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The histone H3 Lys 27 demethylase JMJD3 regulates gene expression by impacting transcriptional elongation

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The histone H3 Lys 27 (H3K27) demethylase JMJD3 has been shown to play important roles in transcriptional regulation and cell differentiation. However, the mechanism underlying JMJD3-mediated transcriptional regulation remains incompletely understood. Here we show that JMJD3 is associated with KIAA1718, whose substrates include dimethylated H3K27 (H3K27me2), and proteins involved in transcriptional elongation. JMJD3 and KIAA1718 directly bind to and regulate the expression of a plethora of common target genes in both a demethylase activity-dependent and -independent manner in the human promyelocytic leukemia cell line HL-60. We found that JMJD3 and KIAA1718 collaborate to demethylate trimethylated H3K27 (H3K27me3) on a subset of their target genes, some of which are bivalently marked by H3K4me3 and H3K27me3 and associated with promoter-proximal, paused RNA polymerase II (Pol II) before activation. Reduction of either JMJD3 or KIAA1718 diminishes Pol II traveling along the gene bodies of the affected genes while having no effect on the promoter-proximal Pol II. Furthermore, JMJD3 and KIAA1718 also play a role in localizing elongation factors SPT6 and SPT16 to the target genes. Our results support the model whereby JMJD3 activates bivalent gene transcription by demethylating H3K27me3 and promoting transcriptional elongation. Taken together, these findings provide new insight into the mechanisms by which JMJD3 regulates gene expression.

[*Keywords*: JMJD3; chromatin; transcriptional elongation]

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Histone modifications regulate gene transcription by affecting chromatin compaction, DNA accessibility, and recruitment of transcription machinery and regulators (Jenuwein and Allis 2001; Goldberg et al. 2007; Li et al. 2007). Multiple lysine residues on histones, including H3K4, H3K27, and H3K36, are methylated by histone methyltransferases and demethylated by histone demethylases. H3K27 trimethylation (H3K27me3) is a repressive histone modification mediated by the histone methyltransferase EZH2 (Cao et al. 2002), which is a component of the Polycomb group (PcG) complex 2 (PRC2) (for review, see Cao and Zhang 2004). H3K27me3 contributes to the recruitment of the PRC1 complex, and together, PRC2 and PRC1 play important roles in the repression of many developmental genes in different biological settings, including embryonic stem (ES) cells (Schuettengruber et al. 2007; Margueron and Reinberg 2011). A recent study demonstrated the coexistence of H3K27me3 with the activating histone modification H3K4me3 (bivalent domains) on key developmental genes, and the bivalent domains are believed to help poise these genes for activation during ES cell differentiation (Bernstein et al. 2006). Consistently, the H3K27me3 demethylase UTX has been shown to be present at low levels or absent from the bivalently marked genes in ES cells (Agger et al. 2007; Lan et al. 2007; Lee et al. 2007). Upon differentiation, UTX and its related H3K27me3 demethylase, JMJD3, are recruited for the activation of specific developmental genes (Agger et al. 2007; Lee et al. 2007; Burgold et al. 2008). Therefore, the H3K27me3 demethylases have been proposed to play an important role in the resolution of the bivalent domains during differentiation (Burgold et al. 2008; Kim et al. 2011). Despite the important roles these enzymes have been shown to play, the molecular mechanism by which

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UTX and JMJD3 regulate gene expression and differentiation remains incompletely understood.

RNA polymerase II (Pol II)-mediated transcription has a close and dynamic relationship with histone modifications and is regulated at multiple steps, including Pol II recruitment to promoters, initiation, elongation, and termination (Nechaev and Adelman 2011). The initiation step has been studied extensively and was thought to be the major step in regulating gene transcription (Margaritis and Holstege 2008). However, more recent genome-wide studies show that Pol II pauses on many genes after initiation (Guenther et al. 2007; Muse et al. 2007; Zeitlinger et al. 2007), and the transition from pausing to productive elongation is another critical step in gene expression regulation (Levine 2011; Nechaev and Adelman 2011). The promoters of genes with poised Pol II are usually associated with H3K4me3 (Guenther et al. 2007; Muse et al. 2007; Zeitlinger et al. 2007). The PRC1 complex has been shown to play a role in the stalling of Pol II at promoters on the bivalently marked genes in ES cells (Stock et al. 2007), although a more recent report shows that bivalent genes bound by both PRC1 and PRC2 have reduced levels of paused Pol II at promoters compared with those bound by PRC2 only (Min et al. 2011). The promoter-proximal Pol II stalling is mediated by the negative elongation factors DSIF (DRB sensitivity-inducing factor) and NELF (negative elongation factor) (Yamaguchi et al. 2002; Wu et al. 2003). p-TEFb phosphorylation of Ser2 on the Pol II C-terminal domain (CTD), NELF, and DSIF promotes the release of Pol II into productive elongation (Peterlin and Price 2006; Cheng and Price 2007; Bres et al. 2008). Pol II-mediated elongation is tightly linked with histone modifications and nucleosome dynamics. The PAF (Pol II-associated factor) complex, whose components include CDC73, is required for H2B ubiquitination and H3K4 methylation (Krogan et al. 2003; Wood et al. 2003). The H3K36 methyltransferase SETD2 has been shown to be associated with Ser2-phosphorylated Pol II (Yoh et al. 2008), and, at least in yeast, H3K36 methylation is important for preventing cryptic transcription initiation within the transcribed regions (Carrozza et al. 2005; Joshi and Struhl 2005; Keogh et al. 2005). Histone chaperons, including SPT6 and SPT16, travel with the elongating Pol II and mediate histone displacement and redeposition to allow Pol II to transcribe through nucleosomes (Belotserkovskaya and Reinberg 2004). Therefore, these different transcriptional regulators work together to help Pol II move through coding regions while maintaining nucleosome integrity.

To address the mechanism by which JMJD3 regulates transcription, we took a proteomics approach and purified proteins that were associated with JMJD3. In contrast to UTX, which has been shown to be associated with H3K4 methyltransferase complexes (Cho et al. 2007; Issaeva et al. 2007; Lee et al. 2007), we found that JMJD3 was associated with the H3K9me2/H3K27me2/H4K20me1-specific demethylase KIAA1718 as well as proteins that are known to be involved in transcription elongation, implicating a role for JMDJ3 in transcriptional elongation. In the differentiating HL-60 cells, both JMJD3 and KIAA1718 were up-regulated and contributed to the differentiation of HL-60 cells. Importantly, these two demethylases appeared to regulate the expression of a common set of target genes, suggesting potential collaboration between the two enzymes, which is consistent with their biochemical association. Genome-wide chromatin immunoprecipitation (ChIP) coupled with deep sequencing (ChIP-seq) showed that JMJD3 and KIAA1718 were colocalized at promoters, transcriptional start sites (TSSs), and coding regions of many genes. ChIP-seq, expression microarray analysis, and genetic rescue experiments with enzymatically inactive JMJD3 support the view that JMJD3 regulates gene expression in both an enzymatic activity-dependent and -independent manner. Further analyses of individual JMJD3/KIAA1718 target genes suggest a role for these enzymes in the resolution of the bivalent domains during differentiation, the facilitation of the release of paused Pol II, and the recruitment of elongation factors such as SPT6 and SPT16 to impact Pol II traveling along the gene bodies. Taken together, these findings suggest the model that JMJD3 and KIAA1718 activate gene transcription, at least in part, by demethylating H3K27me3 and promoting transcriptional elongation.

Results

JMJD3 is associated with proteins involved in transcriptional elongation

To gain insights into how JMJD3 regulates gene transcription, we purified the JMJD3 complex using sequential Flag and HA pull-down from the nuclear extract of a human 293T cell line stably expressing Flag-HA-JMJD3, followed by mass spectrometry. The results revealed the presence of proteins involved in histone modifications, transcriptional regulation, chromosome structural maintenance, and segregation (Fig. 1A; Supplemental Table S1), suggesting that JMJD3 may be involved in multiple chromatin template-based processes. Importantly, we identified proteins involved in transcriptional elongation, including SPT6, SPT16, SETD2, and CDC73 (Fig. 1B). Flag-HA-JMJD3 also pulled down KIAA1718, which possesses multiple substrate specificities, including H3K27me2 (Horton et al. 2010; Huang et al. 2010; Qi et al. 2010; Tsukada et al. 2010), the H3K36 methyltransferase SETD2 (Edmunds et al. 2008), and the chromodomain protein CHD7 (Fig. 1B). To confirm the mass spectrometry results, we performed coimmunoprecipitation. Consistently, our results demonstrated association of endogenous JMJD3 with KIAA1718, SPT6, SPT16, and CDC73 in 293T cells (Fig. 1C). Taken together, our biochemical data suggest that JMJD3 may work with other demethylases, methyltransferases, and elongation factors to regulate transcriptional elongation.

JMJD3 and KIAA1718 regulate HL-60 differentiation toward macrophage-like cells

JMJD3 has been shown to play important roles in differentiation (De Santa et al. 2007; Burgold et al. 2008; Sen et al. 2008; Satoh et al. 2010; Kim et al. 2011). It has also

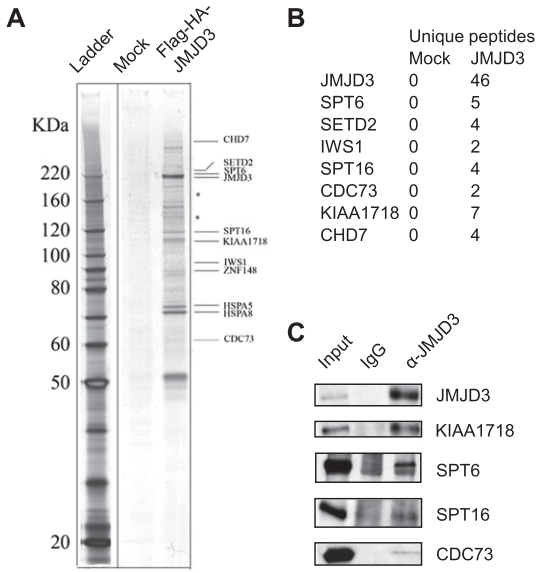

Figure 1. JMJD3 is associated with histone modifiers and proteins involved in transcription elongation. (*A*) Silver staining showing the Flag and HA tandem purification of JMJD3-associated proteins in 293T cells. (*) Degraded Flag-HA-JMJD3. A comprehensive list of proteins can be found in Supplemental Table S1. (*B*) List of JMJD3-associated proteins that may be involved in chromatin modification and transcription elongation. (*C*) 293T nuclear extracts were immunoprecipitated with anti-JMJD3 antibody and immunoblotted with the indicated antibodies.

been shown to be up-regulated in the 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced macrophage-like differentiation of HL-60 cells (Hu et al. 2006). Consistently, we observed an increase of JMJD3 mRNA during the first 24 h in HL-60 cells upon TPA treatment, which mirrored that of the myeloid lineage marker C-FMS (Fig. 2A). At the same time, the mRNA level of KIAA1718 was also elevated, as well as the proteins levels of JMJD3 and KIAA1718 (Fig. 2A). At the protein level, JMJD3 induction appeared to precede that of KIAA1718 (Fig. 2A), but nonetheless, both were induced during HL-60 cell differentiation. To determine whether JMJD3 and its associated proteins play a role in HL-60 cell differentiation, we first investigated whether the interaction of JMJD3 with the above-mentioned components is conserved in HL-60 cells. Consistent with our data in 293T cells, KIAA1718 was coimmunoprecipitated by the JMJD3 antibody in the TPA-treated HL-60 cells (Fig. 2B). Other components, such as SPT6, SPT16, and CDC73, were also coimmunoprecipitated with JMJD3 (Fig. 2B), confirming the association among JMJD3, KIAA1718, and elongation proteins.

To determine whether JMJD3 and KIAA1718 are important for TPA-induced HL-60 differentiation, we established stable cell lines that expressed JMJD3 or KIAA1718 shRNAs, respectively. Western blots show that the expression of both proteins was reduced significantly by two independent shRNAs for each demethylase (Supplemental Fig. S1). Next, we examined the impact of JMJD3 or KIAA1718 knockdown on TPA-induced HL-60 cell differentiation by two different approaches. First, we found that upon 12 h of TPA induction, the mRNA levels of the myeloid differentiation markers CD11b and C-FMS were significantly reduced in all four cell lines infected with either JMJD3 or KIAA1718 shRNAs when compared with the cell line infected with a nonsilencing (NS) control shRNA (Fig. 2C). We selected the 12-h post-treatment because, at this time point, differentiation of HL-60 induced by TPA was readily apparent and the levels of JMJD3 and KIAA1718 were also robustly elevated. Second, when HL-60 cells are induced to undergo macrophage-like differentiation, the cells change from being nonadherent to being attached to the surface of the cell culture plates and form clumps (Rovera et al. 1979). Upon RNAi inhibition of either JMJD3 or KIAA1718, we found significantly fewer attached cells, and the clumps were also smaller (Fig. 2D). Consistently, we also found more suspension cells in the HL-60 cells treated with JMJD3 or KIAA1718 shRNAs (Fig. 2E). Taken together, these data show that both JMJD3 and KIAA1718 play important roles in the TPA-induced HL-60 cell differentiation.

JMJD3 and KIAA1718 regulate common gene targets

To begin to address the underlying molecular mechanism of how JMJD3 and KIAA1718 regulate cell differentiation, we set out to identify gene expression networks regulated by JMJD3 and KIAA1718 during HL-60 cell differentiation by expression microarray analysis of TPA-induced HL-60 cells treated with either NS control or two independent JMJD3 or KIAA1718 shRNAs. From the microarray data, we found that 704 genes were up-regulated by greater than twofold in HL-60 cells treated with TPA. Among this group of genes, the expression of 171 and 139 genes was found to be reduced by either one of the two JMJD3 or KIAA1718 shRNAs, respectively (>20% reduction). When the two gene lists were compared, we found 63 genes regulated by both JMJD3 and KIAA1718, indicating that they are common targets of both demethylases (Fig. 3A; Supplemental Table S2). Gene ontology (GO) analysis shows that genes regulating immune response, plasma membrane components, and cell surface receptor-linked signal transduction, among others, were overrepresented, consistent with a role for JMJD3 and KIAA1718 in HL-60 differentiation (Supplemental Table S3). The regulation of a number of the common targets was further confirmed by quantitative RT–PCR (qRT–PCR) (Fig. 3B).

We next carried out ChIP-seq analysis to identify genes that were directly bound by JMJD3 and KIAA1718 in the TPA-treated HL-60 cells. We identified 58,975 and 27,273 peaks for JMJD3 and KIAA1718 ($P < 10^{-5}$, false discovery rate [FDR] <0.05), respectively. Statistical analyses show that JMJD3 and KIAA1718 are both significantly enriched on gene promoters and the 5′ untranslated region (UTR) (Table 1). Using 1 kb within the TSS as a cutoff, we found that JMJD3 and KIAA1718 bound 8884 and 7057 genes, respectively (Supplemental Table S4). Among them, 6147 genes were bound by both JMJD3 and KIAA1718 (Fig. 3C; Supplemental Table S4). Colocalization of JMJD3 and KIAA1718 was further supported by a high degree of

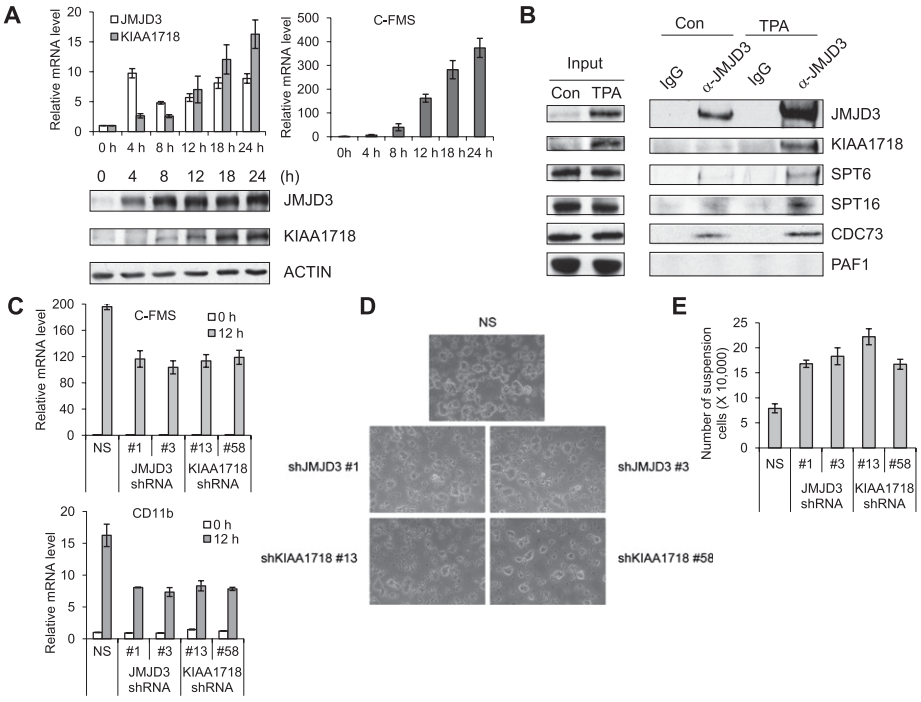

Figure 2. JMJD3 and KIAA1718 regulate TPA-induced HL-60 differentiation toward a macrophage-like phenotype. (*A*) HL-60 cells were treated with 32 nM TPA for different lengths of time, and the mRNA levels of the histone demethylases JMJD3 and KIAA1718 (*left*) and the myeloid marker C-FMS (*right*) were measured by qRT–PCR and normalized to that of GAPDH. The protein levels of JMJD3 and KIAA1718 were measured, and ACTIN was used as a loading control. Results for mRNA data are shown as mean ± SEM from at least three independent experiments. (*B*) Nuclear extracts from HL-60 cells before and after 12 h of TPA treatment were immunoprecipitated with JMJD3 antibody and immunoblotted with the indicated antibodies. Immunoprecipitation of JMJD3 brought down KIAA1718, SPT6, SPT16, and CDC73, while it did not bring down another component of the PAF1 complex, PAF1. (*C*) Stable HL-60 cells infected with NS, two JMJD3 shRNAs, or two KIAA1718 shRNAs were treated with TPA for 12 h and measured for the levels of C-FMS and CD11b mRNAs. Results are shown as mean ± SEM from at least three independent experiments. (*D*) HL-60 cells infected with different shRNAs were treated with TPA for 12 h and then rinsed off of suspension cells with PBS. The cell phenotypes were examined with phase-contrast microscopy. (*E*) HL-60 cells infected with different shRNAs were treated with TPA for 12 h, and the suspension cells were counted. Results are represented as mean ± SEM from triplicates of a representative experiment. The experiments in *D* and *E* were repeated three times with similar results.

correlation of ChIP-seq binding signals between JMJD3 and KIAA1718 data sets (Fig. 3D). When compared with the 63 genes that were found to be commonly regulated by both enzymes via microarray, we found that 41 of them overlapped with the ChIP-seq targets (Supplemental Table S2), suggesting that the majority of the shared transcription targets were directly regulated by JMJD3 and KIAA1718. We further investigated a number of these direct targets, including ZC3H12c, CD44, FAM129B, and CXCL3. In addition to the enrichment of JMJD3 and KIAA1718 around the TSS and in the 5′ UTR, we also found JMJD3- and KIAA1718-binding peaks across the gene bodies, and the majority of these peaks showed overlap between JMJD3 and KIAA1718 (Fig. 3E; Supplemental Fig. S2). Taken together, the expression microarray data and the ChIP-seq analysis both suggest a functional link between JMJD3 and KIAA1718.

JMJD3 and KIAA1718 regulate H3K27 methylation at the common target genes

H3K27me3 is a repressive mark for transcription, and our data show that upon TPA induction, both JMJD3 and KIAA1718 bind to and regulate the expression of a subset of common target genes. We investigated the possibility that JMJD3 is recruited to these genes during differentiation to activate their expression via demethylation of the repressive modification H3K27me3. To do so, we performed ChIP-seq analyses of H3K27me3 in HL-60 cells treated with NS shRNA before and after differentiation, as well as differentiated HL-60 cells treated with JMJD3 shRNA. We obtained 22.7 million, 23.7 million, and 23.1 million reads for the NS shRNA control cells, NS shRNA TPA-treated cells, and JMJD3 shRNA TPA-treated cells, respectively. Using the same criteria, which were applied to the JMJD3 ChIP-seq data set, in the NS HL-60 cells before TPA treatment, we found 4249 genes enriched with H3K27me3, and 1461 of them were bound by JMJD3 after differentiation (Fig. 4A; Supplemental Table S5). Among the 1461 genes, about one third of them (522 genes) showed decreased H3K27me3 signals upon TPA treatment (Supplemental Table S6). Importantly, knockdown of JMJD3 restored the H3K27me3 signal to most of the JMJD3-bound target genes that showed reduction of H3K27me3 during differentiation (458 out of 522, 87.7%) (Supplemental

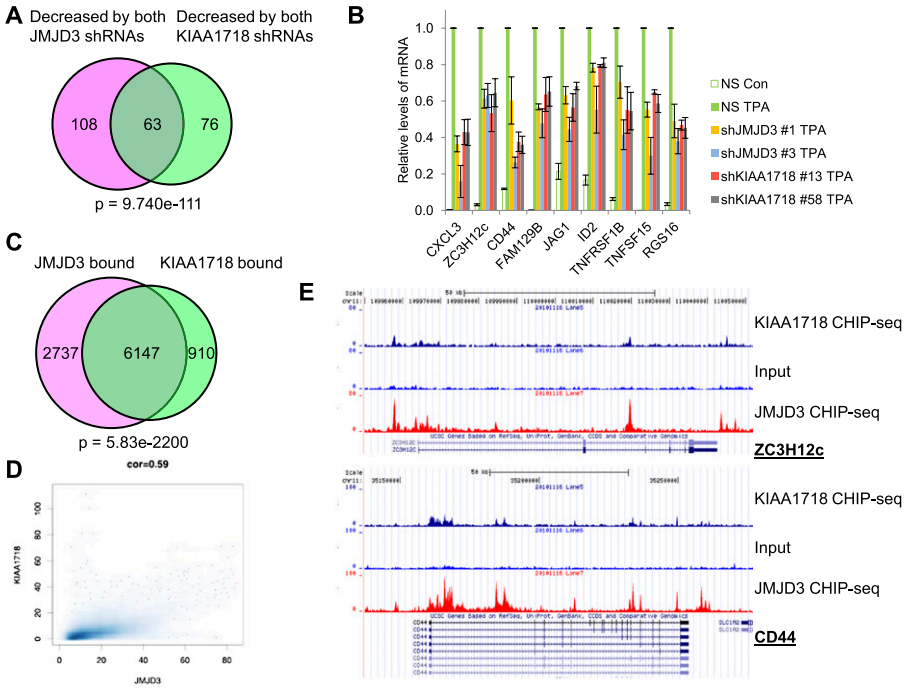

Figure 3. JMJD3 and KIAA1718 share common targets. (*A*) Expression microarray analyses found that there was a significant overlap between genes down-regulated by JMJD3 shRNAs and KIAA1718 shRNAs. The *P*-value was calculated by a hypergeometric probability test. The list of genes can be found in Supplemental Table S2. (*B*) A subset of genes from the common expression microarray list was verified with qRT–PCR. Shown are the relative fold changes of mean ± SEM from three independent experiments. (*C*) Statistically significant overlap between JMJD3- and KIAA1718-bound genes in TPA-treated HL-60 cells. (*D*) The scatter plot shows the correlation between JMJD3 and KIAA1718 peak intensities relative to the input. (*E*) ChIP-seq analysis at the ZC3H12c and CD44 loci.

Table S6). These data suggest that 31% (458 out of 1461) of the H3K27me3-enriched and JMJD3-bound genes are likely to be regulated by JMJD3 via its demethylase activity.

We also compared the total JMJD3-bound genes in the differentiated cells (8884) with the H3K27me3 ChIP-seq data in the untreated cells (Fig. 4A). This analysis identified 16% overlap between the JMJD3-bound genes (1461 out of 8884) in the differentiated cells and the H3K27me3-enriched genes in the untreated HL-60 cells. Taken together, our data support the view that JMJD3 regulates gene expression in an enzymatic activity-dependent and -independent manner.

We next investigated the role of the demethylase activity of JMJD3 and KIAA1718 in regulating the expression of their target genes. To this end, we first examined whether H3K27 methylation was altered during TPA-induced gene activation of the four JMJD3/KIAA1718 direct target genes, *ZC3H12c, CXCL3, CD44*, and *FAM129B*. We observed a significant decrease in H3K27me3 but not H3K27me2 and H3K27me1 during TPA-induced differentiation of HL-60 cells, consistent with a possible involvement of the demethylases (Fig. 4B). Previous studies show that genes poised for activation during differentiation are often marked with the activating mark H3K4me3 in addition to the repressive mark H3K27me3 (Bernstein et al.

Table 1. *Distribution of JMJD3- and KIAA1718-binding sites in TPA-treated HL-60 cells*

	Genome	JMJD3 peaks	*P* (vs. genome)	KIAA1718 peaks	*P* (vs. genome)
Promoter (≤1000 bp)	1.1%	4.3%	3.0×10^{-322}	6.2%	1.1×10^{-175}
Promoter (≤2000 bp)	1.8%	5.8%	1.9×10^{-322}	8.1%	1.6×10^{-178}
Promoter (≤3000 bp)	2.5%	7.1%	1.4×10^{-322}	9.6%	3.4×10^{-182}
5′ UTR	0.4%	1.4%	8.5×10^{-155}	3.4%	5.4×10^{-124}
3′ UTR	1.5%	1.1%	2.4×10^{-11}	1.5%	0.357
Downstream (≤1000 bp)	1.0%	1.1%	0.015	1.4%	4.1×10^{-4}
Downstream (≤2000 bp)	1.7%	2.0%	1.2×10^{-5}	2.4%	1.6×10^{-5}
Downstream (≤3000 bp)	2.4%	2.9%	2.1×10^{-12}	3.2%	1.5×10^{-5}
Coding exon	1.9%	0.9%	3.7×10^{-87}	1.6%	0.041
Intron	42.4%	45.9%	1.1×10^{-62}	45.7%	2.1×10^{-8}
Distal intergenic	46.2%	49.3%	3.9×10^{-48}	52.3%	1.2×10^{-23}

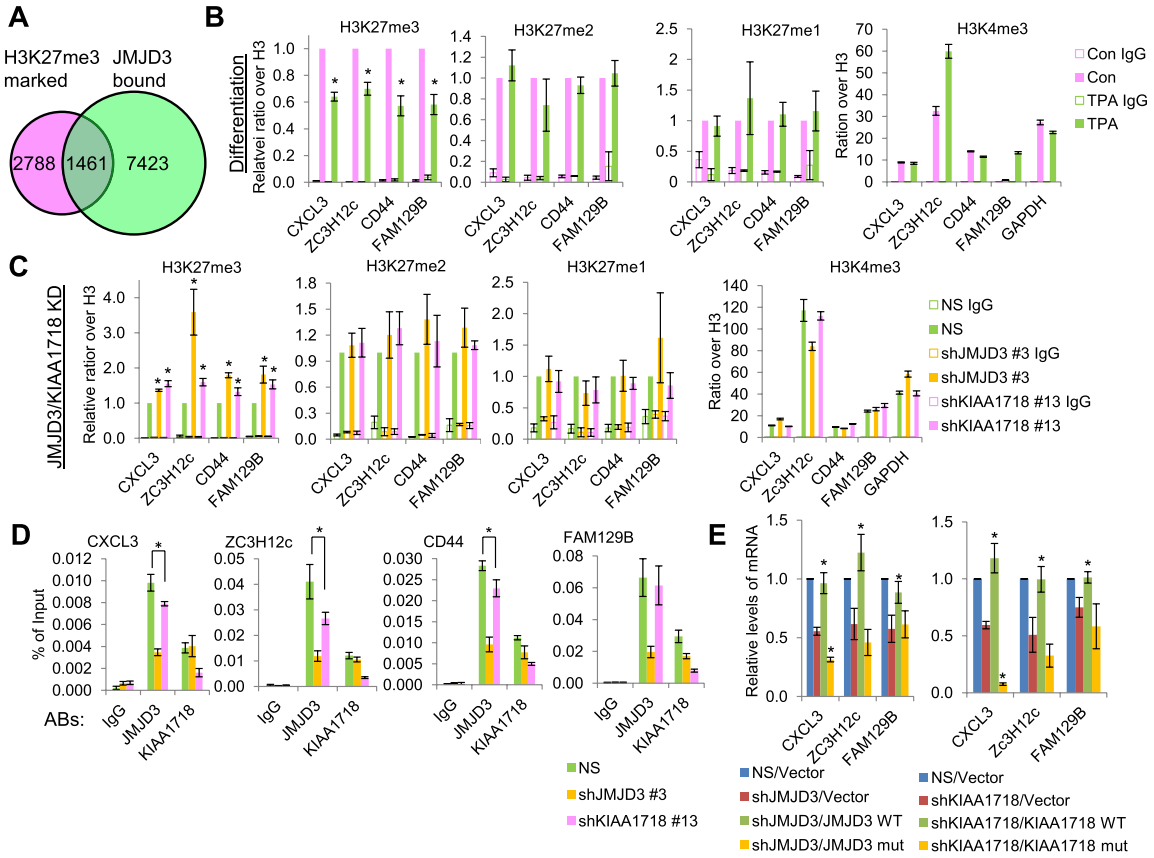

Figure 4. JMJD3 and KIAA1718 regulate H3K27me3 on their targets. (*A*) H3K27me3-enriched genes in NS shRNA control cells that were bound by JMJD3 after TPA-induced HL-60 cell differentiation. (*B*) ChIP-qPCR analysis of H3K27me3, H3K27me2, H3K27me1, and H3K4me3 levels around the TSSs of different JMJD3/KIAA1718 direct targets in HL-60 cells before and after TPA treatment for 12 h. (*C*) The effects of JMJD3 or KIAA1718 knockdown on the levels of H3K27me3, H3K27me3, H3K27me1, and H3K4me3 on different genes in TPA-treated HL-60 cells. The H3K27me intensities in *B* and *C* represent mean ± SEM from at least three independent experiments. (*) $P < 0.05$. The H3K4me3 signals were three determinants from one representative experiment. The experiments were repeated at least twice with similar results. (*D*) Effects of JMJD3 or KIAA1718 knockdown on their enrichment around the TSSs of different genes. The signals are represented as mean ± SEM percentage of the input from three independent experiments. (*) $P < 0.05$. (*E*) HL-60 cells were infected with the indicated shRNA and Flag-HA vectors expressing wild-type or mutant proteins. All three key amino acids important for demethylase activity were mutated for both JMJD3 (H1390A/E1392V/H1470A) and KIAA1718 (H282A/D284V/H354A). The relative mRNA levels of three genes were measured by qRT–PCR. Shown are mean ± SEM from three independent experiments. (*) $P < 0.05$ when compared with cells treated with correspondent shRNA and empty vector.

2006). Indeed, three of the four JMJD3/KIAA1718 target genes discussed here (CXCL3, ZC3H12c, and CD44) were clearly marked with H3K4me3, in addition to H3K27me3, around their TSSs in the untreated HL-60 cells (Fig. 4B), suggesting that they are bivalently marked genes. Interestingly, the fourth JMJD3/KIAA1718 target gene, FAM129B, had no significant enrichment of H3K4me3 at its TSS before TPA induction, but the level of H3K4me3 increased dramatically after TPA induction (Fig. 4B). In the TPA-treated HL-60 cells, reduction of JMJD3 or KIAA1718 increased H3K27me3 on their target genes while having no significant effects on H3K27me2, H3K27me1, or H3K4me3 (Fig. 4C), suggesting that JMJD3 and KIAA1718 both regulate the H3K27me3 level at target genes. Taken together, these data suggest that some JMJD3/KIAA1718 target genes are bivalently marked before activation, and the JMJD3 complex plays an important role in resolving the bivalent marks by demethylating H3K27me3.

Since KIAA1718 does not demethylate H3K27me3 (Huang et al. 2010; Qi et al. 2010; Tsukada et al. 2010), our result that its knockdown increased H3K27me3 suggests that it may impact the H3K27me3 level indirectly. For instance, KIAA1718 may regulate the JMJD3 protein level, affect its localization to the target genes, or help further demethylate H3K27me2, which is a product of JMJD3-mediated demethylation. While exploring these possibilities, we found that knockdown of KIAA1718 significantly decreased JMJD3 localization to the three bivalently marked target genes, *CXCL3, ZC3H12c*, and *CD44* (Fig. 4D). As a control, knockdown of KIAA1718 did not affect the JMJD3 protein level (Supplemental Fig. S3), indicating that reduced JMJD3 occupancy at the TSS was not due to a change in the JMJD3 protein level. Importantly, JMJD3 occupancy of the *FAM129B* TSS, which was not marked by H3K4me3 before TPA induction, was essentially unaffected (Fig. 4D). These data suggest

that KIAA1718 at least partially contributes to the recruitment of JMJD3 to their targets, possibly through physical interaction with JMJD3 and by recognizing H3K4me3 at the target promoters via its PHD domain, which has been shown to bind H3K4me3 (Horton et al. 2010). However, in the case of *FAM129B*, where KIAA1718 did not affect JMJD3 localization, we also observed an increase in H3K27me3 when KIAA1718 was knocked down (Fig. 4C). This raises an additional possibility that KIAA1718 may contribute to JMJD3 function by demethylating its product, H3K27me2. Thus, in the absence of KIAA1718, an increase of H3K27me2 may hamper H3K27me3 demethylation due to a shift in the reaction equilibrium. The involvement of the demethylase activity of both proteins was further supported by reconstituting HL-60 cells treated with JMJD3 or KIAA1718 shRNA with either wild-type or catalytic inactive mutants of JMJD3 and KIAA1718 (Supplemental Fig. S4). As shown in Figure 4E, wild-type JMJD3 and KIAA1718, but not the catalytically inactive mutants, restored the expression of their target genes, suggesting that the demethylase activities are required for both proteins to positively regulate transcription. Taken together, our data suggest that JMJD3 and KIAA1718 may work together to demethylate H3K27me3 at their target genes during TPA-induced HL-60 cell differentiation.

JMJD3 and KIAA1718 regulate target gene transcriptional elongation

Since JMJD3 demethylates H3K27me3 and is associated with proteins involved in elongation regulation, we investigated whether JMJD3- and KIAA1718-mediated H3K27me3 demethylation impacts transcriptional elongation. To address this issue, we first examined the 5′ and 3′ RNA levels of *ZC3H12c* and *CXCL3*. To minimize the impact of accumulating RNA over time, we carried out this experiment at the 4-h time point after TPA induction. We found that in cells treated with the control shRNA, primary transcripts representing the 5′ end for both *CXCL3* and *ZC3H12c* were increased upon TPA treatment; however, the increase of their 3′ end processed transcripts was significantly more dramatic (Fig. 5A,B). When JMJD3 and KIAA1718 were knocked down, transcripts representing the 3′ end were decreased more significantly than transcripts on the 5′ end (Fig. 5A,B).

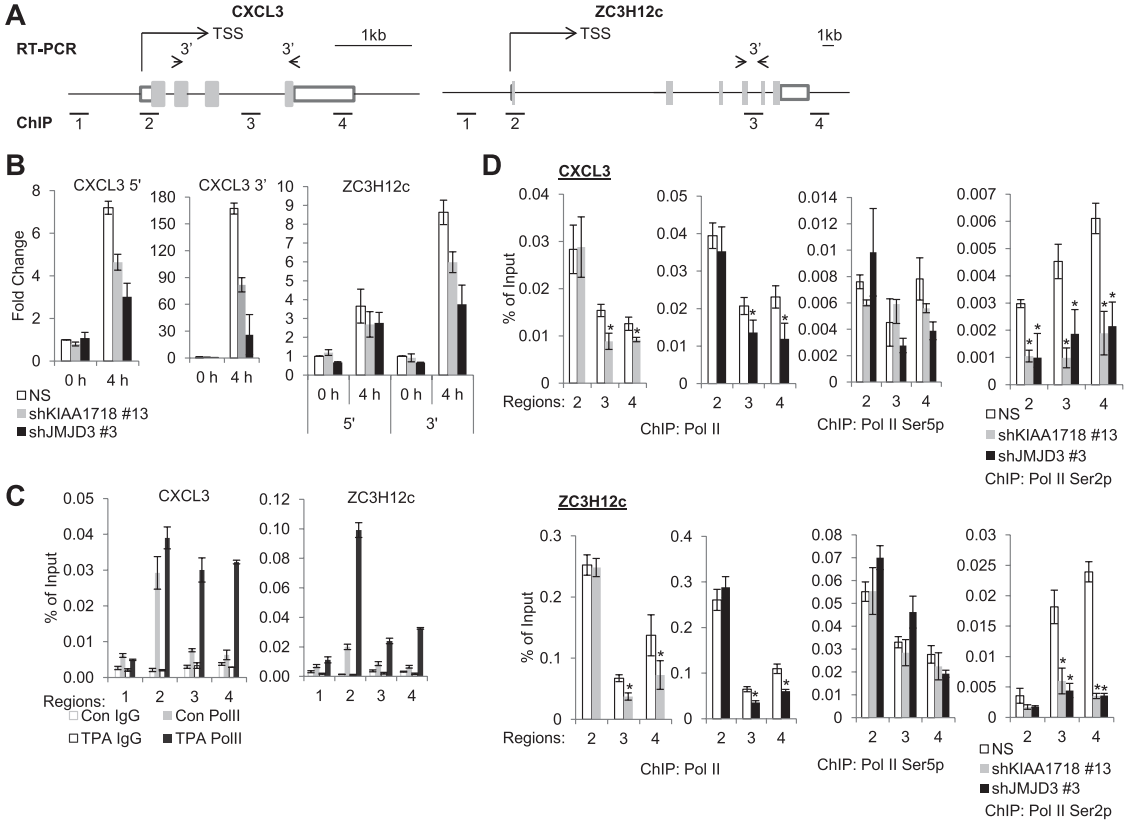

Figure 5. JMJD3 and KIAA1718 regulate Pol II traveling on their targets. (*A*) Schematic diagrams showing the positions of primers used in qRT–PCR and ChIP-qPCR analysis for *CXCL3* and *ZC3H12c*. (*B*) Effects of JMJD3 and KIAA1718 knockdown on the 5′ end and 3′ end transcripts in untreated and 4-h TPA-treated HL-60 cells. Shown are the fold changes (mean ± SEM) from three independent experiments. For the 5′ end, the primers labeled as 2 in *A* were used, and these were located within 100 bp from the TSS. The primers for the 3′ end were at the 3′ end of the genes and crossed introns. (*C*) Quantification of Pol II distribution on *ZC3H12c* and *CXCL3* before and after 12 h of TPA treatment in HL-60 cells. (*D*) Pol II, Pol II Ser5p, and Pol II Ser2p enrichments on different regions of *ZC3H12c* and *CXCL3* in HL-60 cells treated with the indicated shRNAs in the presence of 12 h of TPA treatment. (*) $P < 0.05$. Signals in *C* and *D* are shown as a percentage of the input (mean ± SEM) from at least three independent experiments.

These data suggest that JMJD3 and KIAA1718 may play a positive role in the generation of full-length mRNAs.

Bivalent genes have been shown to have paused RNA Pol II around their TSSs, and their transcription may be regulated at the Pol II release step (Stock et al. 2007; Min et al. 2011). Therefore, we next investigated whether JMJD3 and KIAA1718 regulate transcriptional elongation by examining Pol II localization on the JMJD3/KIAA1718 target genes. Before TPA induction, Pol II was already enriched at the TSSs of *CXCL3* and *ZC3H12c* compared with the upstream and downstream regions (Fig. 5C), suggesting that there was poised Pol II around the TSSs of both genes. After 12 h of TPA induction, there was only a mild increase in Pol II enrichment around the TSS of *CXCL3*. Importantly, there was a remarkable increase in Pol II distribution to the gene body and the 3′ end of the gene body (Fig. 5C). For *ZC3H12c*, there was a significant Pol II increase at both the promoter and gene body (Fig. 5C). The additional recruitment of Pol II around the TSS of *ZC3H12c* is similar to the activation of some Pol II-poised genes reported previously (Hargreaves et al. 2009; Saha et al. 2011) and is likely to support the multiple rounds of RNA synthesis because preassociated Pol II was insufficient. For both *CXCL3* and *ZC3H12c*, knockdown of either JMJD3 or KIAA1718 significantly reduced Pol II distribution throughout the gene body while having no impact on Pol II enrichment at TSSs (Fig. 5D). Furthermore, we found that while knockdown of JMJD3 or KIAA1718 had no significant effects on Pol II Ser5p enrichment, it reduced Pol II Ser2p enrichment across the target genes (*CXCL3* and *ZC3H12c*) (Fig. 5D). These results suggest that one possible mechanism by which JMJD3 and KIAA1718 activate gene transcription is to facilitate the release of the poised Pol II into active elongation along the gene body.

Our biochemical interaction data suggest that JMJD3 interacts with the positive elongation factors SPT6 and SPT16. We next investigated whether JMJD3 and KIAA1718 regulate elongation by recruiting these positive elongation factors to JMJD3/KIAA1718 targets. We performed SPT6 and SPT16 ChIP analysis in differentiating HL-60 cells treated with either JMJD3 or KIAA1718 shRNA. As shown in Figure 6, A and B, reduction of either JMJD3 or KIAA1718 significantly decreased SPT6 and SPT16 localization to *CXCL3* and *ZC3H12c*, but had no discernible effects on a JMJD3/KIAA1718-unbound gene. These data support the idea that the JMJD3 protein complex regulates transcriptional elongation by contributing to the recruitment of positive elongation factors to its targets.

Discussion

JMJD3 has been shown to play important roles in differentiation, but the underlying mechanisms are incompletely understood. Our study shows that JMJD3 is associated with KIAA1718 and the transcriptional elongation

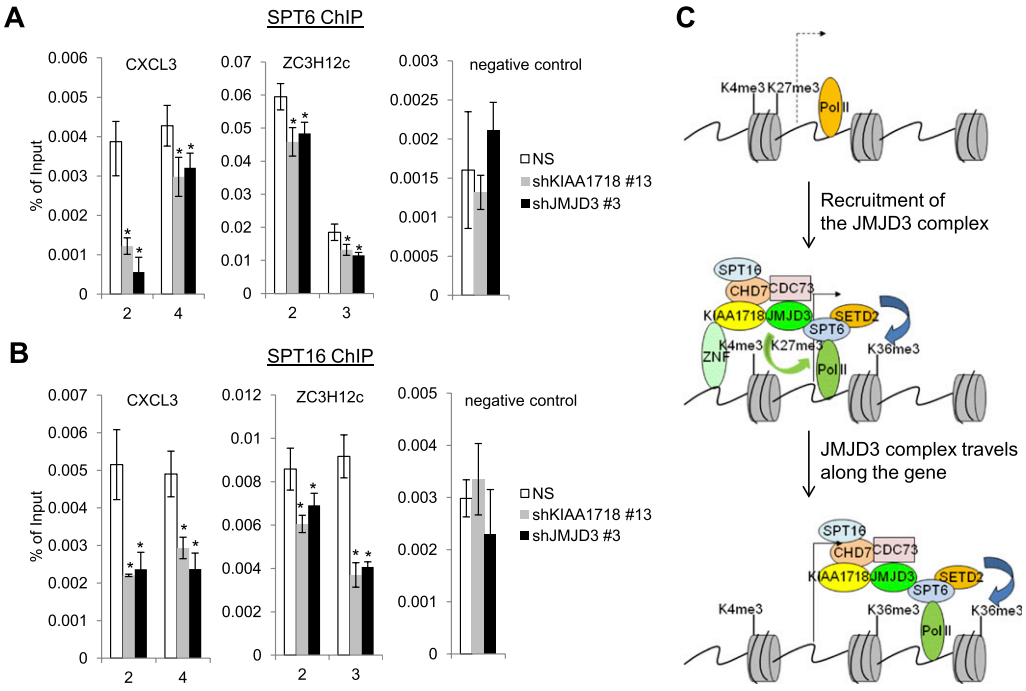

Figure 6. JMJD3 and KIAA1718 contribute to the recruitment of SPT6 and SPT16 to their targets. (*A*,*B*) Effects of JMJD3 or KIAA1718 knockdown on the localization of SPT6 and SPT16 on different regions of *CXCL3* and *ZC3H12c*. The positions of primers are shown in Figure 5A. Results are represented as a percentage of the input (mean ± SEM) from three independent experiments. (*) $P < 0.05$ compared with control shRNA. (*C*) A working model of the mechanism by which JMJD3 activates the expression of bivalent genes. Before bivalent genes are activated, they are bound by promoter-proximal, paused Pol II. When KIAA1718/JMJD3 bind to these genes, they bring additional histone modifiers and transcription elongation factors. Together, the JMJD3 complex demethylates H3K27me3 and helps release Pol II into productive elongation and travel along the gene body.

regulators SPT6, SPT16, CDC73, and SETD2. Our data suggest the model that these different components work together to demethylate H3K27me3 on their direct target genes and facilitate the release of poised Pol II and possibly subsequent transcription through the gene body as well (Fig. 6C). Our findings thus provide new mechanistic insights into how two histone demethylases collaborate to regulate gene transcription.

Collaboration of two H3K27me demethylases

JMJD3 demethylates mainly tri- and di-methylated H3K27 (Agger et al. 2007; Lan et al. 2007; Lee et al. 2007), while KIAA1718 demethylates di- and monomethylated H3K27 (Horton et al. 2010; Huang et al. 2010; Qi et al. 2010; Tsukada et al. 2010). Our finding that these two proteins interact with one another and co-occupy the promoters of multiple developmental genes suggests that they work together to regulate the H3K27 methylation level. Indeed, bioinformatics analysis of the H3K27me3 ChIP-seq data of the undifferentiated HL-60 cells with the genome-wide JMJD3 localization in the differentiated HL-60 cells suggests that JMJD3 regulates a subset of its target genes possibly by demethylating H3K27me3. Interestingly, we note that for the four direct JMJD3/KIAA1718 target genes that we investigated in detail, our ChIP-qPCR data clearly showed that they were marked by H3K27me3 before differentiation, but the ChIP-seq efforts identified only one of them as being marked by H3K27me3. We therefore speculate that our H3K27me3 ChIP-seq analysis may have underestimated the extent of H3K27 methylation in the undifferentiated HL-60 cells and, consequently, the overlap between JMJD3-bound targets and the H3K27me3-enriched genes. This is not unprecedented, as previous studies also documented the discrepancies between genome-wide mapping versus individual ChIP-qPCR results. For instance, a previous study showed that ChIP-qPCR identified many additional targets for the H3K4me3 demethylase RBP2 and the Polycomb protein Suz12, which were simply missed by the ChIP–chip studies (Pasini et al. 2008).

The importance of the demethylase activities in JMJD3/KIAA1718-mediated gene activation was further demonstrated by ChIP-qPCR and rescue data. We speculate that the presence of the two H3K27me demethylases with slightly different methyl group specificities may allow rapid conversion of H3K27me3 to H3K27me0 as part of the transcriptional response to developmental cues. Consistent with this, both proteins are necessary for efficient differentiation of HL-60 cells (and possibly other differentiation processes as well). Previous studies show that KIAA1718 binds H3K4me3 via its PHD domain (Horton et al. 2010; Qi et al. 2010), and this binding event is critical for KIAA1718 to discriminate H3K27me2 versus H3K9me2 substrates (Horton et al. 2010). We speculate that KIAA1718 binding H3K4me3 may be important for localizing not only KIAA1718, but also JMJD3, to a subset of bivalently marked genes. Consistent with this, inhibition of KIAA1718 compromises JMJD3 localization at target promoters (Fig. 4D). It should be noted that mechanisms underlying the recruitment of KIAA1718 and JMJD3 are likely to be complex and involve other factors, such as the DNA-binding transcription factors. Consistently, we found the presence of a number of potential zinc finger transcription factors (ZNF281, ZNF148, and ZNF24) in the JMJD3 complex. Our findings support the idea that DNA-binding factors and the local chromatin environment are both important for the localization of chromatin regulatory proteins, such as KIAA1718 and JMJD3. Taken together, our results suggest that JMJD3 and KIAA1718 collaborate to ensure target specificity and optimal activation of gene transcription.

A possible role for JMJD3 in regulating transcriptional elongation

Our data suggest that for the JMJD3/KIAA1718 targets that are bivalently marked, JMJD3 may regulate their transcription by contributing to the release of poised Pol II into productive transcriptional elongation. Promoter-proximal Pol II pausing has been implicated as an important regulatory step in cell differentiation and signaling transduction (Levine 2011). In the unstimulated HL-60 cells, we observed poised Pol II on a number of bivalent genes. After stimulation, H3K27me3 was removed by JMJD3 and KIAA1718, and the bivalent domains were resolved into H3K4me3, which was accompanied by Pol II traveling into the gene body for productive elongation. In addition to the Pol II ChIP data, our ChIP-seq analysis shows that JMJD3 and KIAA1718 were enriched not only at promoters, but also at the 5′ UTR, supporting the idea that they may regulate the release of poised Pol II and its traveling at least through the 5′ UTR. The traveling of Pol II on the coding regions of target genes may be helped by SPT6, SPT16, and SETD2, which were associated with JMJD3 (Fig. 1). SPT6 has been shown to bind Ser2-phosphorylated Pol II (Yoh et al. 2007) and act together with SPT16 to coordinate nucleosome disassembly and reassembly during transcription (Belotserkovskaya and Reinberg 2004), and their roles in transcriptional elongation have been well established. The interaction between JMJD3/KIAA1718 and these proteins may therefore help bring in Ser2-phosphorylated Pol II and promote Pol II release and traveling through the gene body. Consistent with this view, our data show that JMJD3 and KIAA1718 help recruit SPT6 and SPT16 to some of their bivalent target genes. Taken together, JMJD3/KIAA1718 may regulate transcriptional elongation at multiple steps, including facilitation of release of paused Pol II and Pol II traveling across the gene body.

The biochemical association of JMJD3 with STP6, IWS1, SETD2, and CHD7 is consistent with the previous reports that link these factors to H3K27 methylation regulation. Specifically, Yoh et al. (2008) reported that STP6, IWS1, and SETD2 are in the same complex, and knockdown of SETD2 increases H3K27me3 at the 5′ end of its target genes, consistent with our hypothesis that JMJD3/KIAA1718-mediated H3K27 demethylation and transcriptional elongation may be coupled. Depletion of CHD7, which was also identified in our JMJD3 complex, has

been shown to increase H3K27me3 and reduce Ser2-phosphorylated Pol II on *Drosophila* polytene chromosomes (Srinivasan et al. 2008).

In *Drosophila*, the only reported H3K27me3 demethylase, dUTX, has been shown to be associated with Pol II and colocalized with the elongating form of Pol II on chromatin (Smith et al. 2008). Mammals have two related H3K27me3 demethylases: UTX and JMJD3. The human UTX has been shown to be associated with components of the H3K4 methyltransferase MLL complex (Cho et al. 2007; Issaeva et al. 2007; Lee et al. 2007), while our biochemical and functional data show that the related JMJD3 is associated with another H3K27me demethylase and transcriptional elongation factors. This difference suggests that UTX and JMJD3 may regulate different steps of the transcriptional process, although they both demethylate H3K27me3. Another important difference is that while UTX is abundantly and ubiquitously expressed in many cell types and tissues, JMJD3 is usually lowly expressed, and its expression is elevated in response to development and environmental stimuli (De Santa et al. 2007; Burgold et al. 2008). These features of JMJD3 are consistent with our hypothesis that JMJD3 may have important functions in regulating the expression of Pol II-poised genes.

Materials and methods

Cell culture and reagents

HL-60 cells were from American Type Culture Collection (ATCC) and maintained according to ATCC's instructions. During differentiation, 1×10^6 cells per milliliter were treated with 32 nM TPA (Sigma) as previously reported (Chen et al. 2008) for 12 h if not otherwise specifically indicated. The JMJD3 antibody was raised against His-JMJD3 (amino acids 687–1270) in rabbit by Covance, while the KIAA1718 antibody was raised against His-KIAA1718 (amino acids 540–720) in rabbit by Active Motif, and both were immunoaffinity-purified with the GST-tagged version of the antigens. Antibodies against histones were from Abcam (H3K4me3, AB8580; and H3, AB1791) and Millipore (H3K27me3, 07-449; H3K27me2, 07-452; and H3K27me1, 07-448). The RNA Pol II (N-20x) (sc-899 X) and SPT16 (sc-28734) antibodies were from Santa Cruz Biotechnology. The SPT6 antibody (A300-801A) was from Bethyl Laboratories. The Pol II Ser5p antibody (CTD4H8) was from Millipore, and the Pol II Ser2p antibody H5 was from Covance. The CDC73 antibody was a kind gift from W.F. Simonds.

RNAi, expression microarray analysis, and RT-qPCR

A NS control, two shRNAs against JMJD3, and two shRNAs against KIAA1718 were purchased from Open Biosystems (clones used were as follows: shJMJD3 #1, V2HS_236579; shJMJD3 #3, V2HS_23441; shKIAA1718 #13, V2LHS_201113; and shKIAA1718 #58, V2LHS_200458) and then moved into the retroviral vector pMSCV-PM according to the original publication (Schlabach et al. 2008). Viruses were produced in 293T cells and used to infect HL-60 cells for 48 h, which was followed by puromycin selection. RNA extracted from two biologically independent groups of stable cells subjected to different treatment was sent to the Phalanx Biotech Group for expression microarray analysis. Replicate hybridizations to the Human Whole-Genome OneArray were performed. A subset of genes from microarray results was verified by qRT–PCR. For the rescue experiments, mutations were introduced into all three key residues important for histone demethylase activity, and additional mutations were introduced in the regions targeted by shRNA. Then, the cDNAs were cloned into pMSCV-Flag-HA containing a blasticidin resistance gene. Stable HL-60 cells were infected with overexpression viruses, selected with blasticidin, and then infected with shRNA viruses, followed by puromycin selection. For RT–PCR, RNA was reverse-transcribed (Invitrogen), and PCR was run with SYBR green on a LightCycler 480II (Roche). Primers are listed in Supplemental Table S7.

ChIP-seq and ChIP-qPCR analyses

Chromatin was prepared from HL-60 cells treated with TPA for 12 h as previously described (Lan et al. 2007), except that it was sonicated to ~200 base pairs (bp). Chromatin was incubated with specific antibodies overnight at 4°C and then mixed with protein G Dynabeads (Invitrogen) for 2 h. The beads were washed extensively, de-cross-linked and further treated with RNase A and proteinase K. The mixture was then phenol-chloroform-extracted and precipitated with ethanol. Libraries were constructed from ~50 ng of ChIP DNA or input following Illunima's protocol and sequenced using an Illumina Genome Analyzer. The fastq data were aligned to the human genome (hg19) with Bowtie; those mapped to unique genome locations were kept, and the output data were stored in SAM format. To find the binding sites of the proteins, we ran MACS1.4 with P-value = 10^{-5} to call peaks from the aligned results (Zhang et al. 2008). To get a list of genes bound by proteins or enriched with H3K27me3, first we filtered the MACS result by an FDR of 5% and did a gene center annotation to find the genes within the region of peaks. For qPCR, eluted ChIP DNA was run on a Lightcycler 480II (Roche). The Pol II Ser2p ChIP was carried out as previously described (Brookes et al. 2012). Primers are listed in Supplemental Table S7.

Purification and identification of JMJD3-associated proteins

Flag-HA-JMJD3 and empty vector control cell lines were established by infecting 293T cells with virus expressing these vectors. The tandem affinity purification was performed as described previously (Nakatani and Ogryzko 2003). Briefly, nuclear extractions were incubated with anti-Flag agarose (Sigma) in the incubation buffer (50 mM Tris-HCl at pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 0.1% NP-40, 3 mM β-ME, protease inhibitors), washed extensively with the washing buffer (50 mM Tris-HCl at pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.1% NP-40, 3 mM β-ME), and eluted with Flag peptide (Sigma). Then, the eluate was incubated with anti-HA beads (Santa Cruz Biotechnology), washed, and eluted with HA peptides (Covance). The eluted proteins were subjected to mass spectrometry analysis.

Immunoprecipitation

Nuclear extracts were prepared from 293T or HL-60 cells as described above. The extracts were incubated with specific antibodies as indicated overnight at 4°C, and then protein G beads were added to the mixture. Beads were washed with the washing buffer, boiled in SDS-PAGE loading buffer, and analyzed by Western blot.

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