BMB
reports

The PcG protein hPc2 interacts with the N-terminus of histone demethylase JARID1B and acts as a transcriptional co-repressor

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JARID1B (jumonji AT rich interactive domain 1B) is a large nuclear protein that is highly expressed in breast cancers and is proposed to function as a repressor of gene expression. In this paper, a phage display screen using the N-terminus of JARID1B as bait identified one of the JARID1B interacting proteins, namely PcG protein (Polycomb group) hPc2. We demonstrated that the C-terminal region, including the COOH box, was required for the interaction with the N-terminus of JARID1B. In a reporter assay system, co-expression of JARID1B with hPc2 significantly enhanced the transcriptional repression. These results support a role for hPc2 acting as a transcriptional co-repressor. [BMB reports 2009; 42(3): 154-159]

INTRODUCTION

The human JARID1B (jumonji AT rich interactive domain 1B) gene, also designated PLU-1, encodes for a 1,544 amino acid multi-domain protein that is localized exclusively to the nucleus and is constitutively expressed in 90% of breast cancers (1,2). It was shown that JARID1B had transcriptional repression properties (3). Recently, it was found that JARID1B was an H3K4 demethylase involved in transcriptional repression and breast cancer cell proliferation (4). Methylation of histones is regarded as a stable modification that defines a cell's epigenetic program, which regulates chromatin structure and transcription.

However, the recent discovery of histone demethylases has challenged this view of the stable nature of histone methylation. It was demonstrated that the JmjC-containing JARID1 protein family, including RB binding protein 2 (RBP2, JARID1A), PLU1 (JARID1B) and SMCX (JARID1C), were histone demethylases with specificity for tri- and di-methylated H3K4 (5). Thus, the transcriptional repression function of JARID1B could result from histone demethylation. The detailed mechanism, however, is not clear. Is this demethylation function completed by a complex that includes JARID1B or by JARID1B itself? Are the other JmjC family molecules involved in this process? Moreover, JARID1B can demethylate H3K4 me1, me2 and me3 *in vivo*, but does not target H3K4 me1 containing substrates generated by SET7 *in vitro* (4). These differences suggest that the transcriptional repression function is mediated by molecules besides JARID1B.

In this paper, we identified 35 protein partners that had high binding affinities with the N-terminus of human JARID1B protein. We investigated the interaction between JARID1B and hPc2. We also investigated the effect of hPc2 on the transcriptional repression by JARID1B.

RESULTS

Screening of a phage display library with the N-terminal of JARID1B

To detect proteins that interacted with JARID1B, we performed a phage display assay using a purified recombinant N-terminus of human JARID1B as bait and a human brain cDNA expression library that expressed in-frame cDNAs fused to T7 capsid protein. A list of binding partners is given in Table 1. Among these 35 proteins, we chose the PcG2 protein hPc2 for further investigation based on its previously described transcriptional repression functions (6). hPc2, also known as Cbx4, interacts with other PcG proteins and functions as a transcriptional repressor (7,8). It is noteworthy that other proteins were also detected by JARID1B binding, such as cell cycle regulatory protein CDC20, homeobox transcriptional factor CDX2, transcriptional factor ELF4 and calcium channel related protein CACNA1H. These potential binding partners were not considered further in this initial report.

hPc2 interacts with JARID1B *in vitro* and *in vivo*

To determine if the interaction between JARID1B and hPc2 observed during the phage display assay also occurred *in vivo*, co-immunoprecipitation (co-IP) experiments were performed.

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Funding for this work was provided by the National 973 program (2003 CB515503).

Received 3 June 2008, Accepted 2 July 2008

Keywords: Histone demethylase, hPc2, JARID1B, Transcriptional co-repressor

Table 1. Screening of a phage display library with the N-terminus of JARID1B

IPI number	Gene name	Biological function
IPI00288892	CACNA1H	Calcium channel, voltage-dependent
IPI00329526	CDC20	Acts as a regulatory protein in the cell cycle
IPI00102678	PCNX	Probable maternal-effect neurogenic gene
IPI00216047	SMARCC2	Helicase and ATPase activities
IPI00005565	DGKQ	Eukaryotic diacylglycerol kinase
IPI00291347	CACNA1A	Mediates the entry of calcium ions into excitable cells
IPI00328269	NR4A3	Binds to the B1A response-element
IPI00337426	BMP2K	May be involved in osteoblast differentiation
IPI00239623	VGLL3	Transcription cofactor vestigial-like protein
IPI00016928	CDX2	Caudal type homeobox transcription factor
IPI00010872	CBX4	Promotes SUMO modification, transcriptional repressor
IPI00016467	SLITRK3	Suppresses neurite outgrowth
IPI00294274	AXIN2	Regulator in the Wnt signaling pathway
IPI00019155	FOXC2	Plays a role in the development of mesenchymal tissues
IPI00001413	ONECUT2	Binds to specific DNA sequences
IPI00103655	AUTS2	Unclear
IPI00022438	MAF	Proto-oncogene
IPI00029626	ELF4	Transcription factor (fragment)
IPI00298702	SLC39A6	May act as a zinc-influx transporter
IPI00032408	BRD4	Plays a role in process governing chromosomal dynamics
IPI00160742	RNF43	Membrane protein, biomarker of some cancer cells
IPI00175136	RBM15B	Putative RNA binding protein
IPI00220560	PRDM13	May be involved in transcriptional regulation
IPI00061277	MTPB	P53-binding protein Mdm2-binding protein
IPI00004350	GTF2A1	Transcriptional activation. Interacts with TBP
IPI00101968	DBNL	Acts as a common effector of antigen receptor-signaling
IPI00010832	CECR6	Cat eye syndrome critical region protein
IPI00171950	RHOBTB2	Unclear
IPI00001737	GSH-2	Involved in transcriptional regulation
IPI00328144	TAF2	Serves as co-activator, facilitates complex assembly
IPI00178569	SHOX2	May be a growth regulator
IPI00015404	ARID1B	Involved in transcriptional activation
IPI00027694	HOXA1	Involved in the placement of hindbrain segments
IPI00297933	GRIN2B	NMDA receptor subtype of glutamate-gated ion channels
IPI00030729	MEOX2	Role in mesoderm induction

We co-transfected Flag-tagged hPc2 and HA-tagged JARID1B (N-terminus) into HEK-293T cells. The transfected JARID1B was immunoprecipitated by hPc2 (Fig. 1A), and vice versa (Fig. 1B). To confirm the interaction between JARID1B and hPc2 *in vitro*, we performed pull-down assays. His- JARID1B fusion protein was incubated using an Ni-NTA column, and 500 ng of purified hPc2 protein (with myc tag) was added. After incubating and washing, bound proteins were resolved by SDS/PAGE and visualized by Western blot using anti-myc antibody. The results indicated that His-JARID1B, instead of control plasmids (His), pulled down the hPc2 protein. GST pull down assays also showed that JARID1B (with the his tag) was pulled down when incubated with GST-hPc2 (Fig. 1C). Taken together, these results show that JARID1B interacts with hPc2 both *in vitro* and *in vivo*.

hPc2 interacts with C-terminal JARID1B

To identify the domains responsible for the interaction, we constructed a series of hPc2 deletion mutants for co-IP assays. hPc2 contains 2 previously characterized functional domains (Fig. 1D). The N-terminal region is a highly conserved chromo domain, which binds to histone H3 when methylated at Lys9 (9). The C-terminus contains a conserved COOH box that is involved in transcriptional silencing and binding to other PcG proteins (10, 11). We found that the N-terminus of JARID1B was immunoprecipitated by the C-terminal part of hPc2 (amino acids 414-551; lane 5) rather than by the N-terminus (amino acids 1-227; lane 3). Moreover, the deletion mutant lacking the 29 amino acid C-terminal region (amino acids 1-522; lane 9) lost the capacity to interact (Fig. 1E). These results indicate that the C-terminal region, including the COOH box, is required for the interaction with the N-terminus of JARID1B.

hPc2 acts as a transcriptional co-repressor

It has been previously demonstrated that JARID1B has transcriptional repression properties in GAL4-luciferase reporter gene assays (3,4). To test the effects of hPc2 on JARID1B repression activity, co-transfection experiments with GAL4DBD-hPc2 and JARID1B were performed using this GAL4-luciferase

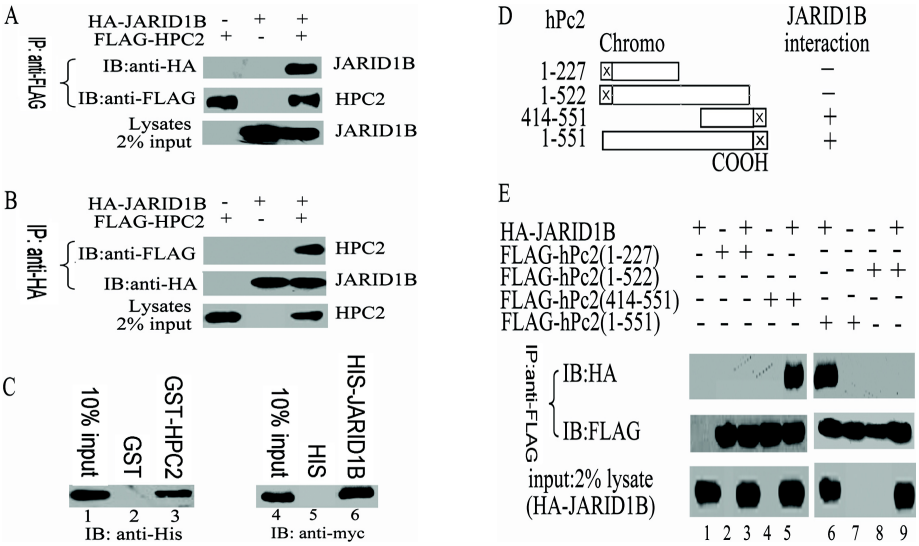

Fig. 1. hPc2 interacts with JARID1B *in vivo* and *in vitro*. (A) Co-immunoprecipitation (IP) of JARID1B with hPc2 expressed from transfected cells. HEK-293T cells were transiently transfected with pcDNA3-HA-JARID1B and pFlag-CMV-HPC2 alone or in combination as indicated at the top. Whole-cell extracts were immunoprecipitated with anti-Flag antibody. The precipitated proteins were analzsed by Western blotting using anti-HA or anti-Flag antibody as indicated. (B) Co-immunoprecipitation of hPc2 with JARID1B from transfected cells. Samples immunoprecipitated with anti-HA antibody were analyzed by Western blotting using anti-FLAG or anti-HA antibody as indicated. (C) Direct interaction of hPc2 and JARID1B: Western-blotting analysis of the His-tagged JARID1B or Myc-tagged hPc2 in fractions obtained from GST or His pull-down assays using GST (lanes 2), His (lanes 5) or His-JARID1B (lanes 6), GST-hPc2 (lanes 3). (D) Schematic representation of hPc2 fragments with their JARID1B-interacting abilities. (E) Several fragments of hPc2 were fused with FLAG tag and transfected into HEK293 cells with or without HA-JARID1B. Whole-cell extracts were immunoprecipitated with anti-Flag antibody. The precipitated proteins were analyzed by Western blotting using anti-HA or anti-Flag antibody as indicated.

reporter system (Fig. 2A). Expression of hPc2 (GAL4BD-hPc2) showed a marked decrease in luciferase activity compared with an empty vector (GAL4BD) in agreement with previous studies (7, 8). Furthermore, the transcriptional repression function of hPc2 was enhanced with increasing amounts of transfection. However, co-expression with JARID1B (equimolar amounts) reproducibly showed a greater repression activity (about 50%) than hPc2 alone (Fig. 2B, compare *white* with *striped black columns*). As controls, JARID1B was either co-expressed with GAL4DBD and GAL4DBD or GAL4DBD-hPc2 was co-expressed with an empty vector. These results clearly showed that hPc2 acted as a co-repressor of JARID1B under these experimental conditions. We also employed a CHIP assay to test if hPc2 could be recruited to JARID1B-binding promoters. Cells were transfected with plasmids, as well as a reporter vector, as indicated in Fig. 2C, and a CHIP assay was performed using specific antibodies. Precipitated DNA was amplified using primers targeting the promoter sequences recognized by JARID1B. The results showed that while no positive band was amplified when precipitated with a pre-immune serum, the promoter sequence was amplified in precipitates with anti-HA-JARID1B, as well as anti-FLAG-hPc2 (Fig. 2C), suggesting that these molecules could form complexes on the promoter (Fig. 2A).

C-terminus of hPc2 is essential for the transcriptional co-repression function

To test the functional relevance of the different domains, additional repression assays were performed. JARID1B was co-expressed with several deletion mutants or the wide type (WT) of hPc2 in luciferase reporter gene assays (Fig. 3A). Transcriptio- nal repression was significantly promoted when co-transfected with the wide type (1-551) or C-terminal part (414-551) of hPc2. When co-transfected with the N-terminal part (1-227) or full length without the COOH domain (1-522) of hPc2, however, the transcriptional activity was similar to transfection with JARID1B alone (Fig. 2B and Fig. 3A). Furthermore, dose-dependent experiments provided evidence that deletion of the COOH domain abolished the co-repression by hPc2 (Fig. 3B). Taken together, these results demonstrate that the C-terminal rather than the N-terminal region of hPc2 was essential for the co-repression function.

DISCUSSION

JARID1B was initially identified as it was significantly overexpressed in breast cancers (1). Recent studies demonstrated that JARID1B was an H3K4 demethylase and had transcriptional repression properties (3, 4). In this study, a human brain cDNA library was screened to identify potential binding proteins for

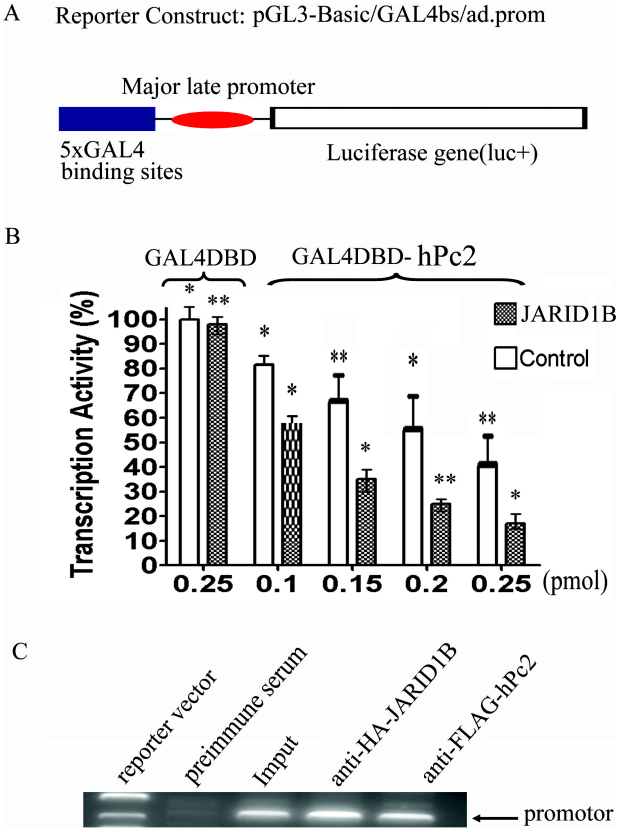

Fig. 2. Transcriptional co-repression by hPc2. (A) Schematic representation of the pGL3 reporter plasmid used in all repression assays. This plasmid contains 5GAL4 DNA-binding sites and the major adenovirus late promoter regulating the expression of luciferase. (B) Co-transfection of pGL3 reporter plasmid with increasing amounts of GAL4DBD-hPc2 without (*white columns*) or with (*gray column*) JARID1B. All values are normalized to the control and determined as the means of triplicate measurements ± the standard error of the mean. *P < 0.01, **P < 0.03. (C) CHIP assay. Forty-eight hours after transfection, cells were collected and subjected to CHIP using the indicated antibodies. Precipitated DNA fragments were amplified by PCR and analyzed using 3% agarose gel electrophoresis. The amplified fragment is indicated by an arrow.

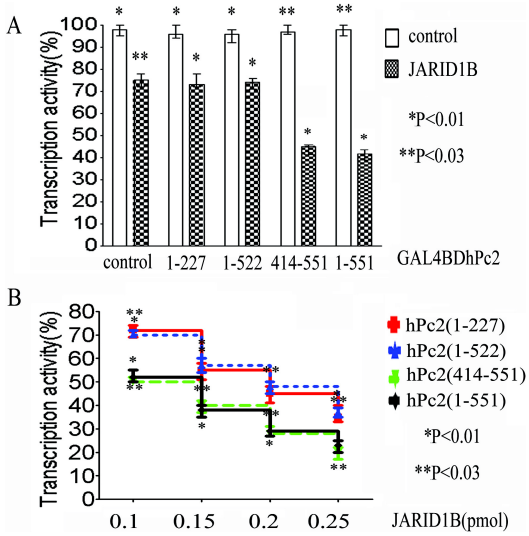

Fig. 3. Transcriptional co-repression by hPc2 requires the C-terminus. (A) Co-transfection of pGL3 reporter plasmid with 0.1 pM of different fragments of hPc2 in the presence of equal pM JARID1B or control plasmids. (B) Co-transfection of pGL3 reporter plasmid with increasing amounts of JARID1B and 0.1 pM of different fragments of hPc2. All values are normalized to the control and determined as the means of triplicate measurements ± the standard error of the mean. *P < 0.01, **P < 0.03.

JARID1B using phage display, as phage display were used as identify new proteins (12). We selected the phage display protein-protein interaction discovery system for two reasons. (a) The human brain cDNA library that we screened contained ~1.5 × 10^7 independent recombinants that were likely to represent even relatively rarely expressed transcripts. (b) Unlike the yeast two-hybrid system (which screens bait/prey partners only under physiological conditions in cytoplasm or nucleus), the phage display system allows selection using a wider range of binding conditions, such as the lower pH and higher Ca^{2+} concentration found within the Golgi apparatus and chromaffin granules (13).

We have further characterized the repression function of JARID1B by identifying potential interacting partner proteins and have explored the functional significance of these interactions using a transcriptional reporter assay. Our results clearly showed that the PcG protein hPc2 interacted with the N-terminus of JARID1B both *in vivo* and *in vitro*. It is interesting that the C-terminus of hPc2 was essential for the interaction and transcriptional co-repression. Studies are underway to further investigate the specificity and structural basis of the JARID1B interacting domain.

hPc2 is a member of the PcG family of proteins that are parts of a cellular memory system responsible for the stable inheritance of gene activity (7, 8, 14). The PcG genes were first identified in Drosophila as suppressors of formation of multi-meric protein complexes (15). In vertebrates, hPc2 can interact with a RING finger protein RING1 and other PcG proteins to form a Polycomb repressive complex (16). Moreover, it has been reported recently that members of the PcG proteins are also recruited to promoters and suppress transcription (17). As we have provided evidence that hPc2 interacted with JARID1B and acted as a transcriptional co-repressor, it is plausible that hPc2 might have been recruited to a functional complex of JARID1B and suppressed transcription. Moreover, recent studies have shown that the PcG protein hPc2 also functions as a SUMO E3 ligase (18), which catalyzes SUMOylation of target proteins, and alters their specific subcellular locations as well as modifying their functions (19). hPc2 may thus also modulate JARID1B through SUMOylation and regulate its functions.

Further studies are underway to test this possibility.

MATERIALS AND METHODS

Mammalian expression constructs

JARID1B cDNA was cloned from MCF-7 cells lysates. The N-terminus of JARID1B cDNA was cloned into pcDNA3-HA. Full-length hPc2 was cloned into pFlag-CMV-2 (with Flag tag) or pcDNA3.0-myc (with myc tag). Different regions of hPc2, namely 1-227, 1-522, 414-551, were cloned into pFlag-CMV-2 by PCR amplification using pFlag-CMV-hPc2 as a template. All PCR-cloned constructs were verified by DNA sequencing.

Protein expression and purification

The full-length hPc2 coding sequences were cloned into pGEX-4T-3 (Amersham Biosciences) for the expressions of the GST fusion proteins in the *E. coli* strain BL21 (DE3). Purification was performed using glutathione-Sepharose 4B (Amersham Biosciences), as described in the manufacturer's instructions, following the induction of the cells with 0.5 mM IPTG (isopropyl β-D-thiogalactopyranoside) at 27°C for 3 h. The N-terminus of JARID1B (with his tag) protein was expressed using the Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, JARID1B cDNA N-terminus (from nucleotide 1 to nucleotide 2525) (with BamHI and SacI) was cloned into a pFastBac vector. The recombinant baculovirus was harvested and used to infect Sf9 cells. One week later, cells were isolated with RIPA buffer(50 mM Tris-base, 1.0 mM EDTA,150 mM NaCl, 0.1% SDS, 1% TritonX-100, 1% Sodium deoxycholate). Ni-NTA resin and column were used to purify the N-termini of JARID1B proteins.

Phage display to identify binding partners of JARID1B

Recombinant JARID1B (N-terminus as "bait") was immobilized in polystyrene wells as per the Novagen protocol. Briefly, protein of N-terminal JARID1B was added as bait and incubated with blocking buffer (0.1 M $NaHCO_3$, 5 mg/ml BSA, 0.02% NaN_3, filter sterilized and stored at 4°C) for 1 h at 4°C. After discarding the blocking solution, wells were washed with TBST (TBS (50 mM Tris-HCl, 150 mM NaCl, autoclaved and stored at room temperature) + 0.1% (v/v) Tween-20). A human brain phage display cDNA library (1.5×10^7 primary cDNA clones; Novagen) in the T7 Select phage vector was amplified in *E. coli* strain BLT 5,615 and applied to the JARID1B-coated wells for 5 h at room temperature, followed by an overnight incubation at 4°C. Non-binding phage was discarded, and plates were washed 10 times with TBST. Bound phage was eluted with eluting buffer (0.2 M Glycine-HCl (PH 2.2), 1 mg/ ml BSA). After final elution, individual/clonal selected phages were identified as plaques in "lawns" of *E. coli* strain BLT- 5615. Plaques were excised and suspended in 100 µL of 10 mM EDTA (pH 8.0). The plaque suspensions were heated at 65°C for 10 min, cooled to room temperature, and centrifuged at 14,000 rpm for 3 min to clarify. Two microliters of each lysate was subjected to PCR using T7 Select UP and T7 Select Down primers (Novagen). An aliquot of each PCR product was run on a 1.2% agarose gel to check the quality of the product and to determine the size of the cDNA insert in the phage clone. PCR products were purified using a PCR purification kit (Qiagen) and sequenced using the T7 Select UP and T7 Select Down primers (Novagen). The resulting cDNA sequences were queried against GenBank, using BLAST-N searches to identify the clones.

Immunoprecipitation and Western blotting

For co-immunoprecipitation experiments, HEK-293T cells were plated in 60-mm-diameter dishes and were transfected with either the mammalian expression constructs pcDNA3-HA-JARID1B and pFlag-CMV-hPc2 or pcDNA3-HA-JARID1B and different deletion mutants of hPc2 (with Flag tag). After 48 h incubation, 5×10^6 transfected cells were washed with PBS and lysed in lysis buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% (v/v) Nonidet P40, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 mM PMSF). After brief sonication (100 W; 10 s on/10 s off; 7 cycles), the lysates were cleared by centrifugation (20,000 g, at 4°C for 20 min). Supernatants were incubated with the anti-Flag or anti-HA antibodies at 4°C for 4 h. Protein G-agarose beads (Roche) and the mixtures were rotated at 4°C overnight. The beads were washed 4 times with lysis buffer and the immunoprecipitated proteins were resolved by SDS/PAGE, transferred to nitrocellulose membranes (Bio-Rad) and probed with antibodies. The ECL (enhanced chemiluminescence) kit (Millipore) was used to visualize immunoreactive proteins. For direct analysis of whole cell extracts, 1×10^6 cells were washed with PBS and lysed in SDS-loading buffer and analyzed by SDS/PAGE and Western blot.

Repression assays

Reporter constructs were made by inserting the major late promoter of adenovirus (as well as 5GAL4 binding sites in the case of the pGL3-Basic/GAL4bs/ad. prom. construct) from pGL5 *luc* (Promega) into the multiple cloning site of pGL3-Basic (Promega), using either the *Nhe*I/*Bst*BI or *Kpn*I/*Hin*dIII restriction sites. Constructs were verified by sequencing. hPc2 full length or different fragments were cloned into the vector pBIND to obtain the construct GAL4DBD-hPc2. Varying amounts of GAL4DBD- hPc2, wild-type or mutant hPc2 with reporter construct were transfected into 70-90% confluent Cos-7 cells. All transfections followed the LipofectAMINE (Invitrogen) protocol according to the manufacturer's instructions. Cells were harvested after 24 h, and luciferase assays were performed using the Dual luciferase reporter assay system (Promega). The empty pBIND vector (GAL4DBD) was co-transfected with the reporter constructs as a control. Data were normalized by assaying Renilla luciferase activity. Luciferase activity from empty pBIND vector was arbitrarily set to 100%. All other measurements are expressed relative to this value. All results are the averages of at 3 three independent experiments. Statistical analysis was performed using Student's *t* test. A value of $P < 0.05$

was considered to be statistically significant.

CHIP assay

Chromatin immunoprecipitation (CHIP) assay was carried out using a kit from Upstate (Milton Keynes, UK) according to the manual provided by the supplier. Briefly, NIH3T3 cells were transfected with expression vectors for GAL4BD-HA-JARID1B, FLAG-hPc2 together with reporter vector. Forty-eight hours after transfection, cells were crosslinked with 1% formaldehyde, disrupted and ultra-sonicated. The cell lysates were immunoprecipitated with anti-HA or anti-FLAG, with a pre-immune serum as a control. The crosslinking of the immunoprecipitates were reversed by heating, and bound DNA was amplified using primers to the promoter containing JARID1B-recognizing sequence. The amplified fragments were analyzed using 1.5% agarose gel electrophoresis.

Acknowledgments

We are grateful to Professor. Hua Han for hPc2 cDNA.

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