Original Article

Kurume Medical Journal, 67, 171-183, 2020

The E2F6 Transcription Factor is Associated with the Mammalian SUZ12-Containing Polycomb Complex

YUKO SHIRAHAMA AND KEN YAMAMOTO

Department of Medical Biochemistry, Kurume University School of Medicine, Kurume 830-0011, Japan

Received 22 September 2020, accepted 14 December 2020
J-STAGE advance publication 2 December 2022

Edited by KOSHI MIMORI

Summary: The Polycomb group protein (PcG) SUZ12 forms Polycomb repressive complexes together with histone methyltransferase EZH2. Although the complexes have been demonstrated to be involved in epigenetic maintenance of gene expression in a transcriptional repressive state, it is unclear how they are recruited to the target genes. Here we report that SUZ12 directly interacts with site-specific transcriptional repressor E2F6 and forms a complex together with EZH2. SUZ12 interacts with E2F6 selectively among the E2F family proteins and E2F6-containing SUZ12-EZH2 complex was biochemically purified from HEK293 cells stably expressing Flag-tagged SUZ12. Chromatin immunoprecipitation assays revealed the target genes of the E2F6-SUZ12-EZH2 complex. Contrary to expectation, the promoter regions of these genes are not or only weakly tri-methylated at histone H3-K27, and their expression is down-regulated by depletion of EZH2. Given that the transactivation function of SUZ12-EZH2 has been previously reported, the inhibitory effect on E2F6-mediated transcriptional repression by physical interaction can be considered a candidate mechanism of gene activation by these PcGs.

Keywords E2F transcription factor, polycomb group protein, histone methylation, chromatin immunoprecipitation, transcriptional regulation

INTRODUCTION

Polycomb group (PcG) proteins have been recognized as transcriptional repressors that play a crucial role in maintaining the correct spatial and temporal expression of homeotic genes during development [1-3]. Several lines of biochemical evidence indicate that PcG proteins function in multimeric protein complexes, Polycomb repressive complexes PRC1 and PRC2, the core components of which are conserved from the fruit fly to humans [4-13]. The PRC2 complexes contain core components EZH2, which possesses methyltransferase activity to histone H3 lysine 9 (H3-K9), H3-K27 and H1-K26, zinc-finger protein SUZ12, and WD-repeat proteins EED and RBBP4/7 (or the *Drosophila* counterparts, Su(z)12, Ezh2, Esc and Nurf55). SUZ12 was shown to be essential for the stability of enzymatic activity of EZH2 in the complexes [14,15].

The tri-methylation of H3-K27 by PRC2 pro-

Corresponding Author: Ken Yamamoto, Department of Medical Biochemistry, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 831-0011, Japan. Tel: +81-942-31-7544, Fax: +81-942-31-4377, E-mail: yamamoto_ken@med.kurume-u.ac.jp

Abbreviations: AEBP2, AE binding protein 2; BRCA1, breast cancer 1; CAMTA2, calmodulin binding transcription activator 2; COASTER, coactivator for steroid receptors; CTIP, CtBP interacting protein; DNMT, DNA methyltransferase; DP1, E2F dimerization partner 1; EED, embryonic ectoderm development; EIF3S10, eukaryotic translation initiation factor 3, subunit 10; EPC1, enhancer of polycomb 1; Esc, extra sex combs; EZH2, enhancer of zeste 2; FNTB, farnesyltransferase; GST, glutathione S-transferase; HA, hemagglutinin; HMT, histone methyltranseferase; HP1, heterochromatin protein 1; HSPC228, hematopoietic stem/progenitor cells; KCNA1, potassium voltage-gated channel, shaker-related subfamily, member 1; MBLR, Mel18- and Bmi1-like RING finger protein; MEP50, methylosome protein 50; MFAP1, microfibrillar-associated protein 1; MYT1, myelin transcription factor 1; Nurf55, nucleosome remodeling factor 55; PAGE, polyacrylamide gel electrophoresis PcG, Polycomb group; Pho, pleiohomeotic; Phol, pleiohomeotic-like; PRC, Polycomb response element; PRE, polycomb response elements; Rb, retinoblastoma; RbAp, Rb-associated protein; RECQL, RecQ protein-like; RT-PCR, reverse transcriptase-polymerase chain reaction; SNF, sucrose nonfermenting; SUZ12, suppressor of zeste 12; SWI, mating-type switch; TA-WDRP, T-cell activation WD repeat protein; TRIM52, tripartite motif-containing protein 52; VEFS, VRN2-EMF2-FIS2-Su(z)12; WNT1, wingless-type MMTV integration site family, member 1; YAF2, YY1-associated factor 2.

vides a binding site for PRC1 on nucleosomes, and the occupation of PRC1 on target genes facilitates the condensation of chromatin structure and the inhibition of chromatin remodeling by SWI/SNF complexes to establish gene silencing [4,8,10,11,16]. In addition to the methyltransferase activity of PRC2 complexes, the catalytic subunit EZH2 interacts with DNA methyltransferase (DNMTs) and associates with DNMT activity [17]. The link of central molecular events in epigenetic transcriptional regulation, histone and DNA methylation, suggests a broad range of molecular functions for PcG proteins in processes of development, hematopoiesis, X chromosome inactivation, and oncogenesis [18-22].

Although an attractive model of gene repression orchestrated by PRCs has been proposed, it remains unclear how the PRC2 are recruited onto the nucleosomes of target genes. Among *Drosophila* PcG genes, Pleiohomeotic (Pho) and Pho-like (Phol) encode sequence specific DNA-binding proteins [23,24] and the binding sites are identified in Polycomb response elements (PRE) that are required for silencing by PcG proteins [23-26]. However, Pho and Phol, and the mammalian counterpart YY1 are not purified as a component of PRC2/3/4 so far. A DNA-binding protein AEBP2 was identified in PRC2 complex [10] but it is not clear how this factor recruits the complex to the specific sites. A previous study reported that Su(z)12 and Nurf55 form a minimal nucleosome-binding module of the PRC2 complexes *in vitro* [27]. This suggests that PRC2 is capable of accessing nucleosomes in a DNA sequence independent manner. In contrast, studies of genome-wide searches for target genes of PRC2 revealed that these complexes are preferentially associated with developmental regulators in embryonic cells [28-32]. This functional bias of the target genes might be explained, in part, by a mechanism in which certain sequence-specific DNA binding proteins transiently interact with a component of PRC2 and invite the complex to the relevant regions of the genome. Such proteins need to be identified to allow a better understanding of the biological functions of PRC2.

In the present study, we found that among the E2F family members, only E2F6 directly interacts with SUZ12 *in vitro* and in over-expressed cells. We biochemically purified an E2F6-containing SUZ12-EZH2 complex on immunoaffinity purification from HEK293 cells expressing Flag-tagged SUZ12. Chromatin immunoprecipitation (ChIP) assays revealed the target genes of this complex. Contrary to expectation, the promoter regions of these genes are not or weakly tri-methylated at histone H3-K27, and their expression is down-regulated by depletion of EZH2. Our data suggest that SUZ12 inhibits, but not intermediates, trans-repression function of E2F6 through physical interaction.

MATERIALS AND METHODS

Plasmid constructs

Details of individual plasmid constructs, which were all verified by sequencing, are available upon request. The human cDNA of SUZ12 has been described before [33]. Human cDNA of E2F1, E2F2, E2F4, E2F5, E2F6 and DP1 were cloned from cDNA of HeLa or MCF-7 cells. We used pCMV-HA or pCMV-Myc (Clontech) to express the proteins in mammalian cells, pGEX-2T (Amersham Biosciences) to express GST-fusion proteins in *E*. coli, and pGADT7 (Clontech) to produce HA-tagged proteins in *in vitro* transcription/translation. Full-length and mutant SUZ12 (amino acid residues 1-599), which lacks the C-terminal half of the VEFS (VRN2-EMF2-FIS2-SUZ12) box, were cloned into pCI-neo (Promega) to generate HEK293 cells stably expressing each protein.

Immunoprecipitation and in vitro binding assays

Non-synchronous COS-7 or HEK293 cells were transiently transfected with indicated plasmids using PolyFect Transfection Reagent (QIAGEN). Co-immunoprecipitation experiments, preparation of GST fusion proteins, and GST pull-down assays were done as previously described [33,34]. In brief, after 36 hours of transfection, the cells were washed with cold phosphate-buffered saline and harvested in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% (w/v) SDS, 1.0% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate). Cell extracts were prepared by incubating at 4 °C for 1 hour followed by centrifugation at 12,000 rpm for 10 minutes at 4 °C. The cell extracts were then incubated with 10 μl of anti-myc, anti-Flag or anti-E2F6 monoclonal antibody-coupled protein G beads for 2 hours at 4 °C. The immunoprecipitates were washed three times with cold RIPA buffer and suspended in 2 × SDS sample buffer. The samples were applied onto an SDS-PAGE and transferred onto nitrocellulose membrane. Western blots were prepared by a standard protocol. Anti-E2F6 (sc-8366, Santa Cruz Biotechnology), anti-Flag antibody M2 (F-9291, Sigma), anti-Myc antibody (9E10, Santa Cruz), and anti-HA antibody (F7, Santa Cruz) were used for immunopre-

cipitation and/or Western blot analysis. For GST pull-down experiments, the full-length E2F6, the E2F6-N (residues 1-173) containing the DNA-binding domain and the leucine zipper motif, the E2F6-C (residues 171-282) containing the marked domain and the E2F6-D (residues 130-240) containing the leucine zipper and the marked domain, were fused to GST and produced in E. *coli* DH5α. *In vitro* translated HA-tagged, full-length SUZ12 and mutant SUZ12 including SUZ12-N (residues 1-402), SUZ12-ZV (residues 403-739) containing Zn-finger motif and VEFS box, SUZ12-N1 (residues 1-201), SUZ-N2 (residues 202-402), SUZ12-Z (residues 403-506) containing Zn-finger, SUZ12-V (residues 537-739) containing VEFS box, and SUZ-C (residues 640-739) were incubated with GST fusion E2F6 proteins in binding buffer (GPB: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10% glycerol, 0.1% NP40, and 0.5 mM DTT) with 100 mM NaCl for 1 hour at 4 °C, washed GPB with 500 mM NaCl, and pulled down SUZ12s were detected on Western blot analysis using anti-HA-tag antibody.

Purification of SUZ12-containing complex

Nuclear extracts from Flag-SUZ12 expressing HEK293 or control HEK293 cells were incubated with anti-Flag M2-agarose beads (Sigma) in binding buffer (20 mM HEPES, pH 7.9, 0.21 M KCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Nonidet P-40, 0.5 mM DTT, and a protease inhibitor cocktail) for 1 hour. The beads were packed into a column, washed with washing buffer (20 mM HEPES, pH 7.9, 10% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.01% Nonidet P-40, and a protease inhibitor cocktail) containing 0.35 M NaCl (buffer W-0.35), and then with buffer W-0.15 as a final step. The bound proteins were eluted with buffer W-0.15 containing 0.4 mg/ml of Flag peptide (Sigma). To isolate the protein complex, the elution fractions 2-4 that contained Flag-SUZ12 were applied to sucrose bed sedimentation. The fractions were loaded onto a 40% sucrose cushion in the buffer containing 20 mM HEPES, pH 7.9, 50 mM NaCl and 1 mM DTT. After ultra-centrifugation at 32,000 rpm for 16 hrs in a TLS-55 rotor (Beckman), the fractions were recovered from the top of the tube. Part of the recovered samples was concentrated by precipitation with 10% TCA. Anti-Flag M2, anti-EZH2 (07-689, Upstate), anti-EED (07-368, Upstate), and anti-E2F6 (sc-22823, Santa Cruz) were used for Western blot analysis.

Chromatin immunoprecipitation

ChIP was performed using the ChIP assay kit (Upstate). Asynchronously growing HEK293 cells were fixed, and the chromatin was sheared to an average size of ~3000 bp. Antibodies used in the ChIP assays included anti-E2F6 (sc-22823), anti-SUZ12 (Ab12201, Abcam), anti-EZH2 (07-689), and anti-histone H3 tri-methylated at K27 (05-851, Upstate). Rabbit normal serum was used as control IgG. The immunoprecipitates were examined by gene-specific PCR. The following primers were used: EIF3S10 forward (5'-CTCACCCTTCCCACCCTAAT-3'), reverse (5'-CAGAGACGGAAAGGAAGGAG-3'); HSPC228 forward (5'-CGGGTCTTCTGTGGGTTCTA-3'), reverse (5'-CTCCGAACTCACCACTTCCT-3'); MFAP1 forward (5'-TCACTGTCGCCTGAGTTTCTT-3'), reverse (5'-GAAGTCGAGTCGGTTTCTGC-3'); TRIM52 forward (5'-CGGAATGTGCAGCACTAGC-3'), reverse (5'-CGACGCAAAATGACGTCTC-3'); MYT1 forward (5'-AGGCACCTTCTGTTGGCCGA-3'), reverse (5'-GCGAACTCCTAAGCCAGCTA-3'); KCNA1 forward (5'-GACCTGCTCGTGTTGAAGC-3'), reverse (5'-ACGTTCTCCCCAGACATCAC-3'); WNT1 forward (5'-ACCCGTCAGCTCTCGGCTCA-3'), reverse (5'-GCACCGCCTCTTATAGTTGC-3'); COASTER forward (5'-GGCGCATGGTTCTTTTCCTC-3'), reverse (5'-GGAAAGAGACGCAAAACCTC-3'); RECQL forward (5'-CTTCCTATTGGCGAAACCTG-3'), reverse (5'-CGAGCAGATCTTTCCGCTAC-3'); FNTB forward (5'-CCTTGCGCTTCAGGACTCTA-3'), reverse (5'-TGCTTCGTTAAGCAACAACG-3'); CAMTA2 forward (5'-GAGGGCTCCTTTCGCTTTCT-3'), reverse (5'-AGCTCGGCCGACTGACTC-3'); TA-WDRP forward (5'-GTTTCCATCTCCAAGGCGTC-3'), reverse (5'-GTTCCGAGGGCCAAGGTC-3'); HP1α forward (5'-TAGACAAGCAGCCAGGAGGT-3'), reverse (5'-GCGCAAAGCTAGGACAAACT-3'); BRCA1 forward (5'-GACGCTTGGCTCTTTCTGTC-3'), reverse (5'-TACCCAGAGCAGAGGGTGAA-3'); CTIP forward (5'-CTCTGGGTCGGAGCGACT-3'), reverse (5'-GCGGGAGACCCAGAAGTAGT-3'); Cyclin A2 forward (5'-ACCCTGTCGCCTTGAATG-3'), reverse (5'-CCGAGGAGGTTGCGAAAG-3'). The genomic regions of the PCR product in ChIP are as follows: EIF3S10, -263 to -15; HSPC228, -183 to 10; MFAP1, -241 to -30; TRIM52, -192 to -22; MYT1, -186 to 83; KCNA1, -151 to 26; WNT1, -150 to 7; COASTER, -87 to 90; RECQL, -178 to 22; FNTB, -61 to 132; CAMTA2, -440 to -251; TA-WDRP, -91 to 76; HP1a, -282 to -89; BRCA1, -406 to -160; CTIP, 268 to 450; Cyclin A2, -255 to -76. The locations are numbered from the transcription start site

according to the following reference sequences: EIF3S10, NM_003750; HSPC228, NM_016485; MFAP1, NM_005926; TRIM52, NM_032765; MYT1, NM_004535; KCNA1, NM_000217; WNT1, NM_005430; COASTER, NM_015555; RECQL, NM_002907; FNTB, NM_002028; CAMTA2, NM_015099; TA-WDRP, NM_139281; HP1α, NM_012117; BRCA1, NM_007294; CTIP, NM_002894.

RNA interference and RT-PCR

The HEK293 cells were transfected with 40 nM of siRNAs in 12-well plates using HiPerFect (QIAGEN). The siRNAs were purchased from B-Bridge International, Inc.: siGL3 (S20C-0200) for control, siE2F6 (THF27A-188), siSUZ12 (THF27A-146), and siEZH2 (THF27A-189). At indicated time points after transfection, the cells were harvested for Western blot analysis or RNA preparation. Total RNA was isolated using the RNeasy mini kit (QIAGEN). Two micrograms of total RNA were reverse transcribed by SuperScript Ⅲ RT and oligo-dT primers (Invitrogen). PCR was performed with Pyrobest DNA polymerase (TaKaRa) for 20-33 cycle amplification. The primer sequences for RT-PCR are as follows: GAPDH forward (5'-TGGCCAAGGTCATCCATGAC-3'), reverse (5'-ATGTAGGCCATGAGGTCCAC-3'); E2F6 forward (5'-CGTTTTGATGTATCGCTGGTT-3'), reverse (5'-TCTGGTTTCTGCTGGAGCTT-3'); SUZ12 forward (5'-TGCAGTTCACTCTTCGTTGG-3'), reverse (5'-GGTTGGCGATGAATATCCTG-3'); EZH2 forward (5'-AGAATGGAAACAGCGAAGGA-3'), reverse (5'-GCATTCACCAACTCCACAAA-3'); EIF3S10 forward (5'-GACAGAGGAAGCTGGCGAAC-3'), reverse (5'-TGAGTCATCACCCCGTCTGG-3'); HSPC228 forward (5'-TGGAAGGCAACATACATCCA-3'), reverse (5'-CTGGGGTTAGACGAACATCC-3'); MFAP1 forward (5'-AGCGCTCTCATGAAGCAACC-3'), reverse (5'-CGCCAAGCATCTCCTTCTAC-3'); TRIM52 forward (5'-GAGGACGAAGATGAAGAGCTG-3'), reverse (5'-TTACTGATTATAGGCCTTGCTGTG-3'); MYT1 forward (5'-GTTTGTCTGGGTGTCCCATT-3'), reverse (5'-TAGGTGAGGTTTCGCTGGTC-3'); KCNA1 forward (5'-CGGGGTCATCCTGTTTTCTA-3'), reverse (5'-CGCAGTTTTGGTTAGCTGTG-3'); WNT1 forward (5'-CGTCTGATACGCCAAAATCC-3'), reverse (5'-GCCTCGTTGTTGTGAAGGTT-3'); COASTER forward (5'-GGACCATTACGACCTGTTCTTG-3'), reverse (5'-GCCTCCTCTTCGGAATGAAC-3'); RECQL forward (5'-TCAGAATAAGTTCAATGGTGGTG-3'), reverse (5'-ACTGCTGTATTAGAAAGTGTGCAA-3'); FNTB forward (5'-TTTGTACTCCCTGAAGCAACC-3'), reverse (5'-CCAGAAGGAGTAGCAGCCATC-3'); CAMTA2 forward (5'-AGTGACGAAGCTCCAAGCAT-3'), reverse (5'-TCCAGTGACAGCCAGTCAAG-3'); TA-WDRP forward (5'-GCCGGTTCTATGTAACAACCT-3'), reverse (5'-TGTTCACTGCCCAGAAGTATTTT-3'); HP1α forward (5'-GACCTGGTGGCCTTAGTCTT-3'), reverse (5'-TCTGGTTCCAGTCCTCTCTC-3'); BRCA1 forward (5'-GGTGTCCACCCAATTGTGGT-3'), reverse (5'-GCCCTAAGCCAACAACAGCC-3'); CTIP forward (5'-GAAGCAAGAGCAGAAGGGAG-3'), reverse (5'-GGAATGTAGCGGAATCGGTG-3'), For the siRNA-ChIP experiments, HEK293 cells were harvested after 72 hours of transfection with the particular siRNAs, and ChIP experiments were performed as described above.

RESULTS

SUZ12 interacts with E2F6 directly and specifically

We focused on the E2F family proteins in the process of searching for site specific DNA-binding factors that interact with SUZ12 because these molecules have been well recognized to control a wide variety of gene transcription including cell division and differentiation to which PcGs also contribute [35]. We found that transcriptional repressor E2F6 interacts with SUZ12 in transient transfection assays (Figure 1A). HA-tagged E2F6 and Myc-tagged SUZ12 were co-expressed in HEK293 and E2F6 was shown to be co-immunoprecipitated with SUZ12 (Figure 1A, lane 4, third panel). We confirmed the interaction by the reverse direction of immunoprecipitation; SUZ12 was co-immunoprecipitated with E2F6 from the cell extract (Figure 1A, lane 4, bottom panel). It has been shown that E2F6 diverges from other E2F-family members on the basis of its structure [36]. Amino acid residues involved in DNA-binding and dimerization activities of the other E2F members are conserved in E2F6, but E2F6 lacks the C-terminal sequences that are responsible for both the interaction with pocket-proteins and transactivation. We further investigated the interaction between SUZ12 and E2F6 with respect to specificity among the family. Co-immunoprecipitation assays using over-expressed HEK293 cells showed that other E2Fs, including E2F1, E2F2, E2F4, and E2F5, do not interact with SUZ12 (Figure 1B). This indicates that there is an E2F6-specific interaction with this PcG protein. To exclude the possibility that this interaction was indirect and was mediated by other proteins, such as EPC1, which was recently shown to interact with both E2F6 and EZH2 [37], we tested interaction

using mutant SUZ12 that lacks the interaction site to EZH2. First, we generated HEK293 cells stably expressing Flag-SUZ12 or mutant SUZ12 that lacks the C-terminal half of the VEFS box (SUZ12ΔVEFS), which is shown to be the important region for interaction with EZH2 [33] and examined interaction with endogenous EZH2. Indeed, SUZ12ΔVEFS did not interact with endogenous EZH2; this again demonstrates that the VEFS box is essential for interaction with EZH2 (Figure 1C, lower panel). Base on this observation, SUZ12ΔVEFS was examined to interact with E2F6 in transient transfection assays. As shown in Figure 1D, E2F6 was co-immunoprecipitated with this mutant SUZ12, indicating, at least, that EZH2 was not involved in the interaction. In addition, the direct interaction was re-confirmed by a GST pull-down assay using *in vitro* translated full-length SUZ12 and GST fusion full-length E2F6 (Figure 1E).

Since there are several functional domains in SUZ12 and E2F6, we next mapped the interaction sites by GST pull-down assays using truncated mutant proteins. We found that interaction requires the dimerization domain for DRTF proteins (DP) which encompasses both the leucine zipper and the marked box motifs, since E2F6-D (residues 130-240) con-

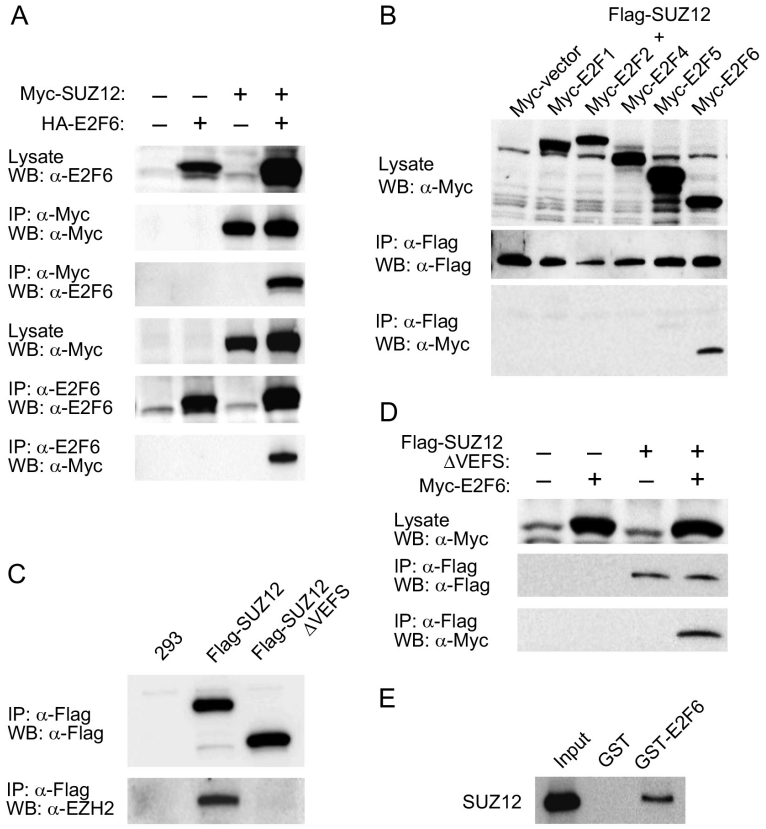

Fig. 1. SUZ12 directly and selectively interacts with E2F6 among the E2F family members. (A) Interaction between E2F6 and SUZ12 in transiently transfected cells. Myc-tagged SUZ12 was co-transfected with HA-tagged E2F6 into COS-7 cells. Cell extracts were prepared and incubated with anti-Myc or anti-E2F6 antibody-coupled protein G beads for immunoprecipitation. The result indicated association of SUZ12 with E2F6 (lane 4 in third and bottom panels). (B) SUZ12 selectively interacts with E2F6 among the E2F family members. Flag-SUZ12 was co-transfected with Myc-E2F1, 2, 4, 5, and 6 into HEK293 cells. The results indicated that only E2F6 is associated with SUZ12 (bottom panel, lane 6). (C) The carboxyl-terminal half of the VEFS box (residues 600-639) in SUZ12 is essential for the interaction with EZH2 in cells. The HEK293 cells stably expressing Flag-SUZ12 or the deletion mutant lacking the C-terminal half of the VEFS box (Flag-SUZ12ΔVEFS) was used. Endogenous EZH2 was associated with wild type SUZ12 but not with SUZ12ΔVEFS (lower panel, compare lanes 2 and 3). (D) EZH2 is not required for the interaction between SUZ12 and E2F6. Myc-E2F6 was co-transfected with Flag-SUZ12ΔVEFS into HEK293 cells. The final immunoprecipitates with anti-Flag beads were analyzed for E2F6 by Western blot using an anti-Myc antibody. The results from (C) and (D) indicate that EZH2 is not involved in the SUZ12-E2F6 interaction. (E) Interaction between E2F6 and SUZ12 *in vitro*. *In vitro* translated HA-SUZ12 was tested for interaction with GST-E2F6 by GST pull-down assay.

taining both motifs clearly interacted with SUZ12 (Figure 2A). On the other hand, the constructs which contained either the leucine zipper (E2F6-N) or the marked box (E2F6-C) showed a weak or background level of interaction *in vitro* (Figure 2A). To date, the components of PRC2/3/4 complexes, namely EZH2, EED, AEBP2, and RbAp48, and HP1, methylosome component MEP50 were shown to directly interact with SUZ12 *in vitro* [14,33,38]. SUZ12 consists of at least two functional sequences that are conserved from plant to mammals: the Zinc-finger motif and the VEFS box domain [39]. With respect to the interaction with E2F6 in this study, *in vitro* experiments have found that multiple sites, including at least two N-terminal parts (SUZ12-N1 and –N2) and a middle part that contains Zinc-finger motif (SUZ12-Z), are involved (Figure 2B). This indicates a mode of interaction that differs from that seen with HP1 and EZH2.

Since SUZ12 shares the interaction domain in E2F6 with DP (Figure 2A), we examined if the interaction interferes with E2F6-DP dimer formation. HEK293 cells were transiently transfected with Flag-SUZ12, Myc-E2F6 and Myc-DP1, and co-immunoprecipitation assays were done using anti-Flag beads. As shown in Figure 3A, lower panel, almost equal amounts of E2F6 and DP1 were co-immunoprecipitated with SUZ12 (lane 8), whereas DP1 was not or weakly detected in the immunoprecipitate with SUZ12 prepared from the cells not transfected with E2F6. These data indicate that SUZ12 does not interfere with E2F6-DP1 interaction, and suggest that SUZ12, E2F6, and DP1 can form a tripartite complex in cells. In addition, we found that endogenous SUZ12 and EZH2 were co-immunoprecipitated with transfected E2F6 in the presence of over-expressed DP1 (Figure 3B, lane 4). Taken together, our data suggest that SUZ12 may have a role in transcriptional regulation of E2F6 through direct interaction.

Isolation of E2F6-containg SUZ12-EZH2 complex

To further investigate if E2F6 is present in the SUZ12-EZH2 complex, immunoaffinity purifications were performed using Flag-SUZ12 expressing cells. Nuclear extracts were prepared from Flag-SUZ12 expressing HEK293 cells or control cells and were loaded onto a Flag-M2 affinity resin, and bound proteins were eluted by an excess amount of Flag peptides. Western blot analyses revealed that E2F6 was co-eluted with Flag-SUZ12 in the elution fractions 2-4 that also contained EZH2 and EED, which are known components of PRC2/3/4 complexes (Figure 4A). The amount of eluted E2F6 was low, since it could be detected in the materials concentrated by TCA precipitation. In previous reports, E2F6 was not observed in the SUZ12-containing complexes isolated from mammalian cells [10,11]. Therefore, it seems likely that a small fraction of SUZ12 is associated with E2F6 in cells. The elution fractions 2-4 were further subjected to sucrose bed sedimentation in order to examine whether E2F6 forms a complex with SUZ12, EZH2, and EED. As shown in Figure 4B, the eluted Flag-SUZ12 was detected in the fractions 6-18 with a peak at fraction 10 or 12 (third panel), whereas free SUZ12 protein generated by *in vitro* translation migrated to fractions 4-14, with a peak at fraction 8 (second panel). When the fractions 6-16 were analyzed for migration of E2F6, EZH2 and EED, fraction 10 contained a low but definite amount of E2F6, as well as EZH2 and EED (Figure 4B). Since free E2F6 was primarily detected in fractions 4-8 with a peak at fraction 6 (Figure 4B, upper panel), our data indicate that E2F6 forms a multi-protein complex with SUZ12, EZH2, and EED, at least through interaction with SUZ12.

Identification of common target genes of E2F6 and SUZ12-EZH2

We next examined whether E2F6 target genes are transcriptionally regulated by EZH2, or whether known SUZ12 or EZH2 target genes are regulated by E2F6. Previous studies have reported several SUZ12 and E2F6 target genes [40,41]. However, we could not find common genes in the two groups. Therefore, we chose several genes from them: EIF3S10, HSPC228, MFAP1, TRIM52, MYT1, KCNA1, and WNT1 as SUZ12 targets, in addition to COASTAR, RECQL, FNTB, CAMTA2, TA-WDRP, HP1α, BRCA1, and CTIP as E2F6 targets. We performed ChIP assays using anti-E2F6, -SUZ12, -EZH2 antibodies, and non-specific IgG control to identify the common target genes in HEK293 cells. The Cyclin A2 promoter was analyzed as a negative control. Since gene silencing mediated by SUZ12-EZH2-containing PRC2/3/4 complexes is associated with tri-methylation of H3-K27 [41], anti-tri-methylated H3-K27 antibody was also included in our assay. All primers were designed within 500 bp upstream or downstream of the transcription initiation sites of each gene to examine the binding of E2F6, SUZ12, and EZH2 near the promoter region.

Consistent with a previous report, we detected binding of SUZ12 to all of the known target genes that were analyzed (Figure 5, lane 4, panels of SUZ12 target genes) [41]. In addition, EZH2 co-localized

with SUZ12 on these promoters (Figure 5, lane 5, the same panels), suggesting that SUZ12-EZH2-containing complexes are involved in transcriptional regulation of these genes. In our assay, we found that E2F6 binds to promoter of four SUZ12 target genes (EIF3S10, HSPC228, MFAP1, and TRIM52) (Figure 5, lane 3, the same panels). Contrary to expectation, tri-methylation of H3-K27 of these four genes is weak or nil in comparison with the other SUZ12 target genes, MYT1, KCNA1, and WNT1 (Figure 5, lane 6, the same panels). As further support for these findings, ChIP for EZH2 gave stronger signals in these three genes than in the other four genes (Figure 5, lane 5, the same panels). Next, we analyzed eight

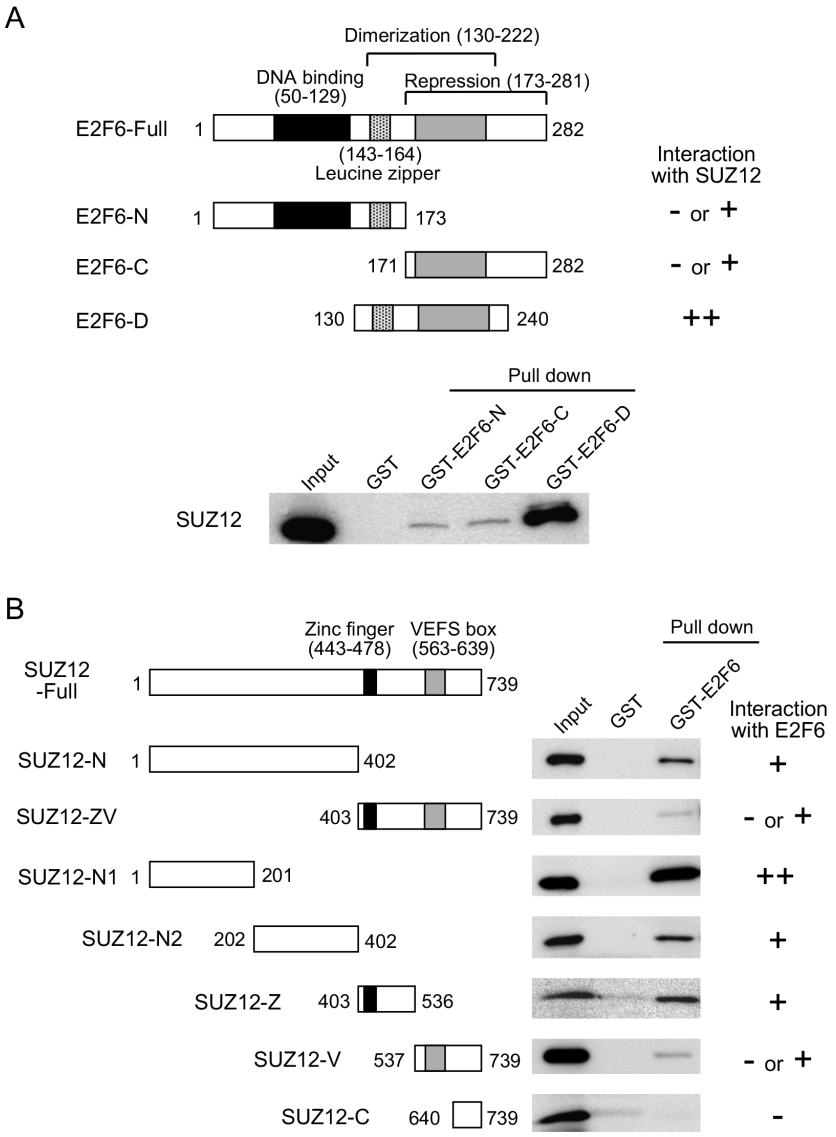

Fig. 2. Mapping of interaction sites in E2F6 and SUZ12. (A) The dimerization domain in E2F6 is involved in the interaction with SUZ12. In vitro translated HA-SUZ12 was tested for interaction with the three truncated mutants of GST-E2F6 (GST-E2F6-N, -C and -D) by GST pull-down assay. The E2F6-N (residues 1-173) contains the DNA-binding domain and the leucine zipper motif. The E2F6-C (residues 171-282) contains the marked domain, and the E2F6-D (residues 130-240) contains the leucine zipper and the marked domain, both of which are known to be required for dimerization with the DP molecule. A summary of interactions is given in the panel on the right of the schematic representation of E2F6 constructs. (B) Multiple sites in SUZ12 are involved in the interaction with E2F6. Mutants of HA-SUZ12 were generated by in vitro translation and tested for interaction with GST-E2F6. The mutants include SUZ12-N (residues 1-402), SUZ12-ZV (residues 403-739) containing Zn-finger motif and VEFS box, SUZ12-N1 (residues 1-201), SUZ-N2 (residues 202-402), SUZ12-Z (residues 403-506) containing Zn-finger, SUZ12-V (residues 537-739) containing VEFS box, and SUZ-C (residues 640-739). Pulled down SUZ12 was detected on Western blot analysis using anti-HA-tag antibody. A summary of interactions is given in the panel on the right.

reported E2F6 target genes and found that E2F6 binds to these genes (Figure 5, lane 3, panels of E2F6 target genes) as has been previously described [40]. We found that, in our assay, SUZ12 and EZH2 bind to the promoter of six genes among them (COASTAR, RECQL, FNTB, CAMTA2, TA-WDRP and HP1α) (Figure 5, lanes 4 and 5, the same panels). As is the case of the SUZ12 target genes to which we detected binding of E2F6, H3-K27 is not at all or only weakly tri-methylated in the six SUZ12-EZH2 bound E2F6 target genes (Figure 5, lane 6, the same panels). We examined di- or tri-methylation of H3-K9, which is also known to occur with EZH2-mediating histone modification, by ChIP assays, but none of them were subjected to this modification (data not shown). To exclude the possibility that E2F6 and SUZ12-EZH2

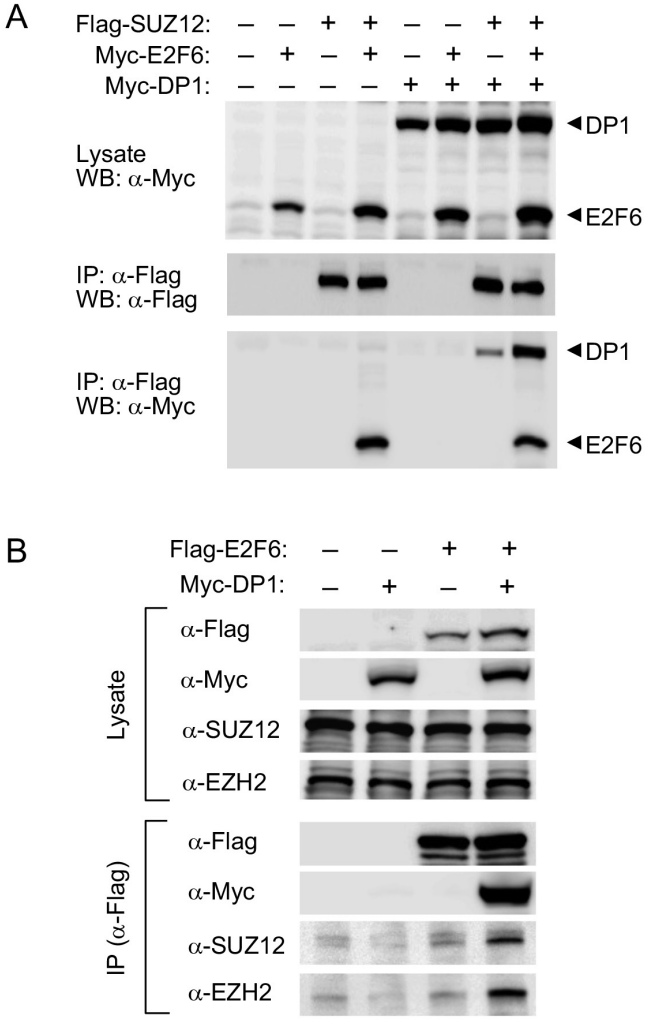

Fig. 3. SUZ12, E2F6, and DP1 can form a tripartite complex. (A) DP1 is associated with SUZ12 in the presence of E2F6 in transiently transfected cells. Flag-SUZ12 was co-transfected with Myc-E2F6 and Myc-DP1 into HEK293 cells. The final immunoprecipitates with anti-Flag beads were subjected to Western blot analysis using an anti-Myc antibody. Almost equal amounts of E2F6 and DP1 were co-immunoprecipitated with SUZ12 (bottom panel, lane 8). (B) Enhancement of association between SUZ12 and E2F6 by DP1. Flag-E2F6 was co-transfected with Myc-DP1 into HEK293 cells. The final immunoprecipitates with anti-Flag beads were tested for co-immunoprecipitation of endogenous SUZ12 and EZH2 with E2F6 by Western blots. The results indicated that endogenous SUZ12 and EZH2 were associated with E2F6 in the presence of DP1.

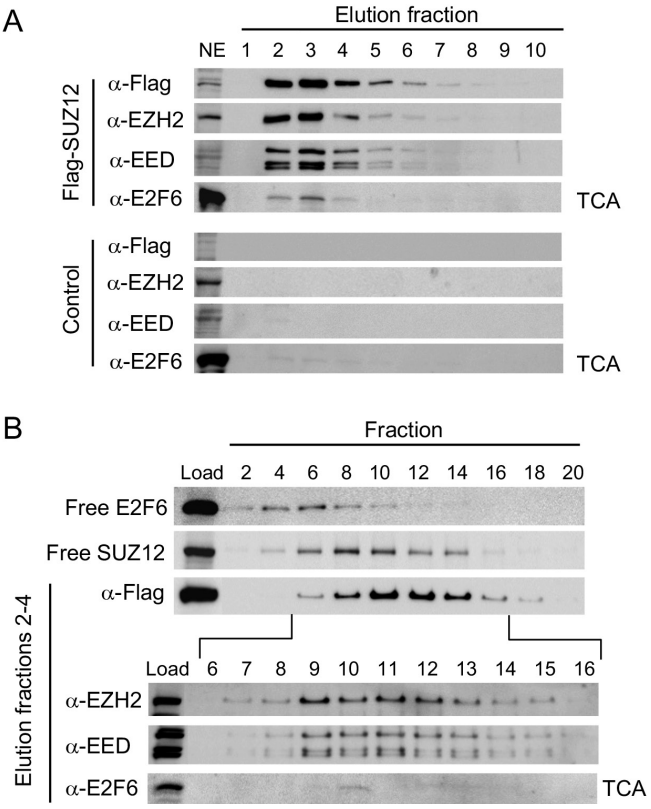

Fig. 4. A purified SUZ12-complex contains E2F6. (A) Western blot for Flag-purified SUZ12-complex. Nuclear extracts were prepared from HEK293 cells stably expressing Flag-SUZ12 or control HEK293 cells, and which were subjected to immunoaffinity chromatography with an anti-Flag-M2 resin. Ten elution fractions were collected and analyzed by Western blot analysis using the indicated antibodies. E2F6 was detected in fractions 2-4 concentrated by TCA precipitation (fourth panel). (B) Sucrose bed sedimentation of Flag-SUZ12-containing elution fractions. The elution fractions 2-4 described in (A) were combined and loaded onto a 40% (v/v) sucrose bed. After ultra-centrifugation, the samples were recovered from the top of the tube and subjected to Western blot analysis using the indicated antibodies. The E2F6 and SUZ12 generated by in vitro translation were used as a control to monitor the migration of free proteins. The results revealed that E2F6, as well as Flag-SUZ12, migrated to different fractions from free proteins, and that they were both co-sedimented with EED and EZH2 at fraction 10.

independently bind to different regions of the same target gene, E2F6 was depleted and ChIP assays were done for the common target genes, EIF3S10, HSPC228, CAMTA2, and TA-WDRP in HEK293 cells. The siRNA efficiently depleted E2F6 after 72 hours of transfection at the mRNA level and at the protein level (Figure 6A and B). As shown in Figure 6C, siRNA for E2F6 led to a reduction of EZH2 binding to the common target genes, whereas binding to KCNA1, a non-E2F6 target, was not affected. Collectively, these results suggest that E2F6 recruits SUZ12-EZH2 onto particular target genes (e.g., EIF3S10) where the complex has a role in transcriptional regulation in a manner that is different from

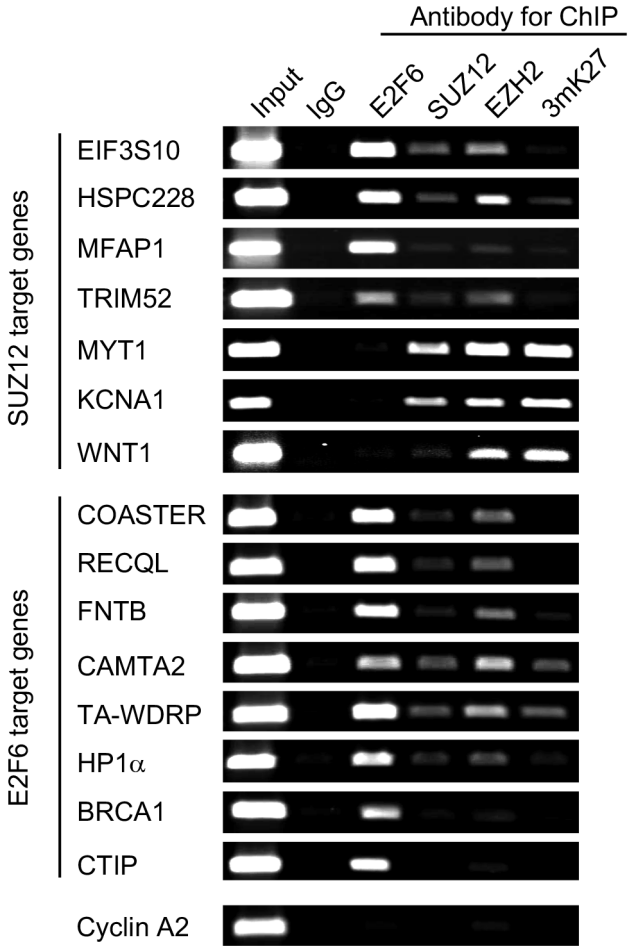

Fig. 5. The promoters bound by both E2F6 and SUZ12-EZH2 are not at all or only weakly tri-methylated at H3-K27. Several SUZ12 and E2F6 target genes were chosen based on the previous reports for the ChIP assays in order to identify genes regulated through the interaction between E2F6 and SUZ12. We tested seven SUZ12 (EIF3S10, HSPC228, MFAP1, TRIM52, MYT1, KCNA1 and WNT1) and eight E2F6 (COASTER, RECQL, FNTB, CAMTA2, TA-WDRP, HP1α, BRCA1 and CTIP) target genes that have been reported previously. The Cyclin A2 gene was analyzed as a negative control. The ChIP assays were performed in HEK293 cells using antibodies to E2F6 (lane 3), SUZ12 (lane 4), EZH2 (lane 5), and tri-methyl H3-K27 (lane 6). Rabbit non-specific IgG was used as a negative control (lane 2). The immunoprecipitated chromatin was analyzed by PCR using primers specific to the promoters of the indicated genes. Details on the primers are available in "Material and Methods". The ChIP assays were repeated five times, and representative results are presented.

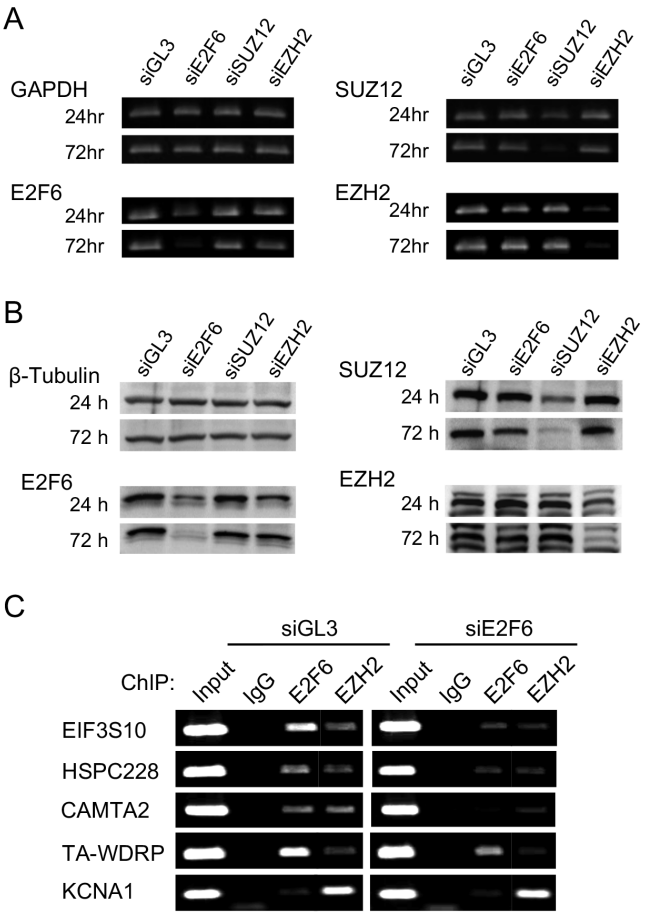

Fig. 6. Depletion of E2F6 by siRNA reduced the binding of EZH2 to the common target genes. (A) RT-PCR analysis of total RNA extracted from HEK293 cells 24 or 72 hours after transfection with 40 nM of siRNA for GL3 (negative control), E2F6, SUZ12 or EZH2. Primers specific to GAPDH mRNA were used in the RT-PCR to ensure that the mRNA was correctly quantitated. (B) Western blot analysis for whole cell extracts from HEK293 cells transfected with siRNA as described in (A). Antibody against β-tubulin was used to demonstrate equal loading of protein samples. (C) ChIP assays were performed using the indicated antibodies in HEK293 cells 72 hours after transfection with siRNA specific for GL3 or E2F6. The common target genes of E2F6 and EZH2 (EIF3S10, HSPC228, CAMTA2 and TA-WDRP), and the EZH2-specific target (KCNA1), which were identified in the experiments presented in Figure 5, were analyzed. The ChIP assays were repeated five times, and representative results are presented.

H3-K27 tri-methylation and H3-K9 di- or tri-methylation.

SUZ12-EZH2 inhibits transcriptional repression of E2F6

Based on ChIP experiments, we found that tri-methylation of H3-K27 of the common target genes for E2F6 and SUZ12 is weak. This might provide information about a unique group of SUZ12 target genes. In order to investigate whether SUZ12-EZH2 regulates E2F6-mediated transcriptional repression, the expression of E2F6, SUZ12, and EZH2 were depleted using siRNA, and a change in the expression level of the target genes described above was examined by RT-PCR. The siRNAs for SUZ12 and EZH2 used in our experiments efficiently depleted SUZ12 and EZH2, respectively, after 72 hours of transfection at the mRNA level and at the protein level (Figure 6A and B). As expected, the depletion of E2F6 resulted in an elevation of the transcripts of its target genes identified in this study (EIF3S10, HSPC228, MFAP1, and TRIM52) and previously reported (RECQL, FNTB, TA-WDRP, HP1α and CTIP) (Figure 7, lanes 1 and 2), though there were some exceptions (COASTER, CAMTA2, and BRCA1). On the other hand, depletion of SUZ12 and EZH2 showed various effects on the alteration of target gene expression. Consistent with previous reports [41], the expression of the genes strongly tri-methylated at H3-K27 (MYT1, KCNA1, and WNT1) was induced by the reduction of SUZ12 or EZH2 (Figure 7, *lanes 3-6*), which were not identified as E2F6 targets in this study. The expression of BRCA1 and CTIP, which were not identified as SUZ12 and EZH2 targets in this study, was not affected. Interestingly, contrary to our prediction, the expression of common targets of E2F6, SUZ12, and EZH2 was not elevated, but rather decreased by the reduction of SUZ12 and EZH2, except that RECQL exhibited the same pattern of expression change as MYT1. Because it is not unlikely that SUZ12-EZH2 directly transactivates transcription, these data may suggest blocking effect on transcriptional repression of E2F6 via physical interaction.

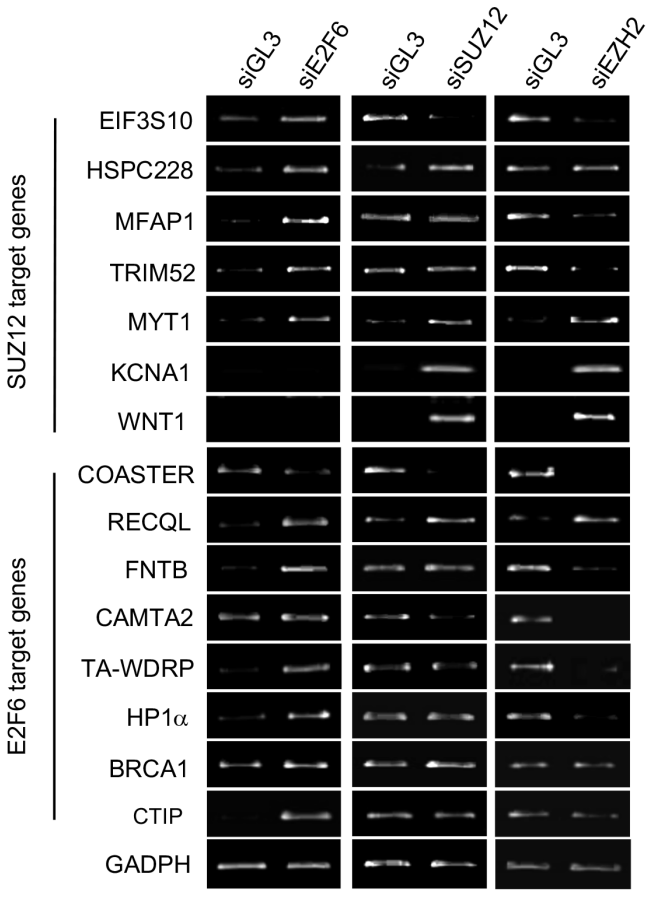

Fig. 7. EZH2 might inhibit E2F6-mediated transcriptional repression. Depletion of E2F6, SUZ, or EZH2 in HEK293 cells resulted in alteration of the expression levels of E2F6 and SUZ12 target genes. Total RNA was extracted 72 hours after transfection with siRNAs, and the mRNA levels of the indicated genes (the same set of genes described in Figure 5) were analyzed by RT-PCR. The assays were repeated five times, and representative results are presented.

DISCUSSION

In this report, we characterized the functional interaction between SUZ12 and E2F6. We isolated E2F6-containing SUZ12-EZH2 complex and identified several target genes of this complex. Since both E2F6 and SUZ12 have been shown to be transcriptional repressors, it was predicted that SUZ12 intermediates transcriptional repression of E2F6. Unexpectedly, reduction of SUZ12 and EZH2 by siRNA resulted in decreased expression of the target genes. Bracken et al. [42] showed that siRNA-mediated depletion of EZH2 and EED in mammalian cells resulted in decreased expression of several genes, although it was unclear whether these genes are directly regulated by EZH2 or not. Other studies reported that a SUZ12-containing complex can activate gene expression of its direct target genes [41,43]. Our findings provide support for the hypothesis that SUZ12-EZH2 complexes can function as a trans-activator for particular genes which, as was identified in this study, might be among the E2F6 target genes.

The precise molecular mechanism of trans-activation by SUZ12-EZH2 is unclear. However, the reciprocal effect of siE2F6 and siEZH2 on gene ex-

pression implies a mechanism in which SUZ12-EZH2 blocks trans-repression of E2F6 through a physical interaction between SUZ12 and E2F6. It was assumed that when the E2F6-SUZ12-EZH2 complex was abrogated by depletion of SUZ12 or EZH2, the blocked repression function of E2F6 was restored, resulting in gene expression being turned off. The EZH2 possesses methyltransferase activity toward H3-K27, H3-K9, and H1-26; however, this activity is regulated by another component of the complexes to which EZH2 belongs [12,14,15,37]. Moreover the EZH2 in the E2F6-EPC1-EZH2 complex does not show H3 methylation activity [37]. In this study, we were not able to examine the E2F6-containing SUZ12-EZH2 complex for HMTase activity because the amount of this complex was extremely low in the purified SUZ12-EZH2 complex. However, since the common target genes of E2F6, SUZ12, and EZH2 showed weak or no tri-methylation at H3-K27, it is likely that the EZH2 on SUZ12 associated with E2F6 could not display methyltransferase activity, at least toward H3-K27.

One may argue that the E2F6-containg SUZ12-EZH2 complex identified in the present study is identical to the E2F6-EPC1-EZH2 [37]. Since it is not known if SUZ12 is involved in formation of E2F6-EPC1-EZH2, we cannot completely distinguish them. However, our experiments showing that EZH2 is not required for an interaction between SUZ12 and E2F6 strongly suggest that the complex in our study diverged from E2F6-EPC1-EZH2. E2F6 has been identified as a component of the complex which consists of PRC1 associated PcG proteins including Bmi1, Mel18, Mph1 and Ring1 [44]. Another E2F6-containg complex, termed PRC1.6 was identified [20,21]. Consistent with biochemical isolation of E2F6-containing PcG complexes, E2F6-deficient mice displayed homeotic transformations that are similar to those observed in PcGs-deficient mice [45-49]. Thus, it is possible that E2F6 can be a component of several PcG complexes, including SUZ12-EZH2, which are formed to function at different stages of cell differentiation and/or proliferation.

The target genes of SUZ12 were uncovered at the genome-wide level using the ChIP-on-chip technique in human and mouse cell lines, embryonic stem cells and human embryonic fibroblasts [28-30,32]. These studies revealed that the genes occupied by SUZ12 contain nucleosomes which are tri-methylated at H3-K27 and are associated with transcriptional repression. On the other hand, the authors also observed that this histone modification was not detected in 20%-25% of SUZ12-bound genes. In addition, it was reported that RNA polymerase Ⅱ occupied 57 of 274 genes which are not tri-methylated at H3-K27 in spite of SUZ12-binding, and that the expression was confirmed in more than half of these genes [30]. This suggests another role for SUZ12 in gene regulation in addition to mediating transrepression by tri-methylation of H3-K27 as a component of PRC2 complex. Although it is as yet unclear whether these genes are targets of E2F6 and could be up-regulated by depletion of SUZ12-EZH2, the findings presented in this study may provide a candidate mechanism of gene activation mediated by SUZ12 and EZH2.

Deregulation of SUZ12 as well as EZH2 gene has been implicated in conferring the phenotype of various tumors including prostate, breast, liver, colon, stomach, bladder and hematological cancer [50-52]. Because our data suggest that SUZ12-EZH2 modulates E2F6 function, expression of a subset of E2F6 target genes might be altered in such tumor cells. Interestingly, both SUZ12 and EZH2 genes are transcriptionally regulated by E2F pathway [40,42]. It has been shown that E2F6 can repress a set of target genes in human cancer cell lines by competing for activating E2Fs [40]. Because it is also believed that E2F pathway is frequently deregulated in tumor cells [53], the interaction between E2F6 and SUZ12-EZH2 presented in this study implicates tumorigenic transcriptional networks: for example, elevated SUZ12-EZH2 blocks E2F6 repression function toward SUZ12 and/or EZH2 genes.

Our results raise questions that remain to be addressed. Because we purified E2F6-containing SUZ12-EZH complex from non-synchronous HEK293 cells, cell cycle-dependent formation of this complex is not clear. It has been shown that another E2F6 containing PcG complex, named E2F6.com-1, could be formed in cells in G0 [54], suggesting E2F6.com-1 regulates its target genes at this cell cycle stage. Previous study indicated that E2F6 binds to the promoters of G1/S-regulated gene by activating E2Fs during S phase of the cell cycles [55]. Thus, it can be postulated that SUZ12-EZH2 interacts with E2F6 to modulate its activity at a particular cell cycle stage. In addition, it remains unclear if E2F6-containing SUZ12-EZH2 complex regulates transcription in a cell type-specific manner or not. Further characterization of this complex is required to understand the biological relevance of the blocking effect of SUZ12-EZH2 on E2F6 trans-repression function.

CONFLICT OF INTEREST: The authors declare no conflicts

of interests.

AUTHORS' CONTRIBUTIONS: KY conceived and planned the experiments. SY and KY carried out the experiment. SY wrote the manuscript with support from KY.

REFERENCES

1. Orlando V. Polycomb, epigenomes, and control of cell identity. Cell 2003; 112:599-606.
2. Levine SS, King IF, and Kingston RE. Division of labor in polycomb group repression. Trends Biochem Sci 2004; 29:478-485.
3. Ringrose L and Paro R. Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu Rev Genet 2004; 38:413-443.
4. Shao Z, Raible F, Mollaaghababa R, Guyon JR, Wu CT et al. Stabilization of chromatin structure by PRC1, a Polycomb complex. Cell 1999; 98:37-46.
5. Francis NJ and Kingston RE. Mechanisms of transcriptional memory. Nat Rev Mol Cell Biol 2001; 2:409-421.
6. Brock HW and van Lohuizen M. The Polycomb group--no longer an exclusive club? Curr Opin Genet Dev 2001; 11:175-181.
7. Simon JA and Tamkun, JW. Programming off and on states in chromatin: mechanisms of Polycomb and trithorax group complexes. Curr Opin Genet Dev 2002; 12:210-218.
8. Czermin B, Melfi R, McCabe D, Seitz V, Imhof A et al. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 2002; 111:185-196.
9. Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A et al. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 2002; 111:197-208.
10. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 2002; 298:1039-1043.
11. Kuzmichev A, Nishioka K, Erdjument-Bromage H, Tempst P, and Reinberg D. Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev 2002; 16:2893-2905.
12. Kuzmichev A, Jenuwein T, Tempst P, and Reinberg D. Different EZH2-containing complexes target methylation of histone H1 or nucleosomal histone H3. Mol Cell 2004; 14:183-193.
13. Kuzmichev A, Margueron R, Vaquero A, Preissner TS, Scher, M et al. Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. Proc Natl Acad Sci U S A 2005; 102:1859-1864.
14. Cao R and Zhang Y. SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell 2004; 15:57-67.
15. Pasini D, Bracken AP, Jensen MR, Lazzerini Denchi E, and Helin, K. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. EMBO J 2004; 23:4061-4071.
16. Francis NJ, Saurin AJ, Shao Z, and Kingston RE. Reconstitution of a functional core polycomb repressive complex. Mol Cell 2001; 8:545-556.
17. Vire E, Brenner C, Deplus R, Blanchon L, Fraga M et al. The Polycomb group protein EZH2 directly controls DNA methylation. Nature 2006; 439:871-874.
18. Blackledge NP, Rose NR, and Klose RJ. Targeting Polycomb systems to regulate gene expression: modifications to a complex story. Nat Rev Mol Cell Biol 2015; 16:643-649.
19. Brockdorff N. Polycomb complexes in X chromosome inactivation. Philos Trans R Soc Lond B Biol Sci 2017; 372:20170021.
20. Schuettengruber B, Bourbon HM, Di Croce L, and Cavalli G. Genome Regulation by Polycomb and Trithorax: 70 Years and Counting. Cell 2017; 171:34-57.
21. Di Carlo V, Mocavini I, and Di Croce L. Polycomb complexes in normal and malignant hematopoiesis. J Cell Biol 2019; 218:55-69.
22. Bracken AP, Brien GL, and Verrijzer CP. Dangerous liaisons: interplay between SWI/SNF, NuRD, and Polycomb in chromatin regulation and cancer. Genes Dev 2019; 33:936-959.
23. Brown JL, Mucci D, Whiteley M, Dirksen ML, and Kassis JA. The Drosophila Polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1. Mol Cell 1998; 1:1057-1064.
24. Mihaly J, Mishra RK, and Karch F. A conserved sequence motif in Polycomb-response elements. Mol Cell 1998; 1:1065-1066.
25. Fritsch C, Brown JL, Kassis JA, and Muller J. The DNA-binding polycomb group protein pleiohomeotic mediates silencing of a Drosophila homeotic gene. Development 1999; 126:3905-3913.
26. Ringrose L, Rehmsmeier M, Dura JM, and Paro R. Genome-wide prediction of Polycomb/Trithorax response elements in Drosophila melanogaster. Dev Cell 2003; 5:759-771.
27. Nekrasov M, Wild B, and Muller J. Nucleosome binding and histone methyltransferase activity of Drosophila PRC2. EMBO Rep 2005; 6:348-353.
28. Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 2006; 441:349-353.
29. Bracken AP, Dietrich N, Pasini D, Hansen KH, and Helin, K. Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev 2006; 20:1123-1136.
30. Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS et al. Control of developmental regulators by polycomb in human embryonic stem cells. Cell 2006; 125:301-313.
31. Tolhuis B, Muijrers I, de Wit E, Teunissen H, Talhout W et al. Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in Drosophila melanogaster. Nat Genet 2006; 38:694-699.
32. Squazzo SL, O'Geen H, Komashko VM, Krig SR, Jin VX et al. Suz12 binds to silenced regions of the genome in a cell-type-specific manner. Genome Res 2006; 16:890-900.

33. Yamamoto K, Sonoda M, Inokuchi J, Shirasawa S, and Sasazuki, T. Polycomb group suppressor of zeste 12 links heterochromatin protein 1alpha and enhancer of zeste 2. J Biol Chem 2004; 279:401-406.
34. Yamamoto K and Sonoda M. Self-interaction of heterochromatin protein 1 is required for direct binding to histone methyltransferase, SUV39H1. Biochem Biophys Res Commun 2003; 301:287-292.
35. Korenjak M and Brehm A. E2F-Rb complexes regulating transcription of genes important for differentiation and development. Curr Opin Genet Dev 2005; 15:520-527.
36. Trimarchi JM and Lees JA. Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol 2002; 3:11-20.
37. Attwooll C, Oddi S, Cartwright P, Prosperini E, Agger K et al. A novel repressive E2F6 complex containing the polycomb group protein, EPC1, that interacts with EZH2 in a proliferation-specific manner. J Biol Chem 2005; 280:1199-1208.
38. Furuno K, Masatsugu T, Sonoda M, Sasazuki T, and Yamamoto K. Association of Polycomb group SUZ12 with WD-repeat protein MEP50 that binds to histone H2A selectively in vitro. Biochem Biophys Res Commun 2006; 345:1051-1058.
39. Birve A, Sengupta AK, Beuchle D, Larsson J, Kennison JA et al. Su(z)12, a novel Drosophila Polycomb group gene that is conserved in vertebrates and plants. Development 2001; 128:3371-3379.
40. Oberley MJ, Inman DR, and Farnham PJ. E2F6 negatively regulates BRCA1 in human cancer cells without methylation of histone H3 on lysine 9. J Biol Chem 2003; 278:42466-42476.
41. Kirmizis A, Bartley SM, Kuzmichev A, Margueron R, Reinberg D et al. Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev 2004; 18:1592-1605.
42. Bracken AP, Pasini D, Capra M, Prosperini E, Colli E et al. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J 2003; 22:5323-5335.
43. Gentile C, Berlivet S, Mayran A, Paquette D, Guerard-Millet F et al. PRC2-Associated Chromatin Contacts in the Developing Limb Reveal a Possible Mechanism for the Atypical Role of PRC2 in HoxA Gene Expression. Dev Cell 2019; 50:184-196.
44. Trimarchi JM, Fairchild B, Wen J, and Lees JA. The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. Proc Natl Acad Sci USA 2001; 98:1519-1524.
45. Storre J, Elsasser HP, Fuchs M, Ullmann D, Livingston DM et al. Homeotic transformations of the axial skeleton that accompany a targeted deletion of E2f6. EMBO Rep 2002; 3:695-700.
46. van der Lugt NM, Domen J, Linders K, van Roon M, Robanus-Maandag E et al. Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. Genes Dev 1994; 8:757-769.
47. Akasaka T, Kanno M, Balling R, Mieza MA, Taniguchi M et al. A role for mel-18, a Polycomb group-related vertebrate gene, during theanteroposterior specification of the axial skeleton. Development 1996; 122:1513-1522.
48. Core N, Bel S, Gaunt SJ, Aurrand-Lions M, Pearce J et al. Altered cellular proliferation and mesoderm patterning in Polycomb-M33-deficient mice. Development 1997; 124:721-729.
49. Takihara Y, Tomotsune D, Shirai M, Katoh-Fukui Y, Nishii K et al. Targeted disruption of the mouse homologue of the Drosophila polyhomeotic gene leads to altered anteroposterior patterning and neural crest defects. Development 1997; 124:3673-3682.
50. Conway E, Healy E, and Bracken AP. PRC2 mediated H3K27 methylations in cellular identity and cancer. Curr Opin Cell Biol 2015; 37:42-48.
51. Comet I, Riising EM, Leblanc B, and Helin K. Maintaining cell identity: PRC2-mediated regulation of transcription and cancer. Nat Rev Cancer 2016; 16:803-810.
52. Yu JR, Lee CH, Oksuz O, Stafford JM, and Reinberg D. PRC2 is high maintenance. Genes Dev 2019; 33:903-935.
53. Kent LN and Leone G. The broken cycle: E2F dysfunction in cancer. Nat Rev Cancer 2019; 19:326-338.
54. Ogawa H, Ishiguro K, Gaubatz S, Livingston DM, and Nakatani Y. A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. Science 2002; 296:1132-1136.
55. Giangrande PH, Zhu W, Schlisio S, Sun X, Mori S et al. A role for E2F6 in distinguishing G1/S- and G2/M-specific transcription. Genes Dev 2004; 18:2941-2951.