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DNA methyltransferase 3B acts as a co-repressor of the human polycomb protein hPc2 to repress fibroblast growth factor receptor 3 transcription

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ABSTRACT

DNA methyltransferase 3B has been demonstrated to mediate gene silencing. The mechanisms how DNA methyltransferase 3B is targeted to specific regions and represses gene transcription, however, are not well understood. Here we show that by using yeast two-hybrid screening, DNA methyltransferase 3B interacts with the human polycomb protein, hPc2. This interaction was verified via co-immunoprecipitation and GST pull-down assay. Sequential deletion analysis showed that the region of DNA methyltransferase 3B responsible for interaction is mapped to the N-terminal regulatory domain. By performing a cDNA microarray analysis in HCT 116 cells, we identified that the expression of fibroblast growth factor receptor 3 is significantly increased upon the small interference RNA-mediated knockdown of hPc2, suggesting fibroblast growth factor receptor 3 as a potential target of hPc2. We further found that DNA methyltransferase 3B enhances hPc2-mediated transcriptional repression of fibroblast growth factor receptor 3, which does not require its de novo methyltransferase activity. Taken together, these results suggest that DNA methyltransferase 3B functions as a co-repressor of polycomb protein in inducing transcriptional repression independent of DNA methylation.

1. Introduction

Cellular memory is required to maintain cell identity over multiple cell divisions. This memory may be regulated via an epigenetic mechanism such as DNA methylation and polycomb group (PcG) proteins which are involved in embryonic development, X chromosome inactivation, and tumorigenesis (Bantignies and Cavalli, 2006). DNA methylation results from attachment of methyl groups to DNA via the activity of DNA methyltransferases (DNMTs); DNMT1, DNMT3A and DNMT3B (Bestor, 2000; Robertson et al., 2000). DNA methyltranferase 3B (DNMT3B) contains the non-catalytic domain in the N-terminus and a catalytic domain with DNA methyltransferase activity in the C-terminus (Robertson, 2001). The non-catalytic domain contains two motifs, PWWP and ATRX, which function in chromosome targeting and protein-protein interactions (Ge et al., 2004; Geiman et al., 2004b; Stec et al., 2000). Specifically, DNMT3B interacts with DNMT1, DNMT3A, SUMO-1, hSNF2H, KIF4A and condensin through its N-terminus results in transcriptional repression (Kim et al., 2002; Kang et al., 2001; Geiman et al., 2004a). In addition, DNMT3B is capable of generating novel methylation patterns with de novo methyltransferase activity, but has no sequence specificity (Hsieh, 1999; Vertino

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et al., 1996; Tajima and Suetake, 1998; Burgers et al., 2002).

PcG proteins were identified as a transcriptional regulator of homeotic gene expression in *Drosophila* (Kennison, 1995; Simon, 1995). They maintain the silent state at specific targets in embryonic development and stem cell renewal (Bracken et al., 2006). PcG proteins interact with one another to form complexes with a family of functionally related proteins (Francis et al., 2001; Pirrotta, 1998). PcG proteins are classified into two groups based on their association with distinct classes of multimeric complexes, referred to as the Polycomb repressive complexes (PRC) (Levine et al., 2004; Lund and van Lohuizen, 2004). The PRC2 complex, which includes EZH2, SUZ12, and EED, was found to be involved in the initiation of gene repression by methylating lysine 27 of histone 3 (H3K27) (Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002). The more diverse PRC1 complex, comprising BMI-1, hPc2, CBX7, and YY1, recognizes trimethylated H3K27 mark via the chromodomain to result in stable gene silencing (Fischle et al., 2003). PcG proteins and DNMTs perform a similar function in transcriptional silencing. Interestingly, EZH2 interacts with DNMTs coupled with the control of DNA methylation (Vire et al., 2006).

In this report, we sought to elucidate the factors that allow targeting of DNMT3B to specific gene promoter by identifying protein that interacts with DNMT3B. Using a yeast two-hybrid assay, we found that DNMT3B interacts with the human polycomb protein, hPc2 in a human fetal brain cDNA library. These studies thus provide evidence for a direct link between DNA methylatransferase and PcG protein and demonstrate how DNMT3B is targeted to specific regions.

2. Materials and methods

2.1. Construction of plasmids

DNMT3B1 and hPc2 were PCR amplified from cDNA plasmids generously provided by Dr. Jae Hoon Chung (Korea Advanced Institute of Science and Technology, Korea) and Dr. David Wotton (University of Virginia, USA), respectively. All constructs were verified by DNA sequencing. In brief, DNMT3B1 and hPc2 PCR products were digested with the appropriate restriction enzymes, and cloned into one or more of the following plasmids, depending on the particular application or assay: pGBKT7 (fusion with the gal4-DNA binding domain for yeast expression) (Clontech, Palo Alto, CA), pcDNA3 with an HA tag (Invitrogen Life Technologies, Carlsbad, CA), pGEX-2T (fusion with glutathione *S*-transferase protein for bacterial expression) (Amersham Biosciences, Piscataway, NJ), and p3XFlag-CMV-7.1 (Sigma–Aldrich, UK). Furthermore, pcDNA Flag-RanBPM was provided by Dr. Elisabetta Bianchi (Institut Pasteur, France). In an effort to generate deletion constructs of DNMT3B, PCR products were digested and cloned into pGEX2T at the BamHI and EcoRI restriction sites. hPc2 deletion constructs were cloned into pDEST 15 Gateway vector (Invitrogen) to express the GST-fusion proteins. The 1.5 kb FGFR3 promoter-pGL2 vector (Promega, Southampton, UK) was kindly provided by Dr. Masataka Nakamura (Tokyo Medical and Dental University, Japan). The Renilla luciferase plasmid (pRL-SV40) (Promega) was used to control for transfection efficiency.

2.2. Yeast two-hybrid assay and β-galactosidase assay

Yeast two-hybrid experiments were conducted as previously described (Kim et al., 2007). DNMT3B was fused in-frame to the GAL4 DNA binding domain in the pGBKT7 vector (Clontech) and transformed into the AH109 yeast strain. The expression of the DNMT3B-bait fusion protein was verified via Western blotting (not shown). They were mated with the Y187 yeast strain which was pre-transformed with a human fetal brain cDNA library in pACT2 vector (Clontech). The transformants were selected on Synthetic Defined (SD) media lacking tryptophan, leucine, adenine, and histidine and the growing colonies were further analyzed for β-galactosidase activity.

2.3. Cell culture and transient transfection

HEK 293T and HCT 116 cell lines were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium and RPMI 1640 medium (WELGENE Inc., Korea), respectively and were supplemented with 10% fetal bovine serum (WELGENE) in a humidified 5% CO_2 atmosphere at 37 °C. DNMT3B knockout HCT116 cell lines were obtained from Dr. Bert Vogelstein (Johns Hopkins University Medical Institution, USA). For transfection experiments, cells were seeded into 60- or 100-mm plates at either 4×10^5 or 8×10^5/plate and then transfected the following day with various plasmids DNAs with Lipofectamine 2000 (Invitrogen), in accordance with the manufacturer's instructions. Transfected cells were collected for further experiments at the indicated times.

2.4. GST pull-down assay

GST fusion proteins were expressed in *Escherichia coli* BL21 and affinity-purified using glutathione-sepharose 4B beads (Amersham Biosciences). GST pull-down assays were carried out by incubating 5–10 μg of GST fusion proteins with 20 μL ^{35}S-labeled in vitro transcribed/translated protein, which was prepared using a Promega TNT T7 Quick Coupled Kit. The binding buffer was 20 mM Tris–Cl (pH 7.4), 0.1 mM EDTA, and 100 mM NaCl. After binding, unbound proteins were removed by 5 washes with NETN buffer [0.5% NP40, 0.1 mM EDTA, 20 mM Tris–Cl (pH 7.4), 300 mM NaCl). The sample buffer was added to the beads and specifically bound proteins were analyzed via SDS-PAGE followed by autoradiography.

2.5. Co-immunoprecipitation analysis and Western blotting

Whole cell extracts were prepared from transfected cells using lysis buffer [50 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1% NP-40, 10 mM NaF and 10 mM sodium pyrophosphate and protease inhibitors]. Cell lysates were then clarified via