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Coordinated Regulation of Retinoic Acid Signaling Pathway by KDM5B and Polycomb Repressive Complex 2

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ABSTRACT

Polycomb repressive complex 2 (PRC2) is a critical epigenetic regulator in many biological processes, including maintenance of cell identity, stem cell self-renewal, differentiation, and deregulation of PRC2 is often observed in human cancers and diseases. Here we report that KDM5B (PLU-1/JARID1B), a histone lysine demethylase of Jumonji family, associates with PRC2 and colocalizes with PRC2 in nuclear bodies, and their physical association is dependent on direct interaction between KDM5B and the SUZ12 component of PRC2. Interestingly, co-occupancy of KDM5B and PRC2 was evidenced at the conserved *cis*-regulatory DNA element on retinoic acid (RA) responsive genes. Transcription readout and in vitro pull-down experiments suggest that KDM5B is an essential co-activator, but not a co-repressor, for the RA signaling, and the interface between KDM5B's JMJC domain and retinoic acid receptor α (RARα) is crucial for RA-mediated gene expression. Detailed chromatin immunoprecipitation assays addressed the seemingly paradox by revealing a biphasic effect of KDM5B on RA-induced gene activation through decoupled H3K4me3 demethylation and PRC2-antagonizing activities. These results demonstrate that KDM5B and PRC2 regulate RA signaling cascade in a cooperative and orchestrated fashion. J. Cell. Biochem. 115: 1528–1538, 2014.

KEY WORDS: KDM5B; PRC2; RETINOIC ACID; RAR; HISTONE MODIFICATION

Epigenetic regulation of gene transcription is achieved by the combinatorial action of various enzymatic complexes with collaborating or opposing activities toward histone tails and chromatin structure. As a highly conserved member of the large repertoire of transcriptional co-regulators, Polycomb group (PcG) proteins play crucial roles in various pathophysiological processes including maintaining cell identity, regulating cell differentiation, and promoting neoplastic progression [Simon and Kingston, 2009; Margueron and Reinberg, 2011; Lanzuolo and Orlando, 2012]. In mammals, PcG-mediated gene silencing involves two major multi-protein complexes, namely polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) [Simon and Kingston, 2009; Margueron and Reinberg, 2011]. PRC2 is composed of three core subunits EZH2, SUZ12, and EED, and WD40-domain proteins RBAP46/48 are also present in the complex [Margueron and Reinberg, 2011]. The SET domain containing protein EZH2 is the catalytic component of PRC2, which deposits methyl groups on histone H3 at lysine 27 and often inhibits gene transcription [Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002]. In addition to the core stoichiometric components, PRC2 recruits a list of proteins essential for its genomic targeting or enzymatic potency under various conditions [Cao and Zhang, 2004; Kuzmichev et al., 2005; Vire et al., 2006; Kim et al., 2009; Zhang et al., 2011].

Dynamic and reversible histone methylation was justified by the biochemical purification of lysine demethylase (KDM) LSD1 and Jumonji family proteins [Shi et al., 2004; Tsukada et al., 2006]. KDM5B belongs to the KDM5 family, a subfamily of Jumonji KDM that specifically targets histone H3 lysine 4. The KDM5 family proteins contain an AT-rich interaction domain (ARID or BRIGHT), a catalytic Jumonji N (JMJN) and Jumonji C (JMJC) domain, a C5HC2-zinc finger domain, and several plant homeobox domains (PHD) that are involved in recognition of histone tail methylation and protein–protein interactions [Yamane et al., 2007]. Aberrant KDM5B

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expression or activity has been found in various cancers, including breast cancer, prostate cancer and melanoma [Lu et al., 1999; Xiang et al., 2007; Roesch et al., 2010].

Concerted action of KMTs and KDMs is critical to establish proper local chromatin states for various gene transcriptional regulation pathways. Despite intensive investigations, how KMTs and KDMs coordinately regulate nuclear receptor (NR)-mediated gene transcription remains poorly understood. Retinoic acid (RA) is a lipid soluble signaling molecule derived from vitamin A (retinol). It exerts important physiological functions in organogenesis, homeostasis, differentiation, and apoptosis [Niederreither et al., 1999; Duester, 2008]. Primarily, RA mediates its biological effects by binding to specific nuclear receptors, retinoic acid receptors (RARs). The RA-RAR complex acts as a transcription factor and initiates gene transcription through recruitment of a number of co-regulators to the RA response DNA element (RARE) [Chambon, 1996; Lefebvre et al., 2005]. Interestingly, PRC2 components have been found to be recruited to many RA target genes in the absence of ligand. Upon RA treatment, the PRC2 binding is attenuated and the H3K27 trimethylation (H3K27me3) is decreased at these RAREs, highlighting the important co-repressor function of PRC2 for RA-mediated gene transcription [Gillespie and Gudas, 2007a,; Kashyap et al., 2011]. Of note, deregulated association of PRC2 with RARs impairs the anti-proliferative effects of RA in breast cancer and acute promyelocytic leukemia [Epping et al., 2005; Villa et al., 2007].

Here we propose KDM5B cooperates with PRC2 to modulate RA signaling pathway. The functional collaboration between KDM5B and PRC2 is dependent on their direct association at the *cis*-regulatory DNA elements on RA target genes. Transcription readout and pull-down experiments demonstrated that KDM5B acts as a co-activator, rather than a co-repressor, of RARα to stimulate RA-induced gene transcription programs. Further chromatin immunoprecipitation and epistasis analysis revealed the orchestrated molecular scenario played by KDM5B and PRC2 for fine-tuning RA-triggered gene transcription.

RESULTS

KDM5B IS PHYSICALLY ASSOCIATED WITH THE PRC2 COMPLEX

In an effort to identify novel epigenetic regulators cooperative with PRC2 in transcriptional silencing, we took a candidate approach to examine the potential interaction between EZH2 and a panel of repressive KDMs [Klose et al., 2006]. We transiently overexpressed individual KDM in a HeLa cell line that stably expresses physiological levels of FLAG-EZH2 fusion protein. EZH2 bound proteins were immunoprecipitated from nuclear extracts using M2 beads and were eluted with FLAG peptides after extensive washes. Among the KDMs we examined, myc-tagged KDM5B showed the most stable and consistent association with EZH2 (Fig. 1A, upper left panel), compared to the undetectable interactions between EZH2 and KDM5C or KDM5D (Fig. 1A, lower panel). The in vivo interaction between EZH2 and KDM5B was also supported by reciprocal immunoprecipitation assays (Fig. 1A, upper right panel). To test whether KDM5B interacts with other components of PRC2, we simultaneously overexpressed equal amounts of FLAG-EZH2, HA-SUZ12, T7-EED, together with myc-KDM5B in HeLa cells, and performed immunoprecipitation assays with antibodies against FLAG-, HA-, and T7-tag. The results suggest all the specific components of PRC2 could interact with KDM5B (Fig. 1B, left panel). Immunoprecipitation with anti-myc antibody further supports this conclusion, as all three subunits of PRC2 are present in the myc-purified fraction (Fig. 1B, right panel). To ensure that the interaction between KDM5B and PRC2 was not particular to conditions of overexpression, we conducted immunoprecipitation experiments in MCF-7 breast cancer cells with endogenous levels of PRC2 subunits and KDM5B. The results showed that all specific components of PRC2 could be efficiently co-immunoprecipitated with KDM5B (Fig. 1C).

Polycomb group proteins are often localized in intensely fluorescent foci in the nucleus under immunofluorescence microscopy, and such foci are termed as PcG bodies [Pirrotta and Li, 2012]. Consistent with the physical interaction between KDM5B and PRC2, immunostaining of endogenous KDM5B and SUZ12 in MCF-7 cells confirmed their co-localization in the PcG bodies (Fig. 1D).

MOLECULAR BASIS OF THE INTERACTION BETWEEN KDM5B AND PRC2

To further confirm the physical association between KDM5B and PRC2, GST pull-down assays were performed to map the interface between KDM5B and the PRC2 complex. Bacterially expressed GST-tagged components of the PRC2 complex were individually purified and incubated with in vitro transcribed/translated myc-tagged KDM5B. The results of these experiments indicated that KDM5B only interacts directly with the SUZ12 component of PRC2, suggesting the recruitment of the PRC2 complex by KDM5B in vivo is through an interaction of KDM5B with SUZ12 (Fig. 2A). Interestingly, although the interaction between wild-type EED and KDM5B was barely detectable, an asparagine-to-isoleucine substitution at amino acid 193 in the second WD40 repeat of EED, a hypomorphic mutation leading to disrupted anterior–posterior patterning of the primitive streak in mice [Shumacher et al., 1996 van Lohuizen et al., 1998], greatly enhanced the binding affinity of EED to KDM5B (Fig. 2B).

KDM5B contains three PHD domains, which have been characterized as the major binding pockets for specific methylated histone lysine residues [Taverna et al., 2007]. We examined the binding preference of KDM5B for histone methylation modifications by using histone peptide binding assays. We purified various GST-KDM5B deletion mutants containing the three PHD domains, and incubated them with different histone H3 trimethylated peptides. The peptide-bound proteins were analyzed by Western blotting and the results indicated that the first PHD domain of KDM5B is capable to read H3K9me3, analogous to its counterpart in KDM5C [Iwase et al., 2007]. Moreover, the third PHD domain of KDM5B, which is missing in KDM5C [Klose et al., 2006] could bind both H3K4me3 and H3K9me3. Importantly, the second PHD domain of KDM5B showed strong binding preference for H3K27me3, providing an additional layer to support the interaction between KDM5B and PRC2 (Fig. 2C).

KDM5B REGULATES RA-MEDIATED GENE TRANSCRIPTION

Genomic targeting of the Polycomb group proteins exhibits a very strong bias for genes controlling development and cell fate decisions

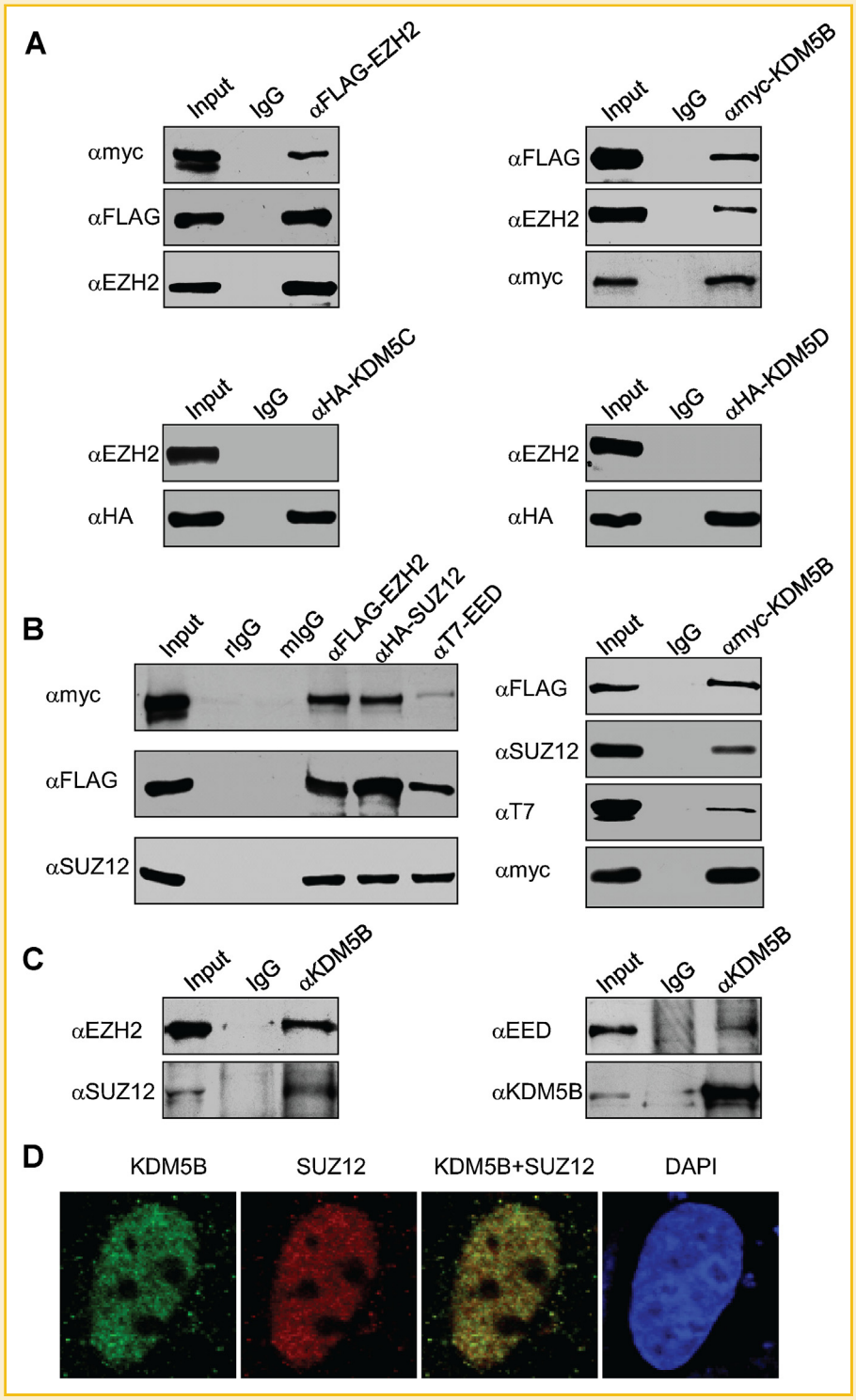

Fig. 1. KDM5B is associated with the PRC2 complex. A: HeLa cells stably expressing FLAG-EZH2 were transfected with a myc-KDM5B construct. Forty eight hours post transfection, the nuclear extract was subjected to immunoprecipitation assay with antibodies against FLAG- (upper left panel) or myc-tag (upper right panel) followed by Western blotting analysis with indicated antibodies. Similar experiments were performed for KDM5C and KDM5D, except the HA-KDM5C or HA-KDM5D constructs were used for transfection and the protein interactions were analyzed with indicated antibodies (bottom panel). B: Equal amounts of FLAG-EZH2, HA-SUZ12, T7-EED, together with myc-KDM5B were simultaneously overexpressed in HeLa cells and immunoprecipitation assays were performed with antibodies against FLAG-, HA-, and T7-tag followed by Western blotting analysis with indicated antibodies (left panel). Reciprocal immunoprecipitation using anti-myc antibody was subjected to Western blotting analysis for detection of PRC2 components (right panel). C: Endogenous Co-IP was performed in MCF-7 cells using antibody against KDM5B. Antibodies used for Western blotting were indicated on the left. The immunoglobulin G (IgG) control was included in each experiment. D: Representative confocal images of indirect immunofluorescence for endogenous KDM5B (green) and SUZ12 (red) in MCF-7 cells. DAPI (4′,6-diamidino-2-phenylindole) staining was used to visualize the cell nucleus.

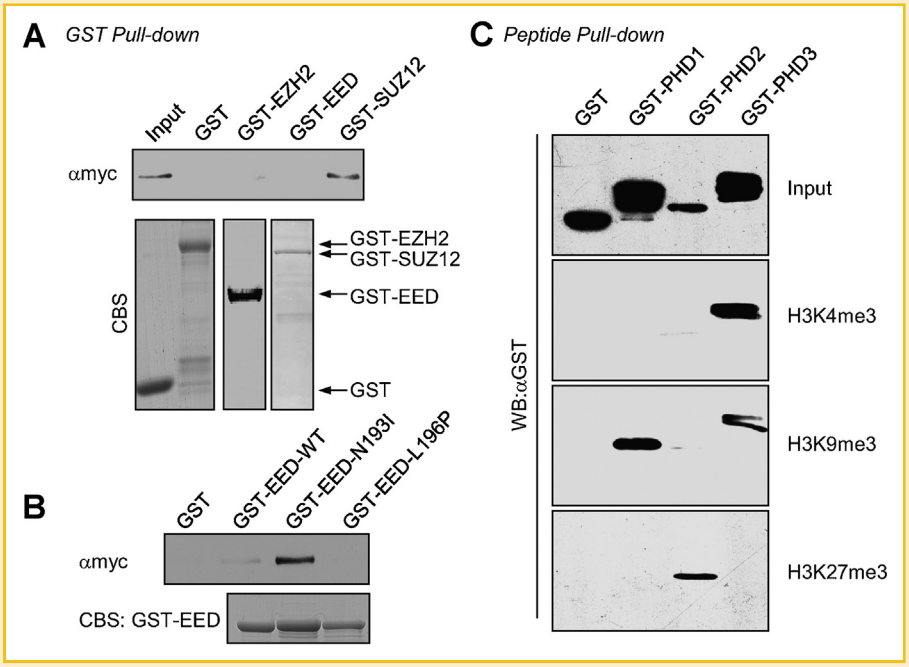

Fig. 2. Molecular basis for the interaction between KDM5B and PRC2. A: Purified GST or GST-EZH2, -EED, -SUZ12 proteins immobilized on glutathione Sepharose 4B beads were incubated with in vitro transcribed/translated myc-KDM5B. After extensive washes, the bound proteins were resolved on SDS–PAGE and detected with anti-myc antibody (top panel). Recombinant GST-EZH2, GST-EED, and GST-SUZ12 fusion proteins were purified by glutathione affinity resin and analyzed by Coomassie Blue Staining (CBS) (bottom panel). B: GST pull-down experiments were performed with in vitro transcribed/translated myc-KDM5B and purified GST, GST fused wild-type EED, or site-specific EED mutants as indicated. The precipitated proteins were examined by Western blotting analysis (top panel). CBS of recombinant GST-EED fusion proteins was shown below. C: Histone peptide binding assays were performed. Each PHD domain of KDM5B (illustrated in Fig. 4A) was fused to GST-tag and purified through glutathione affinity resin. The recombinant GST-PHD fusion proteins were then incubated with biotinylated histone peptides with indicated modifications. Protein-peptide complex was pulled-down with streptavidin resin. The resultant mixture was subjected to Western blotting analysis with anti-GST antibody.

[Bracken et al., 2006]. To investigate the functional importance of the interaction between KDM5B and PRC2, we performed chromatin immunoprecipitation (ChIP) assays to examine the recruitment of KDM5B on the promoters of a particular subset of classic PcG target genes, the homeotic genes HOXA1 and HOXA5 [Bracken et al., 2006; Chen et al., 2007]. The results revealed that both KDM5B and EZH2 were present at the conserved regulatory elements of HOXA1 and HOXA5 (Figs. 3A and 5A). ChIP analysis also revealed concurrent presence of KDM5B and EZH2 on the promoter region of myelin transcription factor 1 (MYT1) [Zhang et al., 2011], a classic PRC2 target gene, but not that of c-Myc, which serves as a negative control gene (Fig. 3A).

Functional convergence of RA signaling cascade and PRC2-mediated gene silencing has been demonstrated by the co-linear activation pattern of the HOX cluster along the anterior–posterior axis [Kmita et al., 2000; Sessa et al., 2007]. Mechanistic studies in embryonic carcinoma and melanoma cells revealed characteristic chromatin structure changes accompanied by dynamic association of PRC2 with RAREs in the presence of RA, further supporting an intimate link between RA signaling and PcG repression [Epping et al., 2005; Gillespie and Gudas, 2007b]. Consistent with these reports, we observed KDM5B together with EZH2 are enriched on RARE at the promoter region of RARβ, a well-established RA target gene, highlighting the involvement of KDM5B in RA/PRC2 cooperation for gene transcription (Fig. 3A).

To further examine the regulatory roles of KDM5B on RA-triggered transcription programs, we performed reporter assays with luciferase reporters driven by either an artificial RARE-containing promoter or the endogenous RARβ promoter. Surprisingly, in contrast to the general repressive function of H3K4 KDMs, KDM5B enhanced RA-mediated transcription in a dose-dependent fashion (Fig. 3B). To understand the underlying mechanism of KDM5B's effect on RA-mediated gene transcription, we next examined whether KDM5B might interact with RARα, the receptor of RA. As shown in Figure 3C, GST pull-down assays indicated that KDM5B interacts with RARα in a RA-independent manner (Fig. 3C). To further demonstrate the positive regulatory effect of KDM5B on RA target genes, we depleted KDM5B in MCF-7 cells with two specific siRNAs (Fig. 3D, right panel) and examined the expression levels of HOXA1 and RARβ. In line with the above results, depletion of KDM5B severely impaired endogenous RA-mediated gene activation as evidenced by the significant decrease of HOXA1 and RARβ levels (Figs. 3D and 5B).

MOLECULAR BASIS FOR THE INTERACTION BETWEEN KDM5B AND RAR

As stated earlier, KDM5B contains multiple domains with various functions (Fig. 4A). To evaluate the contribution of individual module of KDM5B for RA-mediated transcription, we prepared various myc-tagged KDM5B mutants with JMJN, JMJC, BRIGHT,

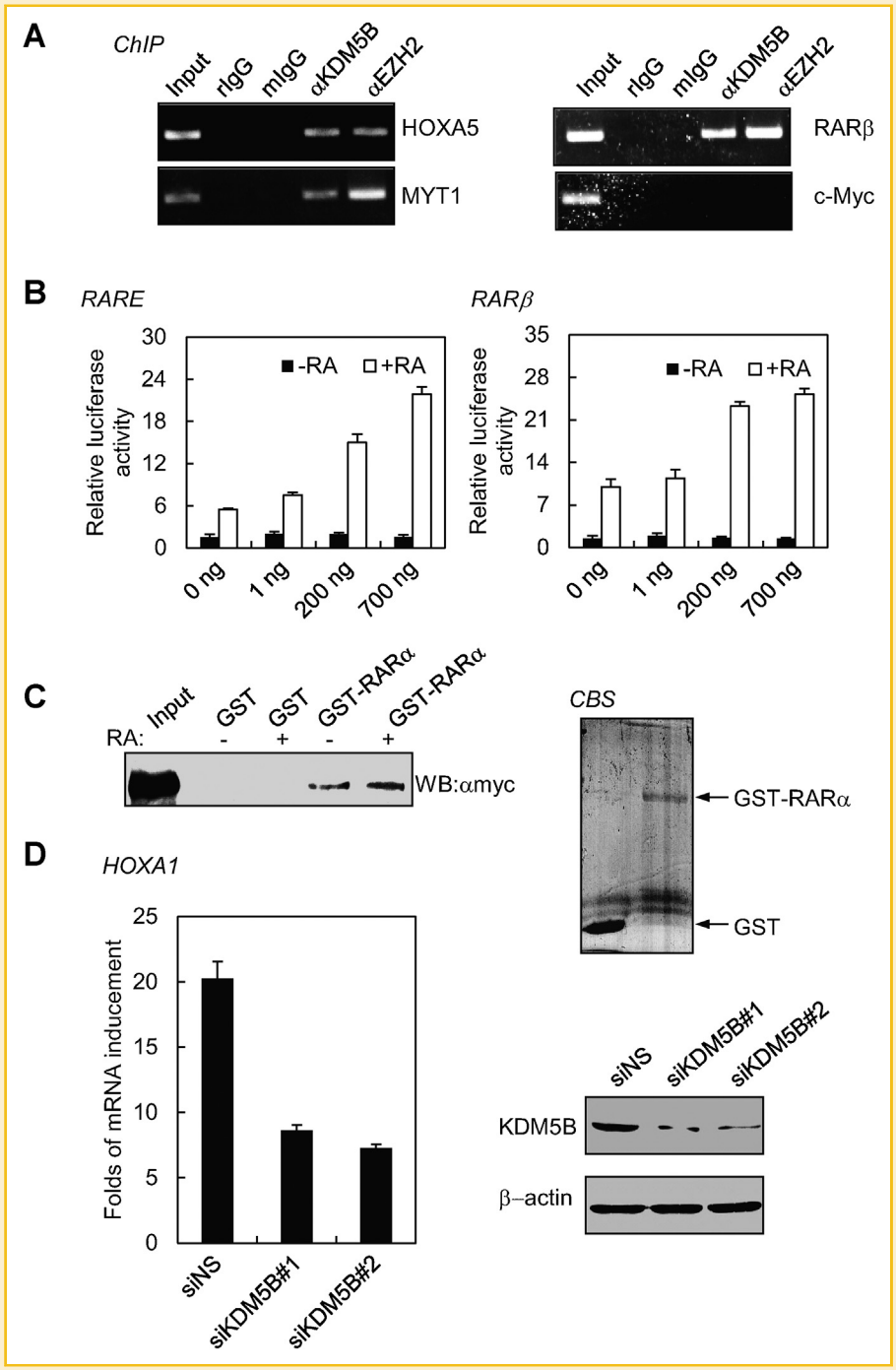

Fig. 3. KDM5B regulates RA-mediated gene transcription. A: ChIP assays were performed in MCF-7 cells with primer pairs to amplify the regulatory elements on HOXA5's RARE, or the promoter regions of RARβ, MYT1, and c-Myc. Specific antibodies against KDM5B and EZH2 together with normal control rabbit or mouse IgG were used to immunoprecipitate the protein-DNA complex. B: Luciferase reporter assays were performed. Increasing amounts of KDM5B together with luciferase constructs harboring RARE or RARβ promoter were transfected in HeLa cells and 48 h later the cells were treated with vehicle (−) or 1 μM RA (+) for additional 24 h before the cells were harvested for luciferase activity assay. C: KDM5B directly interacts with RARα in vitro. GST pull-down experiments were performed with in vitro translated myc-KDM5B and purified GST or GST-RARα in the absence or presence of RA (1 μM) as indicated (left panel). CBS of purified GST or GST-RARα fusion protein was shown in the right panel. D: Knockdown of KDM5B impairs the responsiveness of HOXA1 for RA. MCF-7 cells were transfected with double-stranded control (siNS) or two independent KDM5B RNAi oligonucleotides (siKDM5B#1 and #2), and the cells were cultured with 10% dextran-charcoal-stripped fetal bovine serum for 3 days before treatment with RA (1 μM) for additional 24 h. Quantitative RT-PCR analysis was then performed and mRNA levels of HOXA1 relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) under different conditions were examined. Fold changes of normalized mRNA induction relative to the baseline expression in the absence of RA were used to determine the RA responsiveness of HOXA1 (left panel). Knockdown efficiency for KDM5B was shown in the right panel.

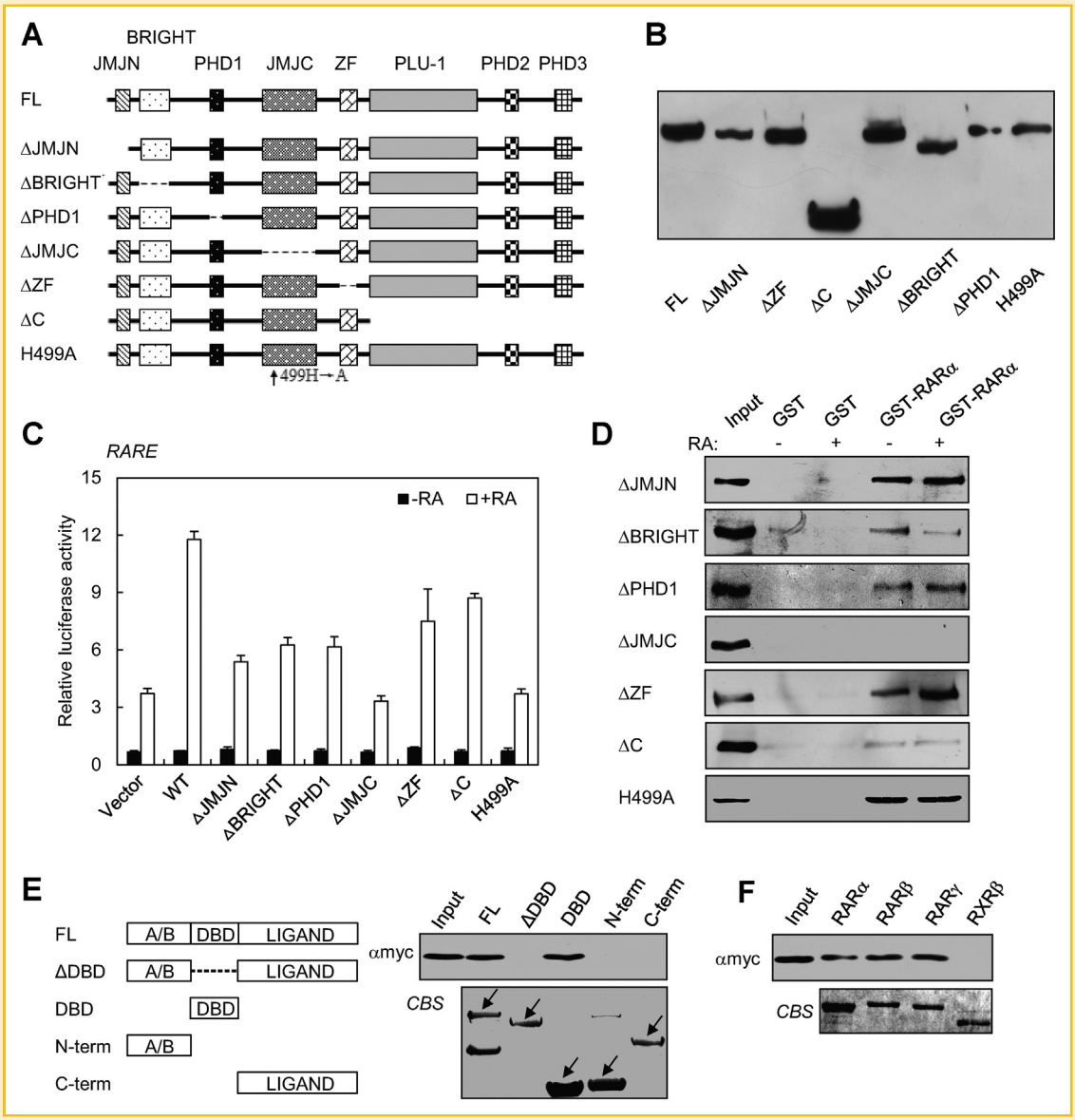

Fig. 4. Molecular basis for the interaction between KDM5B and RAR. A: Schematic diagram showing KDM5B deletion mutants. B: Myc-tagged KDM5B mutants were ectopically expressed in HeLa cells. Their expression pattern was validated through Western blotting analysis using anti-myc antibody. C: Individual KDM5B mutant together with a luciferase construct harboring artificial RARE were transfected in HeLa cells and 48 h later the cells were treated with vehicle (−) or 1 μM RA (+) for additional 24 h. The resultant cells were then harvested for luciferase activity assay. D: Purified GST or GST-RARα were immobilized on glutathione Sepharose 4B beads and then were incubated with various in vitro translated myc-tagged KDM5B mutants in the absence or presence of RA. After extensive washes, the bound proteins were resolved and analyzed by Western blotting assay. E: To determine the domain within RARα necessary for interaction with KDM5B, GST pull-down experiments were performed with in vitro translated myc-KDM5B and purified GST-RARα or various mutants as indicated (upper panel, right). The full-length RARα protein is composed of the N-terminal (A/B) domain, the DBD, and the C-terminal ligand binding domain (Ligand). RARα and RARα mutant proteins were illustrated in the left panel. CBS of purified GST-RARα or mutants was shown in the lower right panel. F: GST pull-down experiments were performed with in vitro translated myc-KDM5B and purified GST-RARα, GST-RARβ, GST-RARγ, or GST-RXRβ. CBS of purified GST-RARα, GST-RARβ, GST-RARγ, and GST-RXRβ was shown in the lower panel.

PHD domain or the entire C-terminal portion deleted (Fig. 4A,B). We performed RARE-luciferase reporter assays using these mutants. The results showed that these mutants impaired the RA-induced gene transcription to varying degrees, in agreement with the indispensible role of the individual domain for DNA/cofactor binding or demethylase activity of KDM5B (Fig. 4C). Among them, the mutant with the JMJC domain deleted (ΔJMJC) completely lost its activation activity on RA-mediation transcription, underscoring the essential role of this domain for the co-activator function of KDM5B (Fig. 4C).

To better understand the modular effects of KDM5B on RARα-mediated transcription, we performed GST pull-down experiments to map the binding interface between RARα and KDM5B. In agreement with the reporter assays, the ΔJMJC mutant of KDM5B completely lost its binding capacity to RARα (Fig. 4D). JMJC domain

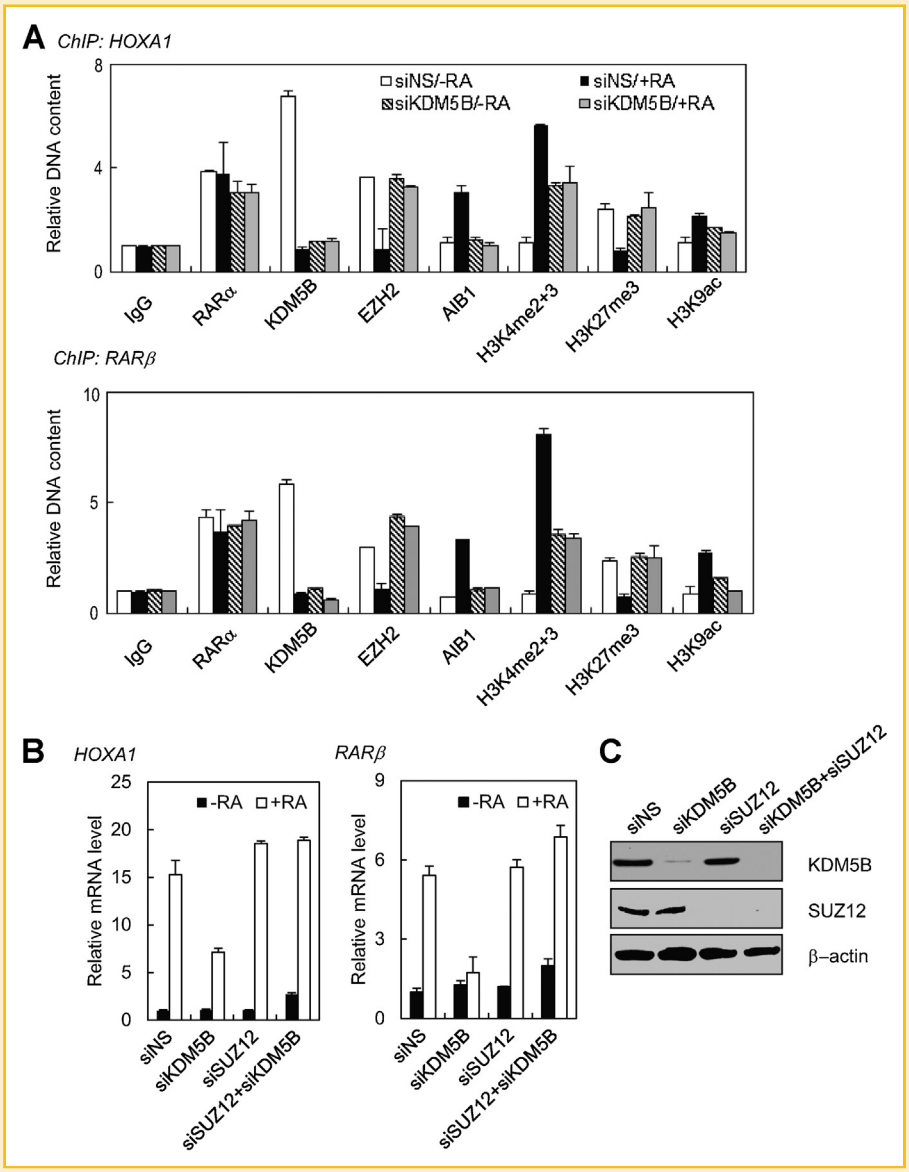

Fig. 5. Biphasic regulation of RA-dependent gene transcription by KDM5B through distinct mechanisms. A: Detailed occupancy patterns of RARα, KDM5B, EZH2, AIB1, H3K4me2/3, H3K27me3, and H3K9ac at the regulatory regions of HOXA1 (upper panel) or RARβ (lower panel). MCF-7 cells were transfected with double-stranded control (siNS) or KDM5B (siKDM5B) RNAi oligonucleotides and were cultured with 10% dextran-charcoal-stripped fetal bovine serum for 3 days before treatment with RA for additional 24 h. ChIP assays coupled with quantitative PCR were then performed to examine the enrichment of specific RARα co-regulators or histone modifications with indicated antibodies. To capture the potential catalytic activities of KDM5B on both H3K4me2 and H3K4me3, a mixture of the two antibodies was applied in a single ChIP experiment. B: Epistasis analysis of RA-responsiveness for HOXA1 and RARβ. MCF-7 cells were transfected with control (siNS) or specific double-stranded RNAi oligonucleotides against KDM5B (siKDM5B), SUZ12 (siSUZ12) or a combination of the two. Normalized mRNA levels of HOXA1 (left panel) and RARβ (right panel) in the absence or presence of RA (1 μM) were examined and presented as relative values compared to their basal levels in siNS cells without RA. C: Knockdown efficiency for KDM5B and SUZ12 was confirmed by Western blotting analysis with indicated antibodies.

is the characteristic domain of Jumonji family of histone demethylases. It contains an enzymatically active pocket that coordinates cofactors Fe(II) and αKG and also confers substrate binding specificity [Klose et al., 2006]. Due to the absence of either Fe(II) or αKG in our experimental conditions, we reasoned that the substrate recognition interface, but not the cofactor binding pocket, of the JMJC domain is critical for the binding between KDM5B and RARα. Accordingly, the KDM5B mutant containing a histidine-to-alanine substitution at amino acid 499 in the JMJC domain, which abolishes the cofactor binding ability of KDM5B but retains its substrate recognition function, remained interacted with RARα efficiently but lost its ability to enhance RA-induced transcription (Fig. 4C,D). We also mapped the domains of RARα that are responsible for the interaction with KDM5B. GST pull-down

experiments indicated that RARα interacted with KDM5B via its DBD domain, which is both necessary and sufficient to mediate the interaction between the two proteins (Fig. 4E). Moreover, we purified RARβ, RARγ, and RXRβ proteins and performed similar GST pull-down assays. The results indicated that KDM5B could directly interact with all three RAR members examined but not with RXRβ (Fig. 4F).

BIPHASIC REGULATION OF RA-DEPENDENT GENE TRANSCRIPTION BY KDM5B THROUGH DISTINCT MECHANISMS

To address the apparent paradox that a H3K4 demethylase functions as co-activator for RA-mediated transcription, we performed quantitative ChIP assays to examine co-regulator dynamics and histone modification changes at the *cis*-regulatory region of RA target genes in the presence or absence of KDM5B. To this end, we transfected MCF-7 cells with control or KDM5B-specific siRNAs, and these cells were further cultured in DMEM containing 10% dextran-charcoal-stripped fetal bovine serum for 3 days before treatment with RA for additional 24 h. In agreement with the general model that unliganded RAR mediates active repression by binding to the RARE of the target gene promoters [Glass and Rosenfeld, 2000], we observed consistent association of RARα on the RAREs irrespective of the presence of RA (Fig. 5A). In contrast, upon RA treatment, both KDM5B and EZH2 were dissociated from the RAREs of the promoter, concomitant with the increased H3K4me2/3 and decreased H3K27me3 levels at this region. By contrast, co-activator AIB1 and the active histone modification marks such as H3K9 acetylation (H3K9ac) were detected more readily at the region (Fig. 5A). These results suggest that the repressive activity of KDM5B is abolished during RA-mediated local chromosomal remodeling.

In concert with the demethylation activity of KDM5B towards H3K4me2/3, removal of KDM5B resulted in increased H3K4me2/3 in the absence of RA, nevertheless to a less degree compared to when both KDM5B was present and RA was applied. Importantly, in the presence of RA, depletion of KDM5B suppressed the acquisition of AIB1 and active H3K4me2/3 and H3K9ac marks on the RAREs (Fig. 5A); meanwhile, KDM5B removal deferred the clearance of EZH2 and repressive H3K27me3 modification from these elements (Fig. 5A).

To further evaluate the transcriptional outcome caused by coordinated KDM5B/PRC2 action, an epistasis analysis with single or combinational knockdown of KDM5B and SUZ12 was performed (Fig. 5B). When RA was absent, depletion of either SUZ12 or KDM5B had little effect on the transcriptional potential of RARβ and HOXA1, and simultaneous removal of both proteins resulted in mild transcriptional activation, underscoring the repressive activity of KDM5B in the absence of ligand (Fig. 5B). By contrast, in the presence of RA, depletion of KDM5B abolished RA responsiveness of RARβ and HOXA1 transcription, suggesting KDM5B acts as a transcriptional activator under this condition. Intriguingly, depletion of SUZ12 completely restored RA-induced gene transcription in KDM5B deficient cells. Together with the occupancy data from ChIP experiments, these results suggested that the major activating function of KDM5B is through antagonizing the repressive PRC2 complex.

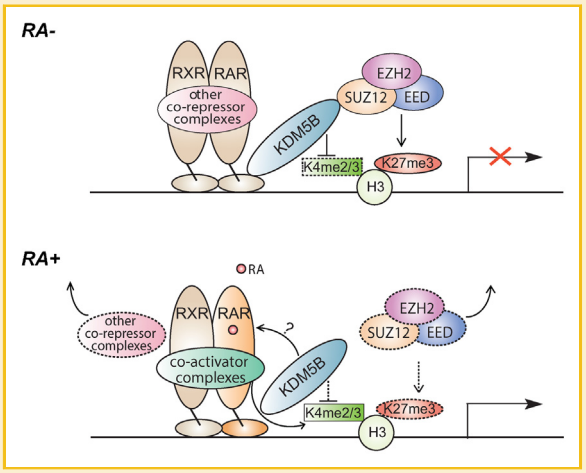

Fig. 6. Model of coordinated regulation of Retinoic acid signaling pathway by KDM5B and PRC2. In the absence of RA, co-binding of PRC2 and KDM5B leads to high level of H3K27me3 and low level of H3K4me2/3 on RARE with target gene transcription being repressed. However, when RA ligand is present, the substrate of KDM5B might be changed to RARα. Such alternation could contribute to a co-regulator exchange strategy and subsequently cause the disassociation of PRC2 at RARE to enable target gene expression.

The above data support a biphasic model regarding to the function of KDM5B in RA-mediated signaling: when the active ligand is absent, KDM5B and PRC2 coordinately demethylate H3K4me2/3 and methylate H3K27me3, leading to gene transcriptional repression; by contrast, when RA is present, the liganded RARα requires KDM5B for co-regulator exchange to unload the repressive PRC2 complex and load the AIB1-contaning co-activators (Fig. 6).

DISCUSSION

By enlisting distinctive chromatin modifiers, the PRC2 complex arsenal has been reported to include histone methyltransferase, deacetylase [Kuzmichev et al., 2005] and DNA methyltransferase [Vire et al., 2006]. In this study, we show KDM5B physically interacts with the PRC2 complex, adding a putative histone H3K4me2/3 demethylation activity to this complex. At the first glance, KDM5B and PRC2 contradict each other regarding to transferring the methyl group to histone tails. However, the chromatin context imposed by distinct reader proteins of H3K4 and H3K27 methylation renders the interplay between KDM5B and PRC2 to be logical for transcription. Indeed, exquisite functional interactions between distinct histone modification enzymes are necessary for ultimate resolving the bivalent H3K4me3/H3K27me3 chromatin structures into unidirectional state during differentiation [Bernstein et al., 2006], as demonstrated by the concerted H3K27 demethylation and H3K4 methylation during HOX gene activation, when mixed-lineage leukemia (MLL) 2/3 complex methylated H3K4 while UTX demethylated H3K27 [Cho et al., 2007; Lee et al., 2007]. Therefore, it is conceivable that a counterpoised complex exists by incorporating H3K4 demethylase and H3K27 methyltransferase in a single PRC2 complex to turn down the poised genes efficiently.

Intriguingly, an embryonic Jumonji family protein JARID2 enriched in stem cells colocalizes with PRC2 on chromatin, and modulates self-renewal of pluripotent cells by coordinated regulation of PRC2 enzymatic activity and target gene occupancy [Peng et al., 2009; Shen et al., 2009; Li et al., 2010; Pasini et al., 2010]. However, due to amino acid substitutions in the catalytic pocket, JARID2 is devoid of enzymatic activity for demethylation. By contrast, KDM5B is capable of performing H3K4me2/3 demethylation function and its coordination with PRC2 in RA cascade is dependent on such enzymatic activity to ensure the active repression of RARα in the absence of RA (Fig. 5). Although it is not totally unexpected that a KDM can function as both an activator and a repressor, as exemplified by the dual demethylase activities of LSD1 towards H3K4 and H3K9 under different chromatin circumstances [Metzger et al., 2005; Klose and Zhang, 2007], it was surprising to find KDM5B is a transcriptional co-activator for RARα (Fig. 3) since its enzymatic specificity for H3K4 could be observed even when RARα is present on RARE (Fig. 5A, notice the increased H3K4me2/3 enrichment when KDM5B was removed and RA was absent). Interestingly, both ChIP results and the epistasis analysis revealed that, removal of KDM5B only lead to partial relaxation of local chromatin structures, as indicated by incomplete restoration of H3K4 methylation and H3K27 demethylation irrespective of RA treatment; while combinational depletion of PRC2 components fully restored the enrichment of active histone modifications to induce RA responsiveness of target genes (Fig. 5). The biphasic manifestation of KDM5B therefore implied the distinction of its enzymatic substrate selectivity in the presence or absence of RA and further raised a possibility that RA-liganded RARα could stimulate KDM5B to unload PRC2 through its enzymatic action on an unidentified substrate other than H3K4 (Fig. 6). Although this postulation cannot be demonstrated at the current stage due to lack of specific antibodies to determine the methylation status of RARα's co-regulators, direct interaction between the demethylase pocket of KDM5B and RARα (Fig. 4) supports the notion that KDM5B might act on RARα enzymatically. In favor of this hypothesis, non-histone methylation was demonstrated to regulate the transcriptional outcome of another member of NR superfamily, namely estrogen receptor α [Subramanian et al., 2008].

In addition to being essential regulators of epigenetic memory and cell fate determination, mammalian PRC2 members have been implicated in regulating cellular proliferation and neoplastic development [Sparmann and van Lohuizen, 2006]. The oncogenic functions of PRC2 complex arise in part from its transcriptional repression of *CDKN2A*-centered tumor suppressor network [Bracken et al., 2006; Li et al., 2013]. However accumulating evidences suggest that aberrant detainment of PRC2 complex in RA cascades, caused by either chimeric PML-RARα fusion in leukemia or tumor antigen PRAME overexpression in melanoma, could lead to constitutive association of co-repressors with RAR and consequently suppress the anti-proliferation and pro-differentiation effects of RA for cancer therapy [Epping et al., 2005; Villa et al., 2007]. The association of KDM5B with the PRC2 complex identified in this manuscript may thus provide at least a clue or a potential pharmaceutical target for antagonizing the repressive effect of PRC2 on RA pathway.

EXPERIMENTAL PROCEDURES

CELL CULTURE AND DRUG TREATMENT

MCF-7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. All-trans-RA was obtained from Sigma-Aldrich and dissolved in ethanol. To trigger a RA-mediated gene transcription, cells were generally treated with 1 μM retinoic acid for 24 h.

ANTIBODIES AND WESTERN BLOTTING ANALYSIS

αKDM5B and αH3K4me2/3 were from Abcam; αEZH2, αSUZ12, αEED, αAIB1, αH3K9Ac, and αH3K27me3 from Millipore; αFLAG and αHA from Sigma-Aldrich; αT7 and αmyc from MBL; and αRARα from Santa Cruz Biotechnology. Western blotting was performed according to the procedures described elsewhere [Zhang et al., 2010, 2011; Li et al., 2013].

CO-IMMUNOPRECIPITATION (CO-IP)

Cells were collected and lysed on ice with lysis buffer containing 0.5% NP40. The lysates were pre-cleared by incubation with protein A/G Sepharose beads (Sigma). The protein complex was then precipitated by a specific antibody together with protein A/G Sepharose beads followed by extensive washing. The resulting materials were analyzed by Western blotting.

FLUORESCENCE CONFOCAL MICROSCOPY

MCF-7 cells were plated into six-well chamber slides. Cells were washed with PBS, fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.1% (v/v) Triton X-100 in PBS, blocked with 0.8% BSA, and incubated with anti-KDM5B and SUZ12 antibodies followed by staining with FITC or RITC-conjugated secondary antibodies. Cells were washed four times and a final concentration of 0.1 μg/ml DAPI (Sigma) was included in the last washing to stain nuclei. Images were visualized and recorded with an Olympus FV1000S confocal microscope.

GST PULL-DOWN ASSAYS

GST pull-down assays were performed as previously described [Zhang et al., 2011]. Full-length KDM5B was transcribed/translated in vitro from the plasmid myc-KDM5B/pcDNA3.1. For protein purification, the following cDNA fragments were amplified by PCR and cloned into pGEX-6P-1 vector: Full length of EZH2, SUZ12, EED; EED mutants N193I or L196P; three individual PHD domains of KDM5B; full length of RARα, RARβ, RARγ, RXRβ; truncated mutations of RARα. These recombinant GST fusion proteins were expressed in *Escherichia coli* strain BL21 and purified through glutathione affinity resin (Amersham Biosciences).

HISTONE PEPTIDE BINDING ASSAY

Five micrograms of purified proteins were incubated with 0.2 μg of biotinylated histone peptide in 100 mL of the binding buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.5% NP-40, and 0.3 mg/mL bovine serum albumin] overnight at 4°C. Protein–peptide complexes were pulled down with streptavidin beads, washed five times, and subjected to Western blotting analysis using anti-GST antibody.

CHROMATIN IMMUNOPRECIPITATION (ChIP)

ChIP experiments were performed according to the protocol described previously [Zhang et al., 2010; Li et al., 2013]. The precipitated DNA was quantified by PCR followed by agarose gel electrophoresis or real-time PCR with the results presented as mean ± SD for triplicate experiments. The primers for ChIP-PCR analysis are listed below: HOXA1 forward: ATTCTTGCATTGTCCATCTGTCA, HOXA1 reverse: GGAAGTGAGAAAGTTGGCACAGT; HOXA5 forward: CTAAATGGCTTTCCCCCTTC, HOXA5 reverse: ACCAATTGTTAGGCCGTCAG; RARβ forward: AAAGAAAACGCCGGCTTGT, RARβ reverse: AGCCCTGCAAAAGCAGACA; MYT1 forward: AGCCCGCTCTTTATGATGG, MYT1 reverse: AAGGTGCCTATGTTTGTCC; c-Myc forward: GCCTCTATCATTCCTCCCTATC, c-Myc reverse: AAACCGCATCCTTGTCCTG.

LUCIFERASE ACTIVITY ASSAY

The luciferase reporters harboring RARE or RARβ promoter were constructed with backbone of pGL3 basic vector. The reports were transfected in 24-well plates using lipofectamine 2000 (Invitrogen). Forty eight hours post-transfection or for an additional treatment with RA, the cells were harvested and luciferase activity was measured with a dual luciferase kit (Promega) according to the manufacturer's protocol. The quantitative results were presented as mean ± SD for triplicated experiments.

QUANTITATIVE RT-PCR (RT-qPCR)

Total cellular RNAs were isolated with the RNeasy kits (Qiagen) and used for first strand cDNA synthesis with the Reverse Transcription System (Promega, A3500). Quantitation of all gene transcripts was done by quantitative PCR using Power SYBR Green PCR Master Mix and an ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA) with the expression of GAPDH as the internal control. The quantitative results were presented as mean ± SD for triplicated experiments. Primers used for RT-qPCR are listed below: GAPDH forward: GAAGGTGAAGGTCGGAGTC, GAPDH reverse: GAAGATGGTGATGGGATTTC; HOXA1 forward: CGCGTTAAATCAGGAAGCAG, HOXA1 reverse: AAAAGTCTGCGCTGGAGAAG; RARβ forward: ATGCTGGATTTGGTCCTCTG, RARβ reverse: TTTGTCGGTTCCTCAAGGTC.

RNA INTERFERENCE

Double-stranded RNA oligonucleotides (siRNAs) were synthesized by Shanghai GenePharma Co., Ltd. The sequences for KDM5B and SUZ12 siRNAs were described previously [Bracken et al., 2006; Yamane et al., 2007]. siRNAs were transfected into MCF7 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The siRNA oligonucleotide sequences are as follows: siKDM5B#1: GGAGAUGCACUUCGAUAUA; siKDM5B#2: CACUGGAGCUAUUCAAUUA; siSUZ12: AAGCUGUUACCAAGCUCCGUG; siNS: UUCUCCGAACGUGUCACGU.

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