control ΔCt and the values obtained for each sample. The n-fold change in gene expression, relative to a control, was calculated as $2^{-\Delta\Delta Ct}$.

ChIP

THP-1 cells (1×10^7) were seeded in 100-mm plates and treated with 0.3 μM alisertib or DMSO. After 24 h, the cells were cross-linked with 1% formaldehyde, which was added to the medium for 10 min at room temperature, followed by the addition of 125 mM glycine for 5 min at room temperature, then lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris [pH 8.1]). The samples were sonicated and immunoprecipitated using the indicated antibodies. The immunoprecipitates were eluted and reverse cross-linked, after which the DNA fragments were purified using a PCR purification kit (Axygen). The precipitated DNA fragments were amplified using specific primers for the *KDM6B* promoter region via qRT-PCR. The following primer set was used: YY1-BS2 (forward, 5′-CTCCCTTTGGGGAAAGCTAA-3′ and reverse, 5′-TGATAAGAGTGCCCGCTACC-3′). The mean Ct and standard error values were calculated from the individual Ct values obtained from duplicates per stage. The normalized mean Ct was estimated as ΔCt by subtracting the mean Ct of the input.

Flow cytometric analysis of cell differentiation

To measure cell differentiation, THP-1 cells (1×10^6) were split into 35-mm dishes and treated with DMSO or 100 ng/ml PMA for 48 h. The cells were trypsinized, washed, and resuspended in cold PBS with 1 mM EDTA, 1% bovine serum albumin, and 10 mM sodium azide for 1 h. Before flow cytometric analysis, the cells were stained with PE-CD11b (12-0118-42) and APC-CD14 (17-0149-42) antibodies (eBioscience) for 30 min, washed using PBS with 1 mM EDTA and 1% bovine serum albumin, then subjected to flow cytometry using a BD Accuri™ C6 cytometer (BD Biosciences).

Luciferase assay

For the transcriptional activity assays, HEK 293T cells (2×10^4) were seeded in 48-well plates and co-transfected with the pGL3-*p21* promoter or pGL3-*KDM6B* promoter reporter plasmid and the indicated DNA constructs using polyethylenimine (Polysciences), or treated with 0.1 or 0.3 μM alisertib, or treated with 2 or 5 μM GSK-J4, for 24 h. After transfection, the cells were collected and subjected to a luciferase assay (Promega). The level of β-galactosidase activity was used to normalize the reporter luciferase. The data are expressed as the means of triplicates. All results shown are representative of at least 3 independent experiments.

Statistical analysis

The data are expressed as the mean ± SEM of 3 or more independent experiments. Statistical significance ($p < 0.05$) was calculated using functions in Microsoft Excel. The differences between the groups were evaluated by one-way analysis of variance, followed by Student's t-test or Bonferroni's test, as appropriate.

RESULTS

AURK-mediated H3S10 phosphorylation levels decreased during leukemia cell differentiation

Despite the wealth of knowledge regarding the pathogenesis of MLL-rearranged AML, few studies have explored histone modification-associated leukemia cell differentiation. We first identified the epigenetic changes during the differentiation of the MLL-AF9 AML cell line THP-1 after treatment with PMA. The differentiation of the THP-1 cells was confirmed by qRT-PCR for cell surface markers of macrophages, such as *CD14* and *CD11b (ITGAM)* (Fig. 1A). In previous study, differentiation and maturation of myeloid leukemia induces heterochromatin density (Smetana et al., 2011). Consistently, we found that the levels of H3K27me2, H3K27me3, and H3K9me2, which were closed chromatin marker, were significantly increased during the THP-1 differentiation (Fig. 1B). Interestingly, we also found that the level of H3S10 phosphorylation was significantly lower in THP-1 cells during differentiation (Fig. 1B). According to previous studies, AURK family proteins mainly regulate H3S10 phosphorylation during cell cycle progression and regulate gene expression during HL-60 differentiation (Crosio et al., 2002; Kim et al., 2016; Ota et al., 2002). We evaluated the expression levels of the AURK family members and found lower mRNA levels of *AURKA* and *AURKB* in PMA-treated THP-1 cells than in control cells (Fig. 1C). Moreover, we detected the down-regulation of AURKA protein upon PMA treatment in THP-1 cells (Fig. 1D). To investigate if the knockdown of AURKA has a role in THP-1 differentiation, we measured the effects of two different target shRNAs specific for *AURKA* on the differentiation of THP-1 cells. Similar to PMA treatment, knockdown of *AURKA* also induced increases in *CD14* and *CD11b* levels (Fig. 1E). Consistent with previous data, we obtained similar results of the levels of *CD14* and *CD11b* (Fig. 1F, left), and global histone modification (Fig. 1F, right) in alisertib-treated THP-1 cells. Overall, these data indicate that PMA treatment reduces AURKA expression and H3S10 phosphorylation in THP-1 cells, and that AURKA may be involved in differentiation.

AURKA regulates the transcription of KDM6B and p21 expression in PMA-treated THP-1 cells

Having determined the induction of THP-1 differentiation by alisertib treatment, we next analyzed the effect of AURKA on the transcriptional regulation of target genes. Using previously generated microarray data (Shechter et al., 2007), we found that leukemia-related gene expression changed during the differentiation of THP-1 cells by PMA. We selected 5 genes, which were up- or down-regulated by PMA treatment in THP-1 cells, and validated their expression levels by qRT-PCR. The transcription levels of *KDM6B*, *RFX2*, and *CDK19* were increased, but the levels of *CPT1A* and *SFRP1* were decreased, in PMA-treated THP-1 cells (Fig. 2A). Because we discovered that several histone modifications, including H3S10 phosphorylation and dimethylation of H3K27 and H3K9, were changed in PMA-treated THP-1 cells (Fig. 1B), we focused on the regulation of *KDM6B* expression in differentiated THP-1 cells. We first analyzed *KDM6B* mRNA levels in alisertib-treated THP-1 cells. As with PMA treatment, alisertib treatment increased *KDM6B* expression levels (Fig. 2B). Next, we determined if AURKA expression decreased the transcriptional activity of KDM6B. We conducted reporter assays using a *KDM6B*-luc construct in 293T cells. Alisertib

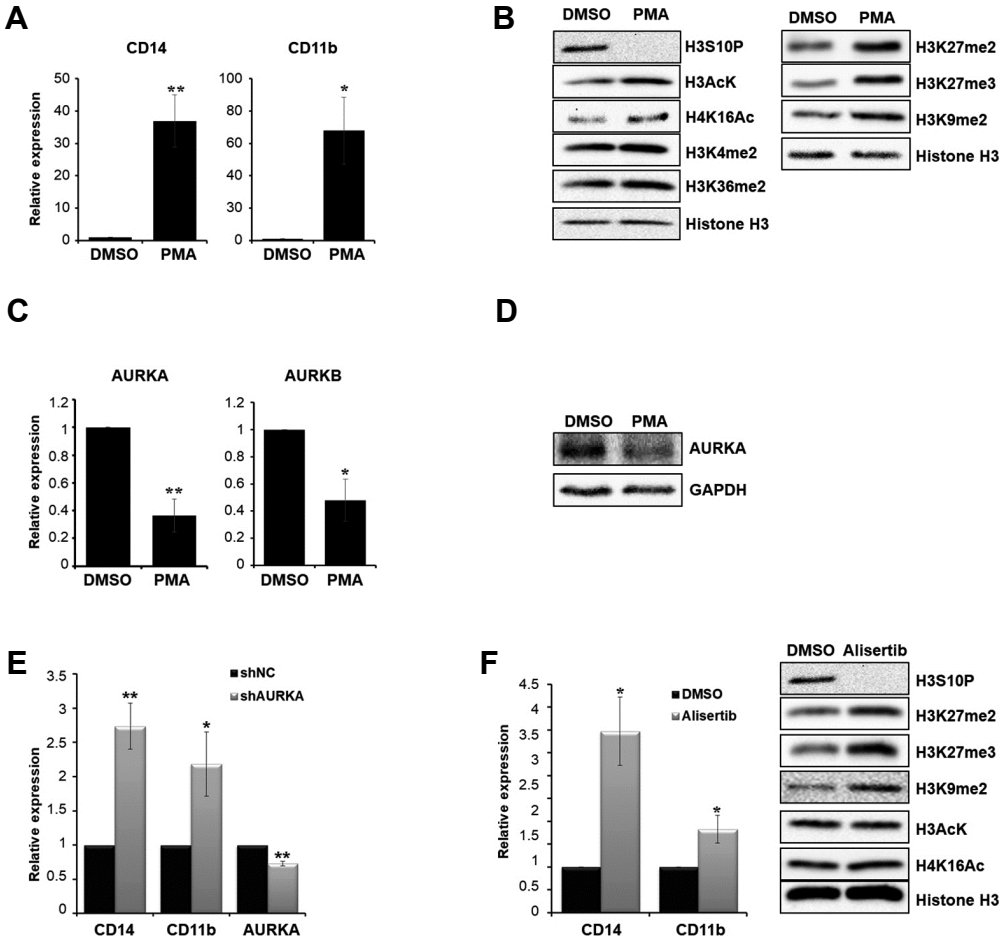

Fig. 1. Decreased levels of H3S10 phosphorylation during leukemia cell differentiation. (A) THP-1 cells were cultivated in the presence of 100 ng/ml PMA or DMSO for 48 h. *CD14* and *CD11b* expression levels were confirmed using qRT-PCR and normalized to *GAPDH*. Results are shown as mean ± SEM, n = 3; $*p < 0.05$, $**p < 0.01$. (B) After 100 ng/ml PMA treatment for 48 h, the levels of histone modifications during THP-1 differentiation were detected by western blot analysis with the indicated antibodies. H3 was used as a loading control. (C) During THP-1 differentiation, *AURKA* and *AURKB* expression levels were evaluated using qRT-PCR and normalized to *GAPDH*. Results are shown as mean ± SEM, n = 3; $*p < 0.05$, $**p < 0.01$. (D) THP-1 cells were grown in 100 ng/ml PMA for 48 h, lysed, then examined by western blot analysis using anti-AURKA antibody. GAPDH was used as a loading control. (E) *CD14* and *CD11b* expression levels were confirmed using qRT-PCR and normalized to *GAPDH* in THP-1 cells in which AURKA was stably knocked down. Results are shown as mean ± SEM, n = 3; $*p < 0.05$, $**p < 0.01$. (F) THP-1 cells were treated with alisertib or DMSO for 48 h. *CD14* and *CD11b* expression levels were confirmed using qRT-PCR and normalized to *GAPDH*. Results are shown as mean ± SEM, n = 3; $*p < 0.05$ (left). The levels of histone modifications were detected by western blot analysis with the indicated antibodies. H3 was used as a loading control (right).

induced transcriptional activity at the *KDM6B* promoter (Fig. 2C). However, AURKA reduced *KDM6B* promoter activity in a dose-dependent manner (Fig. 2D). A previous report showed that ectopic expression of p21 undergoes monocytic differentiation in peripheral blood monocytes (Asada et al., 1999). To determine if *p21* mRNA levels were also regulated by AURKA in THP-1 cells, we performed qRT-PCR in alisertib-treated THP-1 cells. The mRNA levels of *p21* were increased in alisertib-treated THP-1 cells (Fig. 2E). To investigate how AURKA regulates the *p21* transcriptional level, we tested AURKA transcriptional activity at the *p21* promoter in a dose-dependent manner. The promoter activity of *p21* was reduced in AURKA-overexpressed 293T cells (Fig. 2F). While *p21* promoter assay in HEK 293T cells treated with alisertib exhibited inactivation of AURKA enzymatic function contributes to promote *p21* transcription (Fig. 2G). These results suggest that AURKA regulates KDM6B and p21 expression during leukemia cell differentiation.

AURKA repressed transcription of KDM6B via YY1 recruitment in PMA-treated THP-1 cells

In order to determine the mechanism of KDM6B down-regulation by AURKA more precisely, we next searched for transcription factors involved in AURKA-mediated transcript-

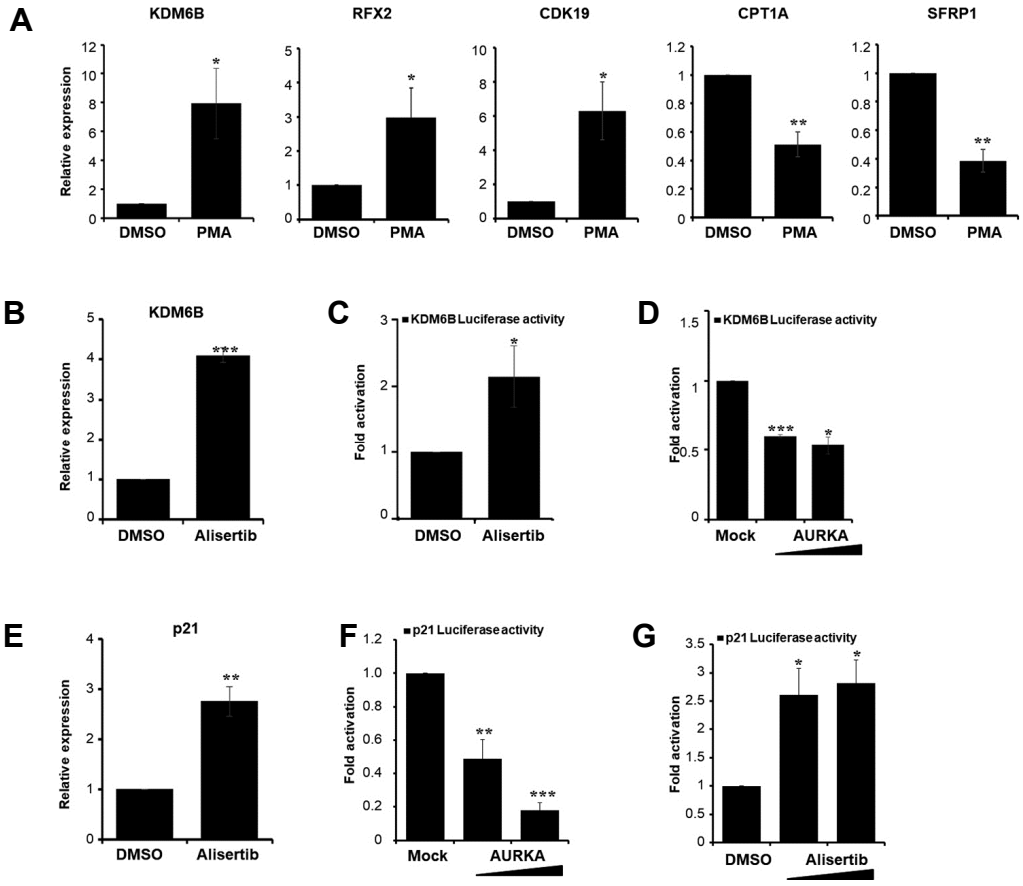

Fig. 2. Transcriptional regulation of *KDM6B* and p21 expression in PMA-treated THP-1 cells. (A) Treatment with 100 ng/ml PMA induced the differentiation of THP-1 cells. After 48 h, the expression levels of *KDM6B, RFX2, CDK19, CPT1A,* and *SFRP1* were quantified using qRT-PCR and normalized to *GAPDH.* Results are shown as mean ± SEM, n = 5; $*p < 0.05$, $**p < 0.01$. (B) THP-1 cells were treated with alisertib or DMSO for 48 h. *KDM6B* expression levels were confirmed using qRT-PCR and normalized to *GAPDH.* Results are shown as mean ± SEM, n = 3; $***p < 0.001$. (C) HEK 293T cells were transfected with the pGL3-*KDM6B* promoter reporter DNA construct. The cells were treated with alisertib or DMSO, and cell extracts were assayed for luciferase activity. The activity was normalized against that of β-galactosidase. Results are shown as mean ± SEM, n = 4; $*p < 0.05$. (D) HEK 293T cells were co-transfected with the pGL3-*KDM6B* promoter reporter and *AURKA* DNA construct. Cell extracts were assayed for luciferase activity and β-galactosidase was used for normalization. Results are shown as mean ± SEM, n = 4; $*p < 0.05$, $***p < 0.001$. (E) THP-1 cells were grown with alisertib or DMSO. After 48 h, we performed qRT-PCR and western blotting to compare the expression levels of p21. GAPDH was used as a control. Results are shown as mean ± SEM, n = 3; $**p < 0.01$. (F) HEK 293T cells were transfected with the pGL3-*p21* promoter reporter and *AURKA* DNA construct. The cell extracts were examined for luciferase activity, which was normalized to β-galactosidase. Results are shown as mean ± SEM, n = 4; $**p < 0.01$, $***p < 0.001$. (G) After pGL3-*p21* promoter reporter was ectopically expressed in HEK 293T cells, the cells were treated with alisertib (0.1 μM or 0.3 μM) or DMSO. After 24 h, the cells were lysed for luciferase analysis and normalized to β-galactosidase. Results are shown as mean ± SEM, n = 4; $*p < 0.05$.

tional regulation. We identified GATA1- and YY1-binding regions in the *KDM6B* promoter using PROMO, which predicts transcription regulatory elements (Fig. 3A) (Messeguer et al., 2002). To determine if AURKA cooperates with GATA1 or YY1 in the regulation of *KDM6B* expression, we investigated the interaction between AURKA and YY1 using co-immunoprecipitation assays in AURKA- and YY1-overexpressing 293T cells, which showed that AURKA reciprocally interacts with YY1 (Fig. 3B). However, we did not detect an interaction between AURKA and GATA1 in the co-immunoprecipitation assay (Fig. 3C). To determine the effects of AURKA and YY1 on *KDM6B* transcriptional activity, we conducted reporter assays using the *KDM6B*-luc promoter construct in 293T cells. As expected, AURKA reduced transcriptional activity at the *KDM6B* promoter, and YY1 caused a further reduction in transcriptional activity (Fig. 3D). Consistent with the interaction assay, GATA1 did not affect *KDM6B* transcriptional activity (Fig. 3E). We next investigated if AURKA and YY1 could bind to the endogenous promoter regulatory regions of the *KDM6B* gene. We conducted ChIP analysis using anti-AURKA and -YY1 antibodies after PMA treatment. The ChIP analysis showed that the recruitment

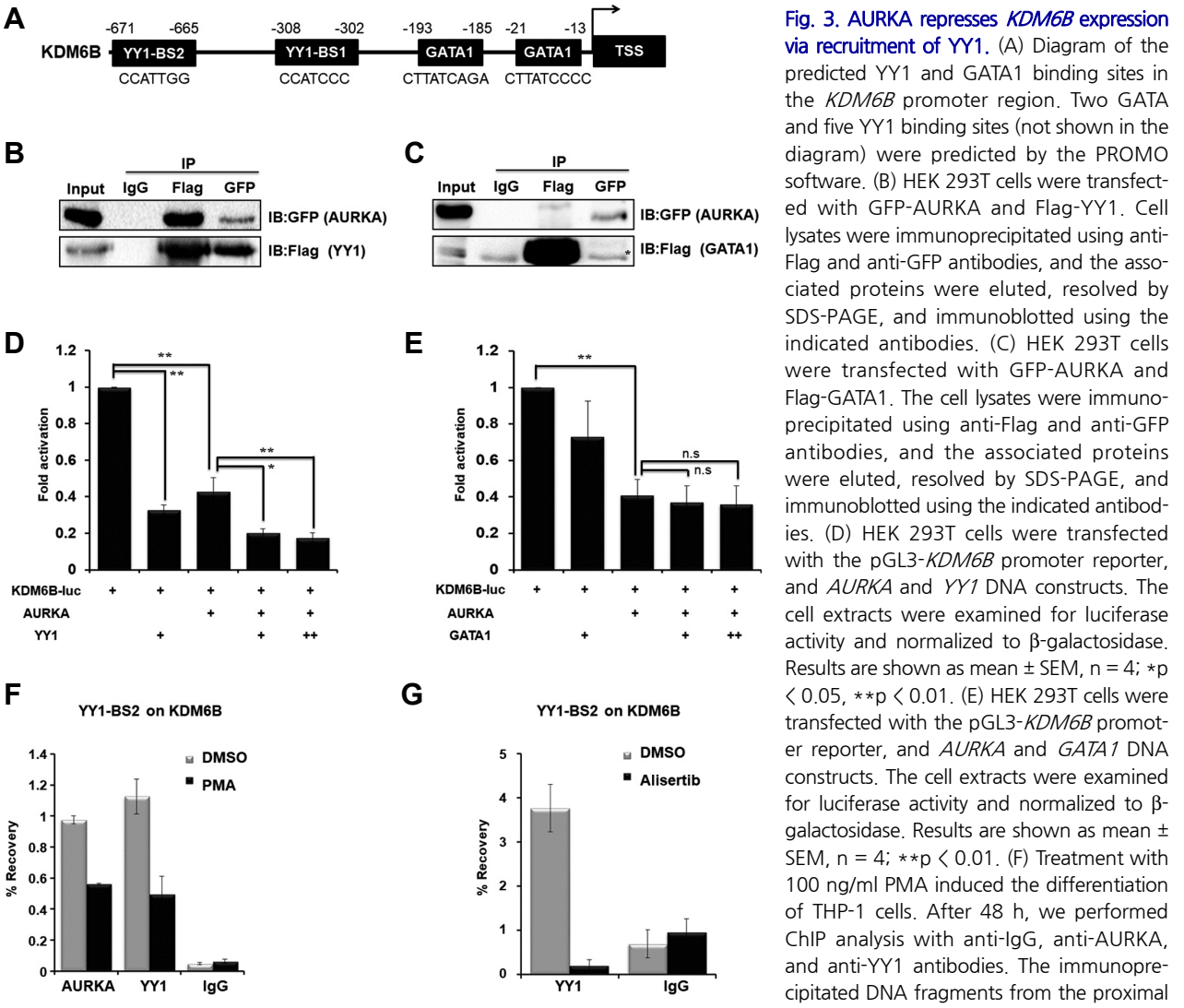

Fig. 3. AURKA represses *KDM6B* expression via recruitment of YY1. (A) Diagram of the predicted YY1 and GATA1 binding sites in the *KDM6B* promoter region. Two GATA and five YY1 binding sites (not shown in the diagram) were predicted by the PROMO software. (B) HEK 293T cells were transfected with GFP-AURKA and Flag-YY1. Cell lysates were immunoprecipitated using anti-Flag and anti-GFP antibodies, and the associated proteins were eluted, resolved by SDS-PAGE, and immunoblotted using the indicated antibodies. (C) HEK 293T cells were transfected with GFP-AURKA and Flag-GATA1. The cell lysates were immunoprecipitated using anti-Flag and anti-GFP antibodies, and the associated proteins were eluted, resolved by SDS-PAGE, and immunoblotted using the indicated antibodies. (D) HEK 293T cells were transfected with the pGL3-*KDM6B* promoter reporter, and *AURKA* and *YY1* DNA constructs. The cell extracts were examined for luciferase activity and normalized to β-galactosidase. Results are shown as mean ± SEM, n = 4; $*p < 0.05$, $**p < 0.01$. (E) HEK 293T cells were transfected with the pGL3-*KDM6B* promoter reporter, and *AURKA* and *GATA1* DNA constructs. The cell extracts were examined for luciferase activity and normalized to β-galactosidase. Results are shown as mean ± SEM, n = 4; $**p < 0.01$. (F) Treatment with 100 ng/ml PMA induced the differentiation of THP-1 cells. After 48 h, we performed ChIP analysis with anti-IgG, anti-AURKA, and anti-YY1 antibodies. The immunoprecipitated DNA fragments from the proximal region of *KDM6B* were analyzed by RT-PCR. Values represent mean ±SD of technical triplicates from a representative experiment. All experiments were performed three times with similar results. (G) After treatment with 0.3 μM alisertib for 24 h, we performed ChIP analysis with anti-IgG and anti-YY1 antibodies. The immunoprecipitated DNA fragments from the proximal region of *KDM6B* were analyzed by RT-PCR. Values represent mean ±SD of technical triplicates from a representative experiment. All experiments were performed three times with similar results.

of AURKA and YY1 to the target gene promoters was decreased in PMA-treated cells (Fig. 3F). Because alisertib has a similar effect to PMA treatment, we tested if alisertib reduced the recruitment of YY1 to the *KDM6B* promoter region. The ChIP analysis suggested that the AURKA inhibitor alisertib dissociated YY1 from the *KDM6B* promoter sites (Fig. 3G). Taken together, these results suggest that *KDM6B* expression levels are induced by the disruption of the enrichment of AURKA and YY1 at the target gene promoter during leukemia cell differentiation.

AURKA regulates PMA-dependent p21 activation via KDM6B pathway during leukemia cell differentiation

A previous report showed that the H3K27 demethylase activity of KDM6B promotes p21 expression in mouse embryonic fibroblasts (Zhao et al., 2013). To gain insight into KDM6B-mediated *p21* expression, we added the KDM6B inhibitor GSK-J4 in PMA-treated THP-1 cells and measured p21 expression. We identified that GSK-J4 markedly diminished p21 level compared to PMA treatment (Fig. 4A). To further determine the mechanisms underlying transcriptional regulation of *p21* via KDM6B, we conducted luciferase reporter assay using *p21*-luciferase reporter system in HEK 293T cells. Ectopic expression of KDM6B accelerated *p21* transcription (Fig. 4B), whereas KDM6B inhibition by GSK-J4 showed reduction of *p21* expression (Fig. 4C). We further investigated if AURKA reduces *p21* transcriptional activity via KDM6B demethylase activity. We found that the promoter

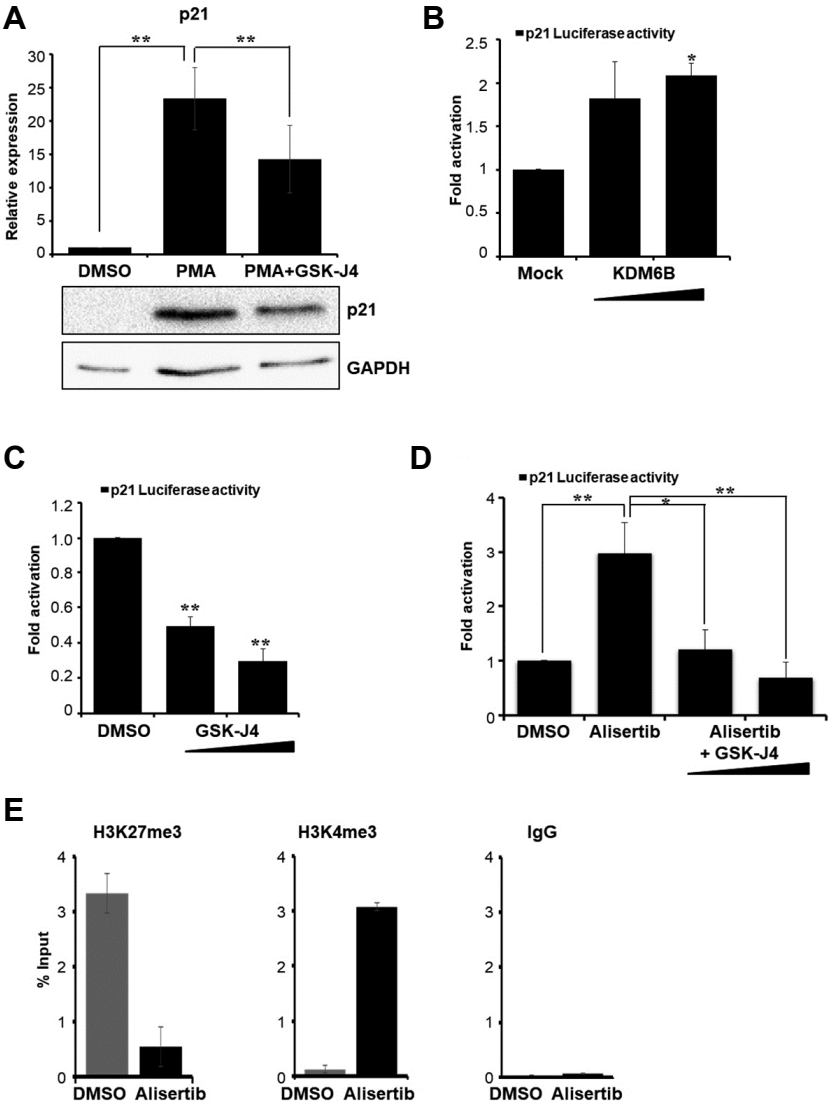

Fig. 4. The p21 expression was regulated by KDM6B during PMA-mediated THP-1 differentiation. (A) THP-1 cells were grown with 100 ng/ml PMA, 5 μM GSK-J4, or DMSO. After 48 h, qRT- PCR was performed to compare the expression level of p21 gene and normalized to GAPDH. Results are shown as mean ± SEM, n = 3; $^{**}p < 0.01$. (B) HEK 293T cells were co-transfected with the pGL3-*p21* promoter reporter and KDM6B DNA construct. Cell extracts were assayed for luciferase activity and to β-galactosidase was used for normalization. Results are shown as mean ± SEM, n = 4; $^{*}p < 0.05$. (C) pGL3-*p21* promoter reporter was ectopically expressed in HEK 293T cells. The cells were treated with GSK-J4 (2 μM or 5 μM) or DMSO. After 24 h, the cells were lysed for luciferase analysis and normalized to β-galactosidase. Results are shown as mean ± SEM, n = 4; $^{**}p < 0.01$. (D) pGL3-*p21* promoter reporter-overexpressed HEK 293T cells were treated with 0.3 μM alisertib, 2 or 5 μM GSK-J4, or DMSO. Luciferase activities of the cell lysates were quantified and normalized to β-galactosidase. Results are shown as mean ± SEM, n = 4; $^{*}p < 0.05$, $^{**}p < 0.01$. (E) ChIP analysis of p21 promoter in alisertib-treated THP-1 cells was conducted using anti-H3K27me3, anti-H3K4me3, and anti-IgG antibodies, and examined via qRT- PCR. The recruitment of each histone modification to the promoter region was normalized to input. Values represent mean ±SD of technical triplicates from a representative experiment. All experiments were performed three times with similar results.

activity of *p21* increased in alisertib-treated THP-1 cells, but the KDM6B inhibitor GSK-J4 disrupted the alisertib-dependent induction of p21 in a dose-dependent manner (Fig. 4D). To further explore the flow of AURKA and KDM6B-dependent alteration of *p21* expression, we estimated the methylation status of p21 promoter via ChIP assay. We observed that H3K27 methylation level was diminished at the *p21* promoter region in THP-1 cells by alisertib treatment (Fig. 4E). These data prove that AURKA functions as a transcriptional regulator of KDM6B and the AURKA-mediated KDM6B suppression subsequently inactivates p21.

KDM6B induces THP-1 cell differentiation

To confirm the role of KDM6B in the differentiation of THP-1 cells, we used the KDM6B inhibitor GSK-J4 in THP-1 cells. The expression levels of *CD11b* and *CD14* were slightly decreased compared to those in untreated cells upon GSK-J4 treatment (Fig. 5A). In addition, GSK-J4 strongly reduced the levels of *CD11b* and *CD14* in PMA-treated THP-1 cells (Fig. 5A). To further confirm that knockdown of KDM6B disrupts PMA-mediated THP-1 cell differentiation, we conducted flow cytometric analysis to measure the expression of the monocyte differentiation markers CD11b and CD14. We observed increased expression of CD11b and CD14 in PMA-treated THP-1 cells compared to control cells, but the expression of CD11b and CD14 did not change after PMA treatment in THP-1 cells in which *KDM6B* was stably knocked down (Fig. 5B). Previously, we confirmed that alisertib induces THP-1 differentiation to monocytes. Therefore, we tested if the inhibition of *KDM6B* repressed alisertib-mediated THP-1 differentiation. Flow cytometric analysis suggested that the inhibition of AURKA increased the expression of CD11b and CD14 by inducing THP-1 differentiation; however, GSK-J4 slightly repressed alisertib-mediated THP-1 differentiation (Fig. 5C). Taken together, these findings suggest

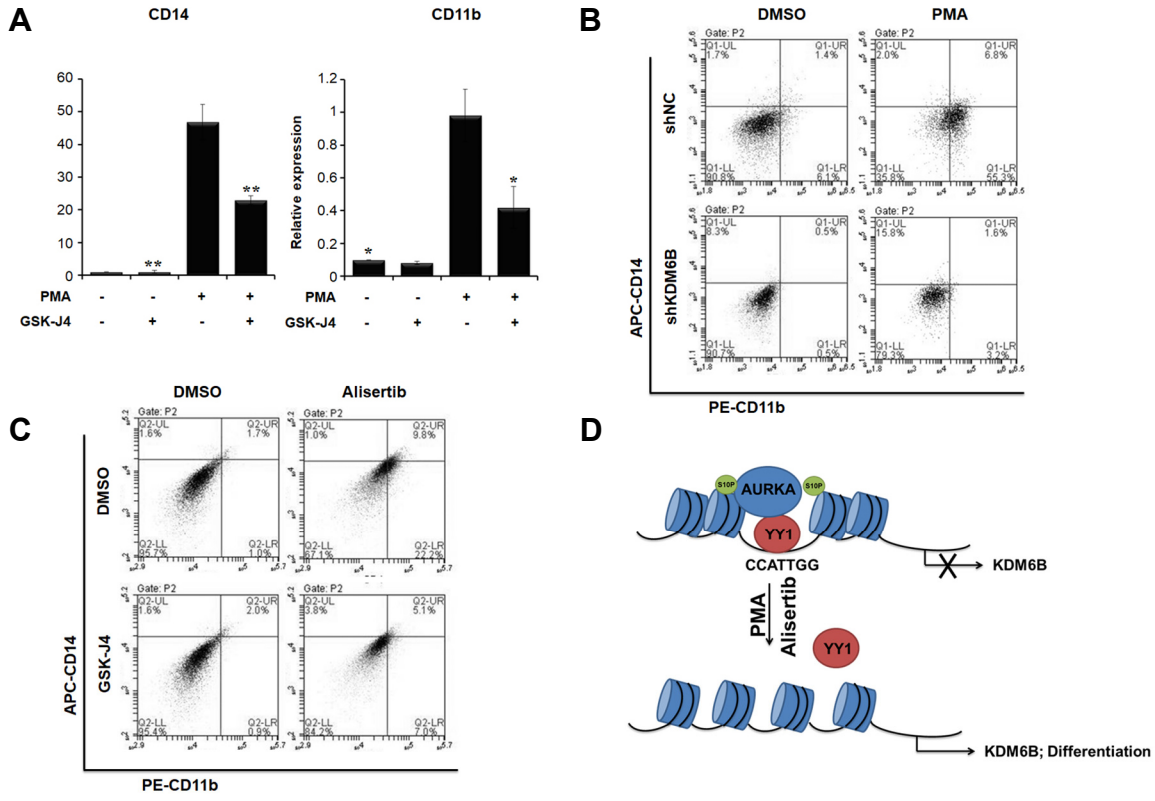

Fig. 5. KDM6B promotes the differentiation of THP-1 cells. (A) THP-1 cells were treated with 100 ng/ml PMA, 2 μM GSK-J4, or DMSO for 48 h. *CD14* and *CD11b* expression levels were confirmed using qRT-PCR and normalized to *GAPDH*. Results are shown as mean ± SEM, n = 3; ⁎p < 0.05, ⁎⁎p < 0.01. (B) We treated negative control (shNC)- and shKDM6B-transfected THP-1 cells with 100 ng/ml PMA for 48 h. The cells were stained with PE-CD11b and APC-CD14 antibodies. The percentage of cells in each quadrant is indicated in the figure. (C) We treated THP-1 cells with 2 μM GSK-J4 or 0.3 μM alisertib for 48 h. The cells were stained with PE-CD11b and APC-CD14 antibodies. The percentage of cells in each quadrant is indicated in the figure. (D) A model of AURKA regulating KDM6B expression in PMA-mediated THP-1 differentiation.

that KDM6B mediates the differentiation of THP-1 cells by a mechanism dependent on AURKA transcriptional regulation.

DISCUSSION

In this study, we showed that AURKA plays a role in THP-1 cell differentiation into monocytes. We found reduced AURKA expression during THP-1 cell differentiation, resulting in increased *KDM6B* and KDM6B-dependent p21 expression levels. We demonstrated the importance of the interaction between AURKA and YY1 for recruitment to the *KDM6B* promoter in PMA-treated THP-1 cells. We also defined the biological significance of AURKA-mediated KDM6B regulation in THP-1 cell differentiation. These results suggest that the loss of AURKA induces the dissociation of YY1 from the *KDM6B* promoter region in PMA-treated THP-1 cells and promotes KDM6B-mediated monocyte differentiation (Fig. 5D).

Previous study showed that Sox6-mediated K562 differentiation increases total hemoglobin content, but which is less than cells treated with hemin (Cantu et al., 2011). Consistently, we found that inhibitor or knockdown of AURKA slightly increases CD11b and CD14 levels (Fig. 1). A change of one factor might be not enough to fully differentiation. So, we hypothesized that AURKA mediated-THP-1 fully differentiation might be associated with regulation of other early response genes together.

AML is characterized by uncontrolled proliferation, cell cycle progression, and DNA hypermethylation (Plass et al., 2008). The DNMT family interact with HDAC1 and HDAC2, suggesting a relationship between histone acetylation and DNA methylation (Fuks et al., 2000; Rountree et al., 2000). DNMT inhibitors, such as decitabine and 5-azacitidine, induce terminal differentiation of AML, with anti-leukemic effects (Chang et al., 2016). Here, we discovered that several histone modifications are altered during the differentiation of THP-1 cells. The induction of H3 acetylation by PMA may inhibit DNA hypermethylation in AML and induce THP-1 differentiation. Previous studies showed that THP-1 cells undergo macrophage differentiation upon PMA treatment, which leads to cell cycle arrest in both G0/G1 and G2/M phase (Sugarman et al., 1995). In addition, accumulating evidence has highlighted the role of p21 in irreversible G1 arrest in MLL-fusion AML (Tonelli et al., 2006). Moreover,

AURKA has kinase activity for CENP-A, TPX2, and p53 and is involved in mitotic entry and bipolar spindle formation (Gautschi et al., 2008). The loss of AURKA results in cell cycle arrest in the G2/M phase (Liu and Ruderman, 2006). Our finding indicated that the differentiation of THP-1 cells by PMA reduces AURKA expression, followed by the induction of KDM6B and p21 expression. These changes in gene expression may be responsible for the cell cycle arrest in both G0/G1 and G2/M phases.

THP-1 cells is an MLL-AF9-driven leukemia; its MLL possesses a DNA-binding domain, but has lost its H3K4 methyltransferase activity. MLL-AF9 recruits DOT1L to the MLL target site, leading to dysregulation of target gene expression, followed by leukemia. Several studies have demonstrated that inhibitors of MLL fusion proteins, including DOT1L, BRD4, MOF, and SIRT, function as potential therapeutic agents (Chen et al., 2015; Valerio et al., 2017; Zuber et al., 2011). In addition, the differentiating agent ATRA has been co-administered with a hyperacetylating agent (an HDAC inhibitor) in attempted epigenetic therapies for AML (Warrell, 1999). Our findings indicate that the irreversible G0/G1 arrest via the KDM6B-dependent p21 pathway is accelerated during THP-1 differentiation, which is consistent with the alleviation of the leukemogenic status of THP-1 cells.

In this study, we discovered that inhibition of AURKA induces differentiation and reduces cell proliferation in THP-1 cells (data not shown), and that the KDM6B inhibitor GSK-J4 disrupts the differentiation of THP-1 cells into monocytes. Our study suggests a mechanism in which AURKA-KDM6B signaling regulates the differentiation of THP-1 cells, which may help identify new therapeutic candidates for leukemia.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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