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The transcriptional repression by NIPP1 is mediated by Polycomb group proteins

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Abstract

NIPP1 is a ubiquitously expressed nuclear protein that represses the transcription of targeted genes. Here we show that the transcriptional repression by NIPP1 is alleviated by the RNAi-mediated knockdown of EED and EZH2, two core components of the Polycomb Repressive Complex 2 (PRC2), and by the overexpression of a catalytically dead mutant of the histone methyltransferase EZH2. NIPP1 is present in a complex with EED and EZH2 *in vivo* and has distinct binding sites for these proteins. These data disclose an essential role for the PRC2 complex in the transcriptional repression by NIPP1.

Keywords: EED; EZH2; NIPP1; Polycomb group proteins; PRC2; Transcriptional repression

1. Introduction

Nuclear Inhibitor of Protein Phosphatase-1 (NIPP1) is a widely expressed protein in metazoans and plants [1]. The phenotyping of NIPP1-deficient mice and cells revealed an essential role for this protein in early embryonic development and cell proliferation [2]. NIPP1 binds to pre-mRNA splicing factors and is required for a late step of spliceosome assembly [3]. In addition, NIPP1 interacts *in vivo* with protein kinase MELK [4], protein phosphatase-1 [5] and the Polycomb group protein EED [6], but the biological implications of these interactions are not known.

Polycomb group (PcG) proteins mediate the silencing of hundreds of genes that are important for embryonic development, cell proliferation and differentiation [7–10]. PcG proteins form two major multimeric complexes, known as the Polycomb Repressive Complexes (PRC) 1 and 2 [11]. The PRC2 complex consists in mammals of a core of the proteins EZH2, EED and SUZ12. Within this complex EZH2 functions as a methyltransferase for Lys27 of histone H3 (H3K27). Trimethylated H3K27 serves as a binding site for the chromodomain-containing Polycomb protein, a component of the PRC1 complex, which promotes gene silencing by poorly understood mechanisms that eventually result in chromatin condensation [12].

Our previous observation that NIPP1 functions as a transcriptional repressor and interacts with EED, a core component of the PRC2 complex, suggested a causal relationship between these findings. Here we show that the transcriptional repression by NIPP1 indeed depends on a functional PRC2 complex. Moreover, we demonstrate that NIPP1 has distinct binding sites for EED and EZH2.

2. Materials and methods

2.1. Materials

The origin of most materials and the details of the transcriptional repression assays have been described previously [6]. The expression vectors for Flag-EZH2 and Flag-EZH2-H689A were a kind gift of Dr. Danny Reinberg (HHMI, New York University). A fragment of human EZH2 (14-CWRKRVKSEYMRLRQLK-30) and human NIPP1 (341-PGKKPTPSLLI-351) coupled to keyhole limpet hemocyanin, were used to generate polyclonal antibodies in rabbits. The antibodies were affinity-purified on the bovine–serum–albumin-coupled peptides linked to CNBr-activated Sepharose 4B.

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2.2. Cell culture

PC-3 human prostate adenocarcinoma cells were cultured as monolayers in 50% Dulbecco's modified Eagle's medium and 50% Ham's F12 with 10% fetal calf serum. U2OS human osteosarcoma cells were cultured in McCoy's 5A medium with 10% fetal calf serum. HEK293T human kidney cells were cultured in DMEM with 10% fetal calf serum. Transfection with plasmid DNA was carried out with Fugene-6 Transfection Reagent (Roche Applied Science).

siRNAs were synthesized by Dharmacon and designed as described by Bracken et al. [13] A scrambled version of a siRNA duplex for NIPP1 (GGAACUCGAACCUCCACGAACAAUU, Invitrogen) was used as a control siRNA. Eight hours after transfection with the indicated siRNA using GeneSilencer siRNA Transfection Reagent (Gene Therapy Systems), the medium was replaced and the U2OS cells were transfected with (1) 50 ng of Gal4, Gal4-NIPP1 or Gal4-NIPP1-(1–324)-V201A/F203A, (2) 100 ng of the reporter plasmid, and (3) 20 ng of the β-galactosidase encoding plasmid. Cells were harvested 36 h later in passive lysis buffer (Promega) and assayed for luciferase and β-galactosidase activities. For the transcriptional repression assay in Fig. 2, COS1 cells were seeded in 24-well plates (6×10^4 cells/well). After 24 h the cells were transfected with (1) 100 ng of the luciferase reporter plasmid, (2) 25 ng of the indicated Gal4-(fusion) encoding plasmids, (3) 50 ng of cDNAs encoding either Flag-mEZH2 or Flag-mEZH2-H689A, and (4) 20 ng of β-galactosidase encoding plasmid.

2.3. Immunoprecipitations

Exponentially growing HEK293T cells, transfected with Flag-NIPP1 (Fig. 3B), and PC-3 cells (Figs. 3A and C) were washed three times with phosphate-buffered saline (PBS) in the presence of 1 mM PMSF. Cells were then lysed in a buffer containing 25 mM Tris–HCl at pH 7.5, 0.3 M NaCl, 0.5% Triton X-100, 0.1% Nonidet P-40, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 5 μM leupeptin, 25 mM NaF and 1 mM orthovanadate. Cell lysis was aided by sonication with three 15-s pulses at 100% amplitude, using the 0.5-mm probe (IKA U50 Control) and further incubation of the suspension on ice for 20 min. The lysates were clarified by centrifugation (10 min at 10,000×*g*) and the supernatants were used for immunoprecipitation. Aliquots of cell lysates (1 mg protein) were pre-cleared with 50 μl of Protein A-TSK (Affiland) beads for 1 h at 4 °C and then incubated overnight at 4 °C with anti-Flag-M2 (Sigma), anti-EZH2 or anti-NIPP1 antibodies. Subsequently, 50 μl protein-A-TSK beads (1:1 suspension) was added for 1 h at 4 °C. After centrifugation the pellet was washed twice with 1 ml PBS plus 0.1% Nonidet P-40 and once with 1 ml 20 mM Tris–HCl at pH 7.5, 250 mM LiCl, 0.1% Nonidet P-40, and subjected to immunoblotting. For the phosphorylase phosphatase assays (Fig. 3D), immunoprecipitates from PC-3 cells were prepared as described above, except that NaF and orthovanadate were omitted from the lysis buffer.

2.4. Histone methyltransferase assay

Two tail peptides of human Histone H3 were synthesized with an additional three basic residues at the C-terminus to ensure binding to the negatively charged P81 paper: 24-AARKSAPATGGVRRR-35, corresponding to H3-(24–35), and 24-AARLSAPATGGVRRR-35, corresponding to H3-(24–35)-K27L. The complexes precipitated with anti-NIPP1, anti-EZH2 or rabbit anti-mouse IgG (DakoCytomation) (Fig. 3A) were incubated for 1 h at 30 °C with 30 μM of the indicated peptide in a buffer containing 50 mM Tris–HCl at pH 8.5, 5 mM $MgCl_2$, 4 mM DTT and 1 μM S-adenosyl-L-[methyl-^{3}H]-methionine (Amersham Pharmacia Biotech). Aliquots were spotted on P81 cellulose phosphate papers, washed 3×10 min in 50 mM carbonate buffer at pH 9, dried and counted.

2.5. GST pull-downs

Mouse EZH2 was cloned into pBleuscript-II-SK under the T7 promoter (pBl-mEHZ2). This plasmid was used as a template for *in vitro* transcription using the rabbit reticulocyte lysate kit (Promega). Bacterially expressed His-tagged EED and the GST-tagged NIPP1-(fragments) were expressed and purified as described [6]. Equimolar amounts of the GST and the GST-fusions of NIPP1-(fragments) were pre-incubated for 1 h at 4 °C with glutathione-agarose beads (Sigma), pre-blocked with TBS containing 1 mg/ml BSA, 0.5% Triton X-100 and 1 mM DTT. After washing with TBS, 0.1% Nonidet P-40 and 1 mM DTT, the beads were incubated for 2 h at 4 °C with *in vitro* translated ^{35}S-Met-EZH2. The beads were washed thrice with 1 ml TBS, 0.1% Nonidet P-40 and 1 mM DTT, and then subjected to SDS-PAGE and autoradiography to visualise the retained EZH2. Equivalent amounts of GST and GST-fusions of NIPP1-(fragments) were also incubated with bacterially expressed His-EED or baculovirus-expressed His-EZH2 for 30 min at 4 °C. Preblocked glutathione-agarose beads were added and an additional incubation for 30 min at 4 °C was performed. Subsequently, the beads were sedimented, washed three times with 1 ml TBS, 0.1% Nonidet P-40 and 1 mM DTT, and probed for the presence of GST-fusions, His-EZH2 or His-EED by Western blot analysis.

2.6. Phosphorylase phosphatase assays

The trypsin-revealed protein phosphatase activities were measured as described previously [6], using ^{32}P-labeled phosphorylase *a* as substrate. One unit of phosphatase released 1 nmol of phosphate/min at 30 °C.

3. Results

3.1. Repression by NIPP1 requires the PRC2 complex

In accordance with our previous data [6], fusions of the Gal4 DNA-binding domain and NIPP1 repressed basal transcription

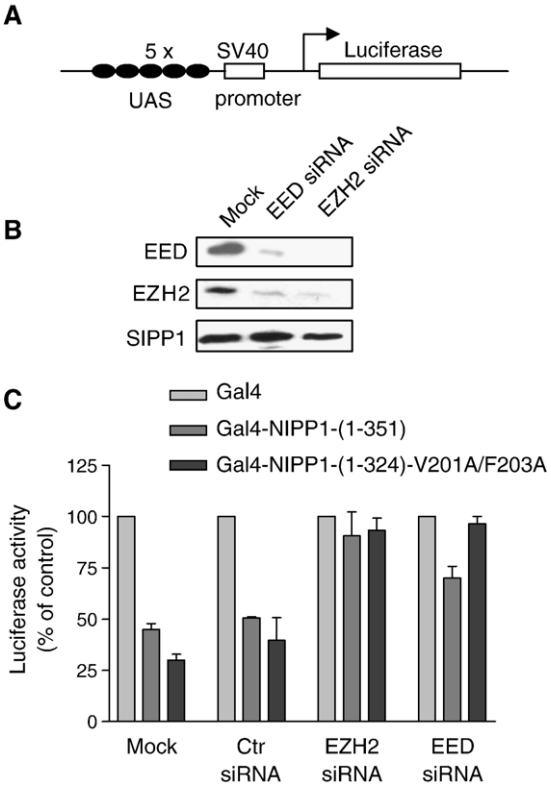

Fig. 1. Transcriptional repression by NIPP1 requires EZH2 and EED. (A) The reporter plasmid contained the luciferase reporter gene, a TATA box and the SV40 promoter. Upstream of the SV40 promoter, five copies of the Gal4-binding site UAS were inserted. (B) Immunoblots of U2OS cell lysates 48 h after transfection with a control siRNA or with siRNA oligos designed to inhibit the expression of EZH2 or EED. SIPP1 is an unrelated nuclear protein serving as a loading control. (C) U2OS cells were co-transfected with the indicated siRNAs, the reporter plasmid, the indicated Gal4-constructs and the reference plasmid encoding β-galactosidase. Two days after transfection, the luciferase activities were determined and normalized for transfection efficiency based on the β-galactosidase activity. The values are expressed as a percentage±S.E. ($n=3$) of the luciferase activities obtained with Gal4.