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Award Number: DAMD17-03-1-0495

TITLE: Characterization of the Role of JJAZ1 in Human Breast Cancer

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REPORT DATE: January 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20050603 192

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2005	Final (1 Jul 03 - 31 Dec 04)

4. TITLE AND SUBTITLE
Characterization of the Role of JJAZ1 in Human Breast Cancer

5. FUNDING NUMBERS
DAMD17-03-1-0495

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

We hypothesized that the upregulation of the PcG protein JJAZ1 (now known as Suz12) in human breast cancers leads to chromatin modification and subsequent changes in gene expression. It is difficult to identify genes regulated by PcG proteins due to the fact that these proteins do not directly bind to DNA, but rather are recruited via one or more site-specific DNA binding proteins. Our overall Aim was to demonstrate that our recently developed method, which combines chromatin immunoprecipitation and promoter microarray analysis, could be used to identify a large set of genes that are regulated by JJAZ1/Suz12. We proposed to develop an antibody to JJAZ1/Suz12 and then to use that antibody in combination with a technique which we have developed called ChIP-CpG that would allow us to identify the chromosomal sites bound by JJAZ1/Suz12 without prior knowledge of its DNA-binding protein partners. We were successful in developing an antibody to Suz12, have demonstrated that the ChIP-CpG assay can identify Suz12 targets in colon cancers, have collected a number of human breast cancer samples, and are currently completing the studies to identify breast cancer-specific JJAZ1/Suz12 target genes.

14. SUBJECT TERMS
Chromatin, Polycomb, Transcription, Microarray, Suz12

15. NUMBER OF PAGES
30

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Attached appendices:
Kirmizis et al. 2003
Kirmizis et al. 2004

Introduction:

We hypothesized that the upregulation of the PcG protein JJAZ1 (now known as Suz12) in human breast cancers leads to chromatin modification and subsequent changes in gene expression. In the past it has been quite difficult to identify genes regulated by PcG proteins due to the fact that these proteins do not directly bind to DNA, but rather are recruited via one or more site-specific DNA binding proteins. Without prior knowledge of the particular site-specific DNA binding protein that recruits a PcG protein to the DNA, standard experiments (such as in vitro DNA site selection experiments) are not useful for studying a PcG protein. Our overall Aim was to demonstrate that our recently developed method (Oberley et al. 2003; Oberley et al. 2004), which combines chromatin immunoprecipitation and promoter microarray analysis, could be used to identify a large set of genes that are regulated by JJAZ1/Suz12. Therefore, we proposed to use a technique which we have developed called ChIP-CpG which would allow us to identify the chromosomal sites bound by JJAZ1/Suz12 without prior knowledge of its DNA-binding protein partners.

Body:

The research accomplishments associated with each of the approved Tasks is detailed in this section.

Task 1: Antibody production and characterization. The goal of this task was to create an antibody to JJAZ1/Suz12 (no antibodies were commercially available when we began our experiments). We successfully completed this task. Using PCR, a 366 bp fragment from the N-terminus and a 369 bp fragment from the C-terminus of Suz12 ORF were generated and cloned into the Pet15b expression vector (cat. 69661, Novagen, Madison, WI, USA). The KIAA0160 (alternative name for JJAZ/Suz12) human cDNA clone, a gift from Dr Takahiro Nagase at the Kazusa DNA Research Institute in Japan, was used as a template. Protein expression and purification were performed using the Bugbuster His-bind Purification Kit (cat. 70793, Novagen) according to manufacturer's instructions. To prepare the JJAZ1/Suz12 immunogen for rabbit injections, the N and C terminal protein fragments were combined in equal amounts to a final concentration of 1ug/ul. Rabbit immunizations and antibody purifications were performed at Covance Inc (Princeton, NJ, USA). We demonstrated that our JJAZ1/Suz12 antibody was successful in detecting JJAZ1/Suz12 protein via Western blot and that it could immunoprecipitate JJAZ1/Suz12 target genes in ChIP assays. See Kirmizis et al. Genes & Devel. 18:1592-605, 2004 (provided in the Appendix) for more details.

Tasks 2 and 3: Identification and confirmation of JJAZ1/SUZ12 target genes. The goals of these tasks were to identify (task 2) and then confirm (task 3) target genes bound by JJAZ1/Suz12 in human breast samples. While waiting to obtain the breast samples, we began our studies using a colon cancer cell line that expressed high levels of JJAZ1/Suz12. Using a ChIP-CpG approach, we identified a large set of target promoters that are bound by JJAZ1/Suz12 in colon cancer. We

confirmed the interaction of JJAZ1/Suz12 with these target promoters using a high density oligonucleotide tiling array. This work was published in Kirmizis et al. Genes & Devel. 18:1592-605, 2004, which is provided in the Appendix. While performing the studies using a colon cancer cell line, we collected a number of human breast tumors and matched control tissue samples. We had previously demonstrated that JJAZ1/Suz12 mRNA could be detected in human breast tumor samples (see Kirmizis et al. Mol. Cancer Therapeutics, 2:113-121, 2003, which is provided in the Appendix.). Using our new JJAZ1/Suz12 antibody we performed a western blot analysis using normal and tumor tissues from four different human breast cancer patients. As shown below, we found that JJAZ1/Suz12 can be detected to varying degrees in all four breast tumor samples (Figure 1). However, other members of the chromatin remodeling complex to which JJAZ1/Suz12 belongs must also be present in order for JJAZ1/Suz12 overexpression to be of functional importance. We found that high levels of another member of the PRC 2 complex (EED) were not always found in human breast tumors. This result suggests that PRC complex may only regulate gene expression in a subset of human breast tumors. The next step will be to perform a ChIP-CpG array using breast tumor tissue from a cancer that has upregulated all the components of the PRC2 complex. These experiments are in progress.

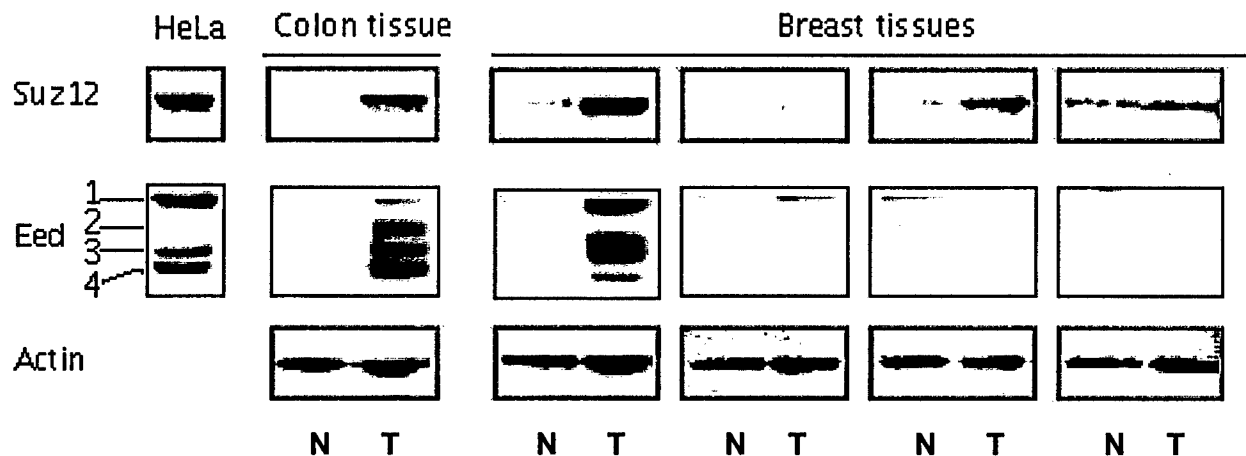

Figure 1: Western blot analysis of Suz12 and EED in human breast tumors. Antibodies to Suz12 and EED (which has four isoforms) were used with normal (N) and tumor (T) tissues obtained from human colon or breast cancer patients. For comparison, the amount of Suz12 and Eed found in HeLa cells is shown. An antibody to actin was used as a loading control.

Key research accomplishments:

1. Production of an antibody to JJAZ1/Suz12
2. Identification of a list of target genes regulated by JJAZ1/Suz12

Reportable Outcomes: (July 1, 2003-Dec. 31, 2004)

1. **Manuscripts**:
   Kirmizis, A., Bartley, S. M., Kuzmichev, A., Margueron R., Reinberg. R., Green, R., and Farnham, P. J. Silencing of human polycomb target genes is associated with methylation of histone H3 lysine 27. Genes & Devel. 18:1592-605, 2004

2. **Abstracts presented at meetings:**
   Kirmizis, A. and Farnham, P.J. Identification and characterization of human SUZ12 target genes. Chromatin meeting. Luxembourg City, Luxembourg. January, 2004.

3. **Oral Presentations by Dr. Farnham:**
   Identification and characterization of SUZ12. Institute of Genetics and Biophysics, Naples, Italy, September, 2003.

   Identification and characterization of human SUZ12 target genes. Chromatin meeting. Luxemburg, January 2004.

   The use of chromatin immunoprecipitation and genomic microarrays to identify target genes of the Polycomb Group protein SU(Z)12. 7th annual MGED (Microarray and Gene Expression Data group) meeting, Toronto, Canada, September, 2004.

   The use of chromatin immunoprecipitation and genomic microarrays to identify target genes of the Polycomb Group protein SU(Z)12. ASBMB meeting on "Transcriptional Regulation by Chromatin and RNA Polymerase II" held at Lake Tahoe, CA in October, 2004.

   The use of chromatin immunoprecipitation and genomic microarrays to identify target genes of the Polycomb Group protein SU(Z)12. MD Anderson Annual Cancer Symposium held at Houston, Texas in October, 2004.
4. **Degrees awarded:**
   Antonis Kirmizis received his Ph.D. based on his work in the Farnham laboratory in July, 2004. His studies were partially funded by this grant.

5. **Development of reagents:** We developed an antibody to JJAZ1/Suz12.

Conclusions:

Our studies have identified the first set of human Polycomb Group Repression Complex (PRC) target genes and have demonstrated the utility of custom oligonucleotide arrays in presenting a detailed profiling of the binding of PRC components to large regions of target promoters. We have also provided evidence in

support of the model that PRC complexes regulate changes in chromatin structure in human cancers via methylation of histone H3.

References:

Kirmizis, A, Bartley, S.M., and Farnham, P.J. Identification of the Polycomb Group protein SU(Z)12 as a new b-catenin target gene which is upregulated in colon tumors. Mol. Cancer Therapeutics, 2:113-121, 2003.

Kirmizis, A., Bartley, S. M., Kuzmichev, A., Margueron R., Reinberg. R., Green, R., and Farnham, P. J. Silencing of human polycomb target genes is associated with methylation of histone H3 lysine 27. Genes & Devel. 18:1592-605, 2004

Oberley, M.J. and Farnham, P. J. Probing chromatin immunoprecipitates with CpG island microarrays to identify genomic sites occupied by DNA-binding proteins. Methods in Enzymology, 371:577-596, 2003.

Oberley, M.J., Tsao, J., Yau, P., and Farnham, P. J. High-throughput screening of Chromatin immunoprecipitates using CpG-island microarrays. Methods in Enzymology, 376: 315-333 2004.

Appendices

Kirmizis, A, Bartley, S.M., and Farnham, P.J. Identification of the Polycomb Group protein SU(Z)12 as a new b-catenin target gene which is upregulated in colon tumors. Mol. Cancer Therapeutics, 2:113-121, 2003.

Kirmizis, A., Bartley, S. M., Kuzmichev, A., Margueron R., Reinberg. R., Green, R., and Farnham, P. J. Silencing of human polycomb target genes is associated with methylation of histone H3 lysine 27. Genes & Devel. 18:1592-605, 2004

Identification of the Polycomb Group Protein SU(Z)12 as a Potential Molecular Target for Human Cancer Therapy[1]

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Abstract

We have previously identified *SU(Z)12* as an E2F target gene. Because many E2F target genes encode proteins that are critical for the control of cell proliferation, we have further characterized the regulation and expression of *SU(Z)12*. To understand the molecular mechanisms responsible for expression of *SU(Z)12* mRNA, we have analyzed the promoter region. We found that the *SU(Z)12* gene is controlled by dual promoters, one of which functions bidirectionally. In addition to the E2F binding site, we have identified two binding sites for T cell factor (TCF)/β-catenin complexes. Using gel mobility shift assays, we demonstrated that both TCF sites can be bound by TCF4. TCF/β-catenin complexes have been shown to be a critical regulator of gene expression in tumors of the colon, breast, and liver. Accordingly, we have used chromatin immunoprecipitation assays to confirm that TCF4/β-catenin complexes are bound to the *SU(Z)12* promoter in colon cancer cells but not in HeLa cells. We next adapted the chromatin immunoprecipitation assay for use with primary colon tumor samples, and, using matched pairs of normal and tumor tissue obtained from several different colon cancer patients, we demonstrate that levels of β-catenin bound to the *SU(Z)12* promoter are increased in colon tumors. Finally, we show that the *SU(Z)12* mRNA is up-regulated in a number of different human tumors, including tumors of the colon, breast, and liver. Recent studies have found that SU(Z)12 is a component of the *Drosophila* ESC-E(Z) and the human EED-EZH2 Polycomb chromatin remodeling complexes. Therefore, we suggest that SU(Z)12, which may modulate the tumor phenotype by changing gene expression profiles, may be a logical target for the design of a new antitumor agent.

Introduction

We had previously identified the human *SU(Z)12* [also known as KIAA0160, *JJAZ1*, and *ChET9* (1–4)] promoter as one of several chromatin fragments that were isolated by virtue of their *in vivo* interaction with E2F transcription factors (4). E2Fs regulate the expression of genes involved in nucleotide metabolism, DNA replication, cell cycle control, apoptosis, DNA repair, and DNA replication (5–8). Many of these E2F target promoters have been shown to be responsive to changes in cell growth conditions. For example, E2F target genes often show increased expression in highly proliferating normal tissues and in certain tumor types (9). In fact, an E2F target promoter has been used to achieve selective killing of tumor cells (10). Much of the proliferation-specific expression of E2F target genes is attributable to their regulation by the Rb[3] tumor suppressor family of proteins, which includes Rb, p107, and p130. Interaction of Rb, p107, or p130 with the E2F factors results in transcriptional repression of target genes. However, in many tumors this interaction is abolished by mutation of the Rb family members or increased expression or activity of cyclin/cdk complexes, which function to phosphorylate Rb and break up the Rb/E2F interaction (11). The loss of Rb-mediated repression allows E2F to activate its target genes to high levels in tumor cells. Tumor-specific expression of E2F target genes may also be achieved via cooperation of E2F family members with nuclear oncogenes. For example, E2F cooperates with Myc to induce cell transformation (9) and, at least in certain cases, it has been suggested that both transcription factors regulate the same promoter (12–14). We have also noted a correlation between E2F target promoters and β-catenin target promoters. For example, the *Myc* and *cyclin D1* promoters, both of which are regulated by β-catenin (15, 16), are also regulated by the E2F family (17, 18). Also, we have shown that the *Myc*, *cyclin D1*, *jun*, and *PPARδ* promoters are all bound by both β-catenin and E2F4 *in vivo*.[4] Although no direct interaction between E2F4 and β-catenin has been reported, it is possible that the two factors cooperate to activate transcription via a mechanism such as transcription factor synergy (19).

The observed coincidence of E2F and β-catenin target promoters suggests that the expression of certain E2F target genes may be altered in cancers caused by the deregulation of β-catenin activity, such as colon cancer. Colorectal cancer is the third leading cause of cancer death in the United States. The majority of colorectal tumors contain mutations in the *APC* tumor suppressor gene (20). Normal APC protein

Received 7/18/02; revised 11/13/02; accepted 11/18/02.

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[1] Supported in part by USPHS Grants CA45240, CA22484, and CA07175.

[2] To whom requests for reprints should be addressed, at McArdle Laboratory for Cancer Research, University of Wisconsin, 1400 University Avenue, Madison, WI 53706. Phone: (608) 262-2071; Fax: (608) 262-2824; E-mail: farnham@oncology.wisc.edu.

[3] The abbreviations used are: Rb, retinoblastoma; APC, adenomatous polyposis coli; RT-PCR, reverse transcription-PCR; PcG, Polycomb group; ESC, extra sex comb; ChIP, chromatin immunoprecipitation; EST, expressed sequence tag; DEN, diethylnitrosamine; TCF, T cell factor; PRC2, polycomb repressive complex 2.

[4] A. Kirmizis, unpublished observations.

has been shown to bind to and down-regulate the level of β-catenin, a component of cell adhesion complexes. Most APC mutations found in colorectal tumors result in truncated APC proteins that lack the region required for down-regulation of β-catenin. Thus, in colon tumors, β-catenin accumulates to high levels and translocates to the nucleus. Yeast two hybrid screens identified β-catenin as an interaction partner of the TCF/Lef family of transcription factors. Overexpression of β-catenin in tissue culture cells can cause transcriptional up-regulation of certain promoters that contain TCF binding sites (16) and a direct fusion of β-catenin to the Lef-1 DNA binding domain can activate transcription (21). Therefore, based on the fact that loss of APC leads to increased β-catenin and that increased β-catenin can cause transcriptional activation, it has been postulated that the abnormal expression of genes regulated by a TCF/β-catenin complex is a critical determinant of the neoplastic phenotype of colon cancer cells (22). To date, several putative β-catenin target genes have been identified by comparing gene expression profiles in populations of cells grown in tissue culture. These genes include *fra-1*, *c-jun*, *c-Myc*, *matrilysin*, *cyclin D1*, *PGHS-2*, and *PPARδ* (15, 16, 23–28). Although the identification of these β-catenin target genes has provided much new insight into colon cancer, it is likely that additional genes important in neoplastic transformation of colon cells remain to be discovered.

We have now expanded our analysis of the *SU(Z)12* gene and have found that the *SU(Z)12* promoter is bound by both E2F4 and TCF/β-catenin complexes. We also show that the levels of β-catenin recruited to the *SU(Z)12* promoter increase in colon tumors, as compared with normal colon tissue from the same patient, and that this increased binding of β-catenin correlates with increased levels of *SU(Z)12* mRNA.

Materials and Methods

RT-PCR Analysis. For each RT-PCR reaction, 100 ng of mouse liver RNA, prepared as described previously (29), was analyzed at a hybridization temperature of 63° for 32 cycles. Human colon and breast RNA, prepared as described previously (30), was a gift from Jeff Ross, who obtained the tissue from the Cooperative Human Tissue Network (which is funded by National Cancer Institute). We note that the normal samples used for these experiments and for the ChIP experiments described below may have slight contamination with the underlying stromal cells. Fortunately, the three-dimensional architecture of the colon tumors allows the tumor samples to be removed from the colon without encroaching on the adjacent stromal cells. For each RT-PCR reaction using human RNA, performed as described previously (31), 100 ng of RNA was analyzed at a hybridization temperature of 59° for 28 cycles. Primers used in the reactions are listed in Table 1; all of the primers used in RT-PCR, PCR, cloning, and gel shifts were obtained from the University of Wisconsin Biotechnology Center. All of the work using human tissues for either RNA analysis or ChIP experiments was performed under guidelines of the NIH and the University of Wisconsin human subjects Institutional Review Board.

Primer Extensions. RNA was prepared from HeLa cells as described previously (32). When using primer A, 10 μg of HeLa mRNA was included in the reaction; when using primer B, 5 μg of HeLa mRNA was included. The sequences of primer A and primer B are listed in Table 1. Primer extensions were performed as described previously (33, 34).

ChIP. ChIPs using cultured cells were performed as described previously (4). For the analysis of colon tissue, the ChIP protocol required several modifications.[5] Briefly, the protocol required mincing the tissue in PBS, cross-linking in formaldehyde for a longer time than used for tissue culture cells, and then processing with a Medi-machine to achieve single cells. Antibodies used were to RNA polymerase II (Santa Cruz Biotechnology; sc-899), E2F4 (Santa Cruz Biotechnology; sc-866X), TCF4 (Santa Cruz Biotechnology; 8631X), TCF3/4 (Upstate Biotechnology; 05-512), and β-catenin (Transduction Laboratories; C19220). The sequences of the primers used in the ChIP assays are listed in Table 1.

Plasmid Constructs. Promoter reporter constructs were prepared by PCR using primers spanning the indicated regions. The sequence of the primers used to clone the promoter constructs is listed in Table 1. Each primer also contained a *Hin*dIII site at the 5′ end to facilitate cloning of the PCR fragments into the *Hin*dIII site in pGL2 basic (Promega Inc., Madison, WI). Genomic human DNA was used as a template for the PCR reactions. Each fragment was cloned in both orientations to allow an analysis of bidirectional promoter activity.

Transient Transfections. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's media (Life Biotechnology, Inc., Grand Island, NY), supplemented with 5% bovine calf serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin, and incubated at 37°C in a humidified 5% CO2 incubator. Transient transfections using the calcium phosphate method were performed as described previously (32). For the analysis of promoter activity, 2 μg of reporter constructs plus 13 μg of sonicated salmon sperm DNA was transfected into proliferating cells, which were harvested 48 h after transfection; experiments were performed in triplicate, and each transfection experiment was carried out in duplicate plates.

Electromobility Shift Assays. *In vitro* TCF/β-catenin DNA-binding activity was assayed using electromobility shift assay competition experiments. Approximately 6 μg of HT29 whole cell extract, prepared as described previously (35), was incubated with 0.3 μg of sonicated herring sperm DNA and 2 μl of binding buffer [50 mM HEPES (pH 7.4), 300 mM KCl, 5 mM EDTA, 5 mM DTT, 11.5% Ficoll] in a total volume of 18 μl for 15 min at room temperature. About 40,000 cpm of the [γ-^{32}P]ATP-labeled optimal TCF (Opt-TCF) oligonucleotide (36) was then added to the binding reactions, and the incubation continued for an additional 20 min. The double-stranded oligonucleotides used as competitors were either the unlabeled probe or oligonucleotides based on sequences from the *SU(Z)12* promoter (TBS1, TBS1mt, TBS2,

[5] The several modifications required by ChIP protocol are described in detail at http://mcardle.oncology.wisc.edu/farnham/.

Table 1 Primer sequences

All of the primers are listed in the 5′ to 3′ orientation. The consensus TCF site (a/t,a/t,caaag) is underlined in each oligonucleotide used in the gel shift experiments.

Assay	Primer sequence
RT-PCR (human)	
Left	cttacatgtctcatcgaaactcc
Right	ggctggaagctcttcattgaca
RT-PCR (mouse)	
Left	gccgaaaatggagcacgtccaggc
Right	cacaagcaggacttccagggtaac
Primer extension	
A	ccaaccgctcgccccagcaggctcc
B	ctgtgttggtttctcaaaggcctgg
Cloning	
Upstream promoter	
Left	tacgataagcttggtggcgagcgcctgtaatc
Right	tacgataagctttgtctcctcagagccccgct
Downstream promoter	
Left	tacgataagctttcggctagccggagcctgct
Right	tacgataagcttcttcttcaccggtaacgccg
ChIP:	
SU (Z) 12	
Left	tcaccctaccctggcctcgct
Right	tcgctaaaccgctcgctgggt
Myc	
Left	acagacgcctcccgcacggg
Right	ccacaccgagaacgcactgc
Cyclin D1	
Left	cggactacaggggagttttgttg
Right	tccagcatccaggtggcgacgat
H2A	
Left	ggagcagacttcgctggctctg
Right	gcgcaactcactacgagcaacc
B-Myb	
Left	ggtgttcgctatgtgggata
Right	ctcctcgctcgcaggaactg
Gel shift	
Opt-TCF	ggtaagatcaaaggg
Proximal	
TBS1	agcttcgcgaattcagttcaaagaggcggccgggca
TBS1mt	agcttcgcgaattcaggccagagaggcggccgggca
Distal	
TBS2	agctttgtgtctatcgttcaaagcaagacctggcca
TBS2mt	agctttgtgtctatcggccagagcaagacctggcca

TBS2mt) and were included in the first incubation at a 10-fold molar excess to the labeled probe. The sequences of the oligonucleotides used in the binding reactions are listed in Table 1. For supershifts, 1.5 μl of mouse anti-β-catenin monoclonal 15B8 ascites fluid (Abcam Ltd.; ab6301–100), 1 μg of TCF4 antibody (Santa Cruz Biotechnology; 8631X), or 1 μg of HNF3γ antibody (Santa Cruz Biotechnology; 5360X) were included in the first incubation. The reactions were electrophoresed for ~3 h on a 4.5% polyacrylamide gel that had been pre-electrophoresed for 30 min.

Results

Dual Promoters Drive Expression of *SU(Z)12* mRNA. A full-length cDNA corresponding to the human *SU(Z)12* mRNA has previously been deposited in GenBank (accession no. D63881; Ref. 1). Inspection of the collection of ESTs and cDNAs that map to the location of the *SU(Z)12* gene, as determined using the University of California-Santa Cruz human genome database,[6] indicated that several other groups have cloned mRNAs that have 5′ ends mapping near the 5′ end of the original cDNA. However, two ESTs extend slightly upstream of the cloned cDNA and several end between 300 and 400 nucleotides downstream of the 5′ end of the cloned cDNA. Interestingly, the shorter human ESTs have 5′ ends mapping near the 5′ end of the corresponding mouse cDNA (GenBank accession no. BF658962). These results suggested that there may be two different transcription start sites, one located near the 5′ end of the human cDNA clone and one located near the 5′ end of the mouse cDNA clone. We noted that consensus Sp1 and E2F sites can be found at distances appropriate to serve as promoter elements for mRNA coming from both putative start sites (see Fig. 1). Therefore, as a first step in characterizing the transcriptional

[6] Internet address: http://genome.cse.ucsc.edu/.

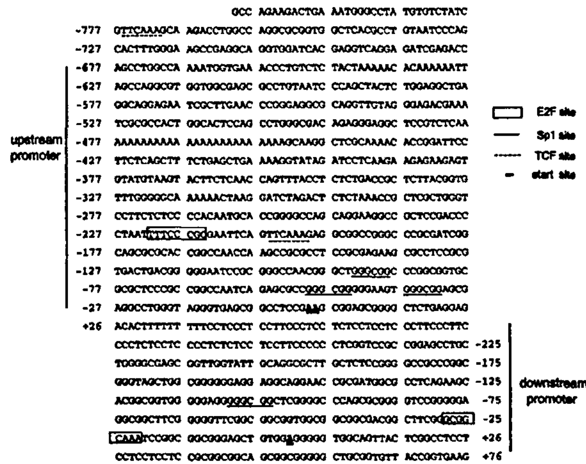

Fig. 1. Sequence of the *SU(Z)12* promoter. The genomic sequence representing the *SU(Z)12* promoter region is shown. Indicated are the consensus E2F, Sp1, and TCF sites. *Bold type* and *underlined*, the positions of the transcription initiation sites, as determined by primer extension analysis. *The numbers to the left of the sequence* are calculated relative to the upstream start site; *the numbers to the right of the promoter* are calculated relative to the downstream start site.

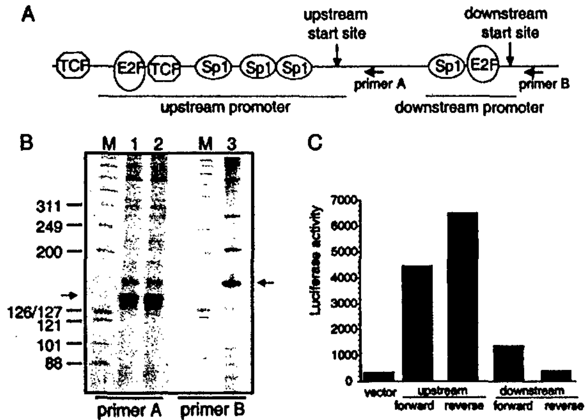

Fig. 2. Dual promoters drive expression of *SU(Z)12* mRNA. *A*, schematic of the human *SU(Z)12* promoter. Shown are the relative locations of the consensus Sp1, E2F, and TCF sites, the location of the primers used for primer extension analyses, the approximate location of the upstream and downstream transcription initiation sites, and the fragments used in panel C for promoter analyses. *B*, primer extension analysis was performed using HeLa cell RNA and primer A and primer B. *Lanes 1* and *2*, two different reactions using the same primer and RNA samples. *Lanes M*, DNA markers (sizes shown *to the left of the panel*). *Arrow on the left*, the upstream transcription initiation site; *arrow on the right*, downstream transcription initiation site. The gel representing the *right panel*, containing samples analyzed by *primer B*, was exposed longer than the left panel so that an easily visible signal could be obtained. *C*, the *upstream* and *downstream* promoter fragments were cloned upstream of the luciferase cDNA in both orientations. The plasmid constructs were transiently transfected into NIH 3T3 cells, and luciferase activity was monitored as described previously (32). The vector (pGL2) sample was obtained by transfection of the luciferase reporter plasmid lacking an inserted promoter fragment. In each case, the *forward* orientation indicates that the fragment is placed in the orientation found in the genome, relative to *SU(Z)12* mRNA.

regulation of the *SU(Z)12* gene, we used primer extension analysis to map the 5′ end of the *SU(Z)12* mRNA from human cells.

To map the 5′ end of the human *SU(Z)12* mRNA, two different primers were prepared; mRNAs beginning in the region corresponding to the 5′ end of the cloned human cDNA should be easily mapped using primer A, and mRNAs beginning in the region corresponding to the 5′ end of the cloned mouse cDNA should be easily mapped using primer B. Using primer A, we found strong signals that corresponded to a position ~11 bp upstream of the cloned human cDNA (Fig. 2*B*). Interestingly, this transcription start site maps to the same position as one of the longest ESTs (GenBank accession no. BG655887). Using primer B, we detected a slightly weaker signal (Fig. 2*B*) that maps to a position near the 5′ end of the shorter ESTs (*e.g.*, GenBank accession no. AA356424). To ensure that signals from primer B were not weaker because of problems with labeling the primer and/or inefficient hybridization of that particular primer, we prepared another primer complementary to the sequences adjacent to primer B; again, weaker signals were obtained using this primer (data not shown). Therefore, it appears as though human *SU(Z)12* mRNA is produced from two start sites, with the upstream region corresponding to the majority of the mRNA.

The results of the primer extension analyses, which indicate that transcription initiates just downstream of both sets of E2F and Sp1 sites, suggest that perhaps two different promoter regions are used to drive expression of *SU(Z)12* mRNA. To test this hypothesis, we cloned genomic fragments spanning both the upstream and downstream start sites in front of the luciferase cDNA. The promoter-reporter plasmids were then transfected into cells, and luciferase activity was monitored (Fig. 2*C*). We found that the upstream fragment, which corresponds to position −617 to +26 relative to the upstream start site mapped by primer extension, does mediate promoter activity and that this fragment functions bidirectionally. Interestingly, the first mammalian promoter shown to be regulated by E2F was the bidirectional *dhfr/msh3* locus (37). Other bidirectional E2F-regulated promoters include *thymidylate synthase* and *RanBP1/Htf9* (38, 39). We also note that in a recent screen for novel E2F-regulated promoters that identified 68 different loci, 15% of the promoters are clearly bidirectional (6). However, an analysis of the human genome suggests that a large percentage of human promoters, not just E2F target promoters, are bidirectional in nature (40). We have not further characterized the possibility that other transcripts may exist in the cell that are initiated in the opposite direction to the *SU(Z)12* mRNA. We found that the downstream fragment, which corresponds to position −243 to +76 relative to the downstream start site mapped by primer extension, also mediates promoter activity; in this case, only the forward direction is active. The relative promoter strength of the upstream *versus* downstream promoter fits well with the relative amounts of mRNA as determined by primer extension. Other E2F-regulated genes, such as human *E2F3*, *c-Myc*, human mitochondrial *glycerol phosphate dehydrogenase*, and human *uracil-DNA glycosylase* (41–44), have been shown to be controlled by dual promoters. Thus, we conclude that the *SU(Z)12* mRNA

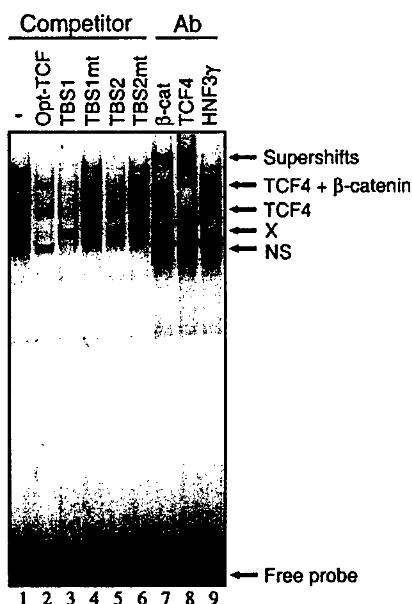

Fig. 3. Identification of TCF/β-catenin binding sites in the *SU(Z)12* promoter. An oligonucleotide containing a previously characterized consensus TCF site was used as a probe for the gel shift assays (36). The probe was incubated with HT29 whole cell extracts, alone or with a 10-fold excess of unlabeled probe (*Lane 1*), an oligonucleotide containing the proximal TCF binding site (*TBS1*) of the *SU(Z)12* promoter (*Lane 2*), an oligonucleotide containing a mutated TBS1 (*Lane 3*), an oligonucleotide containing the distal TCF binding site (*TBS2*) of the *SU(Z)12* promoter (*Lane 4*), or an oligonucleotide containing a mutated TBS2 (*Lane 5*). Antibodies that recognize β-catenin (*Lane 7*) or TCF4 (*Lane 8*) were incubated with the probe and whole cell extracts to identify the bands containing the TCF/β-catenin complexes. A control antibody, HNF3γ was added to the reaction in *Lane 9*. *NS*, a nonspecific band; *X*, a band representing an uncharacterized, but specific, DNA/protein complex.

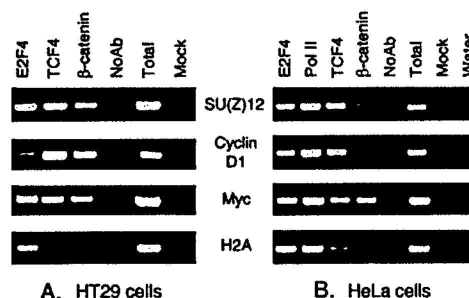

Fig. 4. ChIP analysis of the *SU(Z)12* promoter region. ChIP experiments were performed using the HT29 colon cancer cell line (*A*) or HeLa cells (*B*) and the indicated antibodies or a no-antibody control. The precipitated chromatin was monitored using primers specific for the *SU(Z)12*, *cyclin D1*, *c-Myc*, or *histone H2A* promoters. A mock immunoprecipitation reaction was also performed in which chromatin was omitted at the beginning of the experiment. Also, a control PCR reaction was performed in which only water (no immunoprecipitated sample) was added.

is driven from two promoters and that the bidirectional upstream promoter is the major promoter for *SU(Z)12* mRNA.

TCF and β-Catenin Are Recruited to the *SU(Z)12* Promoter. Sequence analysis of the *SU(Z)12* upstream promoter revealed two consensus binding sites for the TCF family of transciption factors, located at −206 and −776, relative to the upstream transcription start site. TCF family members do not contain transactivation domains. Rather, the coactivator β-catenin interacts with TCF family members and the TCF/β-catenin complex activates transcription. β-catenin is normally present in very low amounts in cells and is sequestered in membrane and/or cytoplasmic complexes (22). However, there are large amounts of nuclear β-catenin in colon cancer cells. We have examined the ability of the two consensus TCF sites in the *SU(Z)12* promoter to bind to TCF/β-catenin complexes using a gel mobility shift assay (Fig. 3). A previously characterized consensus TCF site was used as a probe. Incubation of this probe with extract prepared from HT29 colon cancer cells resulted in an upward shift of the probe, creating bands that are shown to be specific for binding of TCF factors attributable to competition by consensus (Fig. 3, *Lanes 2*, *3*, and *5*), but not mutated (*Lanes 4* and *6*) TCF sites. Inclusion of antibodies in the gel shift reaction identifies one band as free TCF4 and one band as a TCF4/β-catenin complex (*Lanes 7* and *8*); the other bands are likely to be other TCF family members alone or in complex with β-catenin. However, to date, we have not been able to identify which TCF family members compose the remaining bands. Importantly, inclusion of oligonucleotides corresponding to the distal and proximal TCF sites from the *SU(Z)12* promoter demonstrates that each of these sites has the potential to bind to TCF/β-catenin complexes *in vitro*.

Although gel mobility shift assays provide information as to whether a factor has the capability of binding to an isolated consensus site *in vitro*, they do not prove that a particular promoter is bound by a particular protein in living cells. However, the ChIP assay does provide a method to determine whether a particular promoter is bound by a specific transcription factor under biologically relevant *in vivo* conditions. To determine whether the *SU(Z)12* promoter is bound by TCF family members in living cells, we performed ChIP experiments using the HT29 colon tumor cell line (Fig. 4*A*). Antibodies to E2F4, TCF4, and β-catenin were used, along with a no-antibody control. As positive controls, we monitored occupancy of the *Myc* and *cyclin D1* promoters because both promoters have previously been shown to be E2F (17, 18) and TCF/β-catenin (15, 16) target genes. We also monitored the *histone H2A* promoter because it has been shown to be an E2F target gene (45) but has not been reported to be regulated by β-catenin. We used antibodies to E2F4 and TCF4, rather than antibodies to other E2F or TCF family members, because our preliminary experiments showed that these family members are easily detected on known target genes in colon tumor cell lines (data not shown). We found that, as expected, E2F4, TCF4, and β-catenin were bound to the *cyclin D1* and *c-Myc* promoters, whereas only E2F4 was bound to the *H2A* promoter (Fig. 4). As shown in Fig. 4*A*, we found that E2F4, TCF4, and β-catenin can be detected on the *SU(Z)12* promoter in HT29 cells. Interestingly, when similar experiments were performed using chromatin from HeLa cells (which are derived from a cervical cancer), we found that only the *c-Myc* promoter was bound by β-catenin (Fig. 4*B*). Unlike the *Myc* promoter, the *SU(Z)12* and *cyclin D1* promoters were only bound by β-catenin in the colon tumor sample. These results support

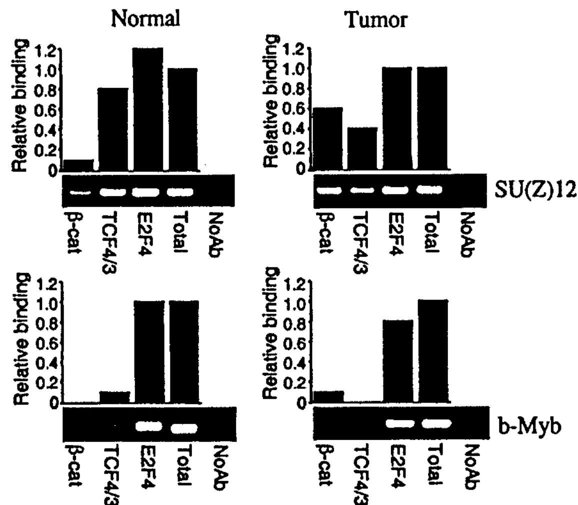

Fig. 5. Levels of β-catenin binding to the *SU(Z)12* promoter increase in colon tumors. ChIP experiments were performed using normal colon tissue or colon tumor tissue from the same patient, along with antibodies that recognize β-catenin, both TCF3 and TCF4, and E2F4, as well as a no-antibody control. Primers specific for the *SU(Z)12* and *b-Myb* (a well-characterized E2F target gene) promoters were used. The relative amount of binding of the different transcription factors was calculated by subtracting the slight signal in the no-antibody lane, then dividing the signal specific for the different factors by the signal obtained using a fixed aliquot of the input chromatin (*Total*).

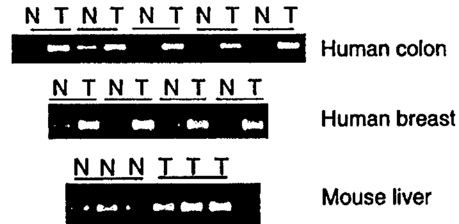

Fig. 6. *SU(Z)12* mRNA is up-regulated in tumors. RT-PCR analysis was performed on RNA isolated from normal (*N*) colon and colon tumors (*T*) from five different patients, normal (*N*) breast and breast tumors (*T*) from four different patients, and normal (*N*) liver from mice not treated with DEN and three different mouse liver tumors (*T*) from mice treated with DEN, using primers specific for the human or mouse *SU(Z)12* mRNA. All of the samples were also analyzed by RT-PCR for *GAPDH* mRNA to ensure that the RNA was correctly quantitated and of high quality (30, 31).

the conclusion that the *SU(Z)12* promoter is bound by E2F and TCF/β-catenin complexes, and they also show that recruitment of β-catenin to a TCF target promoter can be influenced by cell type.

Levels of β-Catenin Binding to the *SU(Z)12* Promoter Increase in Colon Tumors. The ChIP assays performed above used cultured cells. If *SU(Z)12* is a biologically relevant β-catenin target gene, then we would expect that there should be little β-catenin bound to the promoter in normal colon tissue, but the levels would be increased in colon tumor samples. To test this hypothesis, we adapted the ChIP assay for use with surgical samples (details concerning the differences between assays performed using tissue culture cells *versus* surgical samples can be found at our web site).[7] We performed ChIP experiments using samples of matched normal colon and colon tumor from a cancer patient (Fig. 5). As a control, we monitored levels of E2F4. Although, as described above, the activity of E2F family members increases in certain tumors, the levels of E2F4 have not been shown to be influenced by neoplastic transformation. We found that the amounts of E2F4 bound to the *SU(Z)12* promoter and to the *b-Myb* promoter were similar in normal *versus* tumor tissues. In contrast, we found that levels of β-catenin bound to the *SU(Z)12* promoter increased in the tumor, as compared with the adjacent normal tissue. We have used several different matched normal *versus* tumor samples from various colon cancer patients. In all of the tumors in which we observed binding of β-catenin to the *Myc* promoter, we also found β-catenin bound to the *SU(Z)12* promoter (data not shown). Thus, we concluded that, in colon tumors, the *SU(Z)12* promoter is as effective in recruiting β-catenin as is the *Myc* promoter, a previously documented TCF/β-catenin target. Taken together, the gel shift analysis and the ChIP experiments indicated that the *SU(Z)12* promoter is occupied by TCF in normal cells and by TCF/β-catenin complexes in colon tumor cells.

We have noticed that the signal obtained using the TCF family member antibody is always slightly weaker in the tumor samples. This could be caused by a decreased accessibility of the antibody to the TCF family member caused, in turn, by the presence of the large β-catenin protein. Alternatively, in the tumor samples, TCF4 could be replaced by a different TCF family member. We have attempted to address this possibility by the use of antibodies to Lef-1, a TCF family member that has been reported to be increased in colon tumors (46). We did not enrich for the *SU(Z)12* promoter using the Lef-1 antibody in ChIP experiments (data not shown). However, we cannot conclude that Lef-1 did not replace TCF4 on the *SU(Z)12* promoter because none of the other TCF target promoters that we analyzed bound Lef-1, and thus we lacked a positive control.

SU(Z)12 mRNA Is Up-Regulated in Human Tumors. The experiments described above indicate that the *SU(Z)12* promoter is bound by high levels of β-catenin in colon tumors. If the recruitment of β-catenin to the *SU(Z)12* promoter is functionally significant, we would expect that levels of *SU(Z)12* mRNA would be increased in tumor types that are known to be associated with increased β-catenin activity. Therefore, we have investigated the expression of *SU(Z)12* in multiple different colon, breast, and liver tumors, all of which have been correlated with alterations in β-catenin activity (20, 47, 48). We found that *SU(Z)12* showed increased expression in five different human colon tumors, as compared with the normal colon tissue taken from the same patient (Fig. 6). These results, in combination with the analysis of four additional colon tumors (data not shown), indicated that *SU(Z)12* mRNA was more abundant in the tumor tissue in eight of nine colon cancer patients tested. In one of the patients, we found that *SU(Z)12* mRNA was high in both the normal and the tumor sample; it is possible that in this one

[7] Internet address: http://mcardle.oncology.wisc.edu/farnham/.

case, the normal sample was contaminated with tumor cells. Similarly, *SU(Z)12* mRNA was increased in four different human breast tumors. Because of the difficulty in obtaining human liver tumors samples, we have examined the expression of murine *SU(Z)12* in mouse liver tumors that were created by treatment of mice with the carcinogen DEN (see Ref. 31 for details). We found that *SU(Z)12* mRNA was increased in three different mouse liver tumors. Thus, increased expression of *SU(Z)12* mRNA occurs frequently in tumors derived from colon, liver, and breast.

Discussion

We have shown that the *SU(Z)12* promoter binds to TCF/β-catenin complexes *in vitro* and *in vivo* and that the levels of β-catenin binding to the *SU(Z)12* promoter increase in colon tumors, as compared with matched normal tissue. We also showed that the levels of *SU(Z)12* mRNA are increased in tumors known to be associated with high levels of nuclear β-catenin, namely tumors derived from colon, breast, and liver tissues. Thus, we conclude that *SU(Z)12* is a new β-catenin target gene whose expression is increased by recruitment of β-catenin in colon tumors. Interestingly, a recent report has shown that a portion of the *SU(Z)12* gene (called *JJAZ1* in that study) is fused to a gene from chromosome 7 in endometrial stromal tumors (2). In these tumors, most of the *SU(Z)12* coding sequences are fused in frame to the 5′ end of another protein called JAZF1. Therefore, the production of *SU(Z)12* mRNA in these endometrial stromal tumors is under the control of the *JAZF1* promoter, not the *SU(Z)12* promoter. Thus, *SU(Z)12* is activated by translocation in one tumor type (endometrial) and by β-catenin-mediated regulation in another tumor type (colon). Additional investigations are ongoing to understand the mechanisms by which the breast- and liver-tumor-specific increases in *SU(Z)12* mRNA are attained; it is possible that increased binding of β-catenin to the *SU(Z)12* promoter in these tumors types will also be observed.

Other previously identified β-catenin target genes include *fra-1*, *c-jun*, *Myc*, *matrilysin*, *cyclin D1*, *PGHS-2*, and *PPARδ* (15, 16, 23–25). Most of these genes have been identified as β-catenin targets using cell culture experiments in which levels of β-catenin and/or APC have been artificially altered. Although we have shown that the *Myc*, *cyclin D1*, *jun*, and *PPARδ* promoters are bound by β-catenin in colon tumors (Fig. 5 and unpublished data[8]), the other candidate target genes have not yet been validated using an *in vivo* binding assay. It is likely that aspects of the culture conditions used or even the process of establishing cell lines from the tumor samples may alter transcription factor binding profiles. Accordingly, we find that not all β-catenin target promoters show similar binding patterns in all cell types. For example, we detect β-catenin on the *Myc* promoter in both HeLa cells and in colon cancer cell lines. However, β-catenin is bound to the *cyclin D1* promoter only in the colon cancer cell line. Similar to the *cyclin D1* results, we found that β-catenin can be detected on the *SU(Z)12* promoter only in the colon tumor cells. We do not yet understand the molecular mechanisms responsible for mediating the specificity of recruitment of β-catenin to different promoters in different tissues. Possible models include the interchange of β-catenin with γ-catenin or the exchange of β-catenin with a transcriptional repressor such as groucho (49). It is also possible that tissue-specific recruitment of β-catenin may be enhanced by interaction with other site-specific transcription factors.

SU(Z)12 has homology to a group of transcriptional repressors called PcG proteins (3). PcG proteins, along with their counterparts called trithorax group proteins, which act as positive regulators of transcription, are involved in maintaining cellular identity during development and differentiation. The mechanism by which PcG and trithorax group proteins exert their control is via chromatin remodeling. Recent studies have shown that the *Drosophila* ESC-E(Z) chromatin remodeling complex consists of ESC, enhancer of Zeste [E(Z)], NURF-55, and SU(Z)12 (50). The human counterpart of the *Drosophila* ESC-E(Z) chromatin remodeling complex has also recently been purified. This complex, called EED-EZH2 (or *PRC2*), also contains SU(Z)12 (51, 54). Both the *Drosophila* and the human PcG complexes can methylate histone H3 on lysine 27. This methylation was shown to cause transcriptional repression of a target gene, confirming that SU(Z)12 is a component of a transcriptional repression/chromatin remodeling complex.

One hallmark of cancer is the loss of differentiation that occurs as normal cells undergo neoplastic transformation. PcG and trithorax group proteins have previously been found to be dysregulated in tumor cells of hematopoietic origin and it has been postulated that this dysregulation is important in causing and maintaining the neoplastic phenotype. A recent study has shown that EZH2, another component of the EED-EZH2 complex, is up-regulated in prostate cancers (52). Confirming the role of the EED-EZH2 complex in transcriptional regulation, ectopic expression of EZH2 led to transcriptional repression of a set of genes. Importantly, enforced down-regulation of EZH2 led to growth inhibition of a prostate cancer cell line. We have now shown that the mRNA of the PcG protein SU(Z)12 is increased in colon, breast, and liver cancers. We predict that SU(Z)12 target genes will also be deregulated in such cancers. Gene expression profiling of normal *versus* tumor colon tissue revealed 19 mRNAs that are increased at least 4-fold and 47 mRNAs that are decreased at least 4-fold in adenocarcinomas, as compared with normal tissue (53). It is interesting that a large number of genes were shown to be down-regulated in the tumors, given the fact that SU(Z)12 is part of a transcriptional repressor complex. The genes shown to be down-regulated in colon cancer are good initial candidates for SU(Z)12 target genes, as are the genes identified to be repressed by EZH2 (52).

Our future studies will focus on identifying SU(Z)12 target genes, using both a candidate gene approach and global screening methods. The studies that have demonstrated a requirement for EZH2 in the proliferation of prostate cancer cells suggest that targeting SU(Z)12 for inactivation in colon, breast, or liver tumor cells may also lead to the inhibition of proliferation. Therefore, we are also performing a functional

[8] Unpublished observations.

analysis of the SU(Z)12 protein with the goal of eventually designing a SU(Z)12-specific antitumor agent.

Acknowledgments

We thank Jeff Ross (University of Wisconsin, Madison, WI), and Carrie Graveel (University of Wisconsin), for mRNA samples.

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Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27

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Polycomb group (PcG) complexes 2 and 3 are involved in transcriptional silencing. These complexes contain a histone lysine methyltransferase (HKMT) activity that targets different lysine residues on histones H1 or H3 in vitro. However, it is not known if these histones are methylation targets in vivo because the human PRC2/3 complexes have not been studied in the context of a natural promoter because of the lack of known target genes. Here we report the use of RNA expression arrays and CpG-island DNA arrays to identify and characterize human PRC2/3 target genes. Using oligonucleotide arrays, we first identified a cohort of genes whose expression changes upon siRNA-mediated removal of Suz12, a core component of PRC2/3, from colon cancer cells. To determine which of the putative target genes are directly bound by Suz12 and to precisely map the binding of Suz12 to those promoters, we combined a high-resolution chromatin immunoprecipitation (ChIP) analysis with custom oligonucleotide promoter arrays. We next identified additional putative Suz12 target genes by using ChIP coupled to CpG-island microarrays. We showed that HKMT-Ezh2 and Eed, two other components of the PRC2/3 complexes, colocalize to the target promoters with Suz12. Importantly, recruitment of Suz12, Ezh2 and Eed to target promoters coincides with methylation of histone H3 on Lys 27.

[*Keywords*: Suz12; histone methylation; polycomb; Eed; RNA interference; chromatin immunoprecipitation]

Supplemental material is available at http://www.genesdev.org.

Received March 3, 2004; revised version accepted April 27, 2004.

The Polycomb Group (PcG) proteins are negative regulators of transcription that were initially discovered in *Drosophila* (for review, see Jacobs and van Lohuizen 1999; Simon 2003). The PcG proteins, in conjunction with their positively acting counterparts known as Trithorax Group (TrxG) proteins, can maintain heritable transcription patterns of the homeotic (Hox) genes during development and differentiation (for review, see Jacobs and van Lohuizen 1999; Orlando 2003). Accordingly, genetic studies demonstrated that mutations in PcG proteins result in flies having transformed body segments because of inappropriate expression of Hox genes (Jurgens 1985). In addition to regulating developmental decisions, mammalian PcG proteins have been implicated in hematopoiesis, X-chromosome inactivation, and control of cell proliferation (for review, see Jacobs and van Lohuizen 2002). PcG proteins do not function alone but, instead, are assembled into multimeric complexes. The first PcG complex to be biochemically purified was named Polycomb repressive complex 1 (PRC1) and contains the PcG proteins polycomb (PC), polyhomeotic (PH), posterior sex comb (PSC), and dRING, among other polypeptides (Shao et al. 1999). Recent studies have biochemically defined another *Drosophila* polycomb complex, known as PRC2 or ESC-E(Z), whose core subunits are the PcG proteins Extra sex combs (Esc), Enhancer of zeste [E(z)], and Suppressor of zeste 12 [Su(z)12] and the histone-binding protein NURF55 (Czermin et al. 2002; Muller et al. 2002; Tie et al. 2003). Complexes similar to *Drosophila* PRC2 have also been purified from mammalian cells and consist of the human PcG proteins embryonic ectoderm development (Eed), the HKMT-Ezh2, Suz12, and the histone-binding proteins RbAp46 and 48 (Cao et al. 2002; Kuzmichev et al. 2002). Interestingly, four different forms of Eed exist in mammalian cells, and all can interact with Ezh2 and Suz12 resulting in distinct PRC complexes known as PRC2 and PRC3 (Kuzmichev et al. 2004; Pasini et al. 2004).

The PcG complexes have been proposed to control gene activity via transcriptional repression. Recently, in

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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1200204.

vitro assays have provided insight concerning the mechanism underlying PcG-mediated transcriptional repression. Characterization of the PRC2/3 complexes indicates that each complex contains an intrinsic histone lysine methyltransferase (HKMT) activity that is mediated by the SET [Su-(var)3–9;E(z);Trithorax] domain of Ezh2 (Kuzmichev et al. 2002). In vitro, the Ezh2 protein within the PRC2/3 complexes can methylate Lys 9 (H3-K9) and Lys 27 (H3-K27) of histone H3 and Lys 26 of histone H1 (H1-K26), depending on whether the oligonucleosomes contain histone H1 (Kuzmichev et al. 2004). The efficiency with which the different histones (H1 or H3) are methylated depends on the specific form of Eed present in the complex (Kuzmichev et al. 2004). The PRC2 complex contains the longest form of Eed (Eed1) and methylates both H1-K26 and H3-K27. However, PRC2 preferentially methylates H1-K26 when nucleosome arrays contain histone H1. The PRC3 complex, which contains the two shortest forms of Eed (Eed3/4), methylates H3-K27. The intermediate form of Eed (Eed2) is also present in yet another distinct PRC complex (A. Kirmizis, R. Margueron, A. Kuzmichev, P. Farnham, and D. Reinberg, unpubl.). Based on these and previous discoveries, a model for the mechanism of PcG-mediated transcriptional silencing has been proposed. The PRC2 or PRC3 complex is thought to first catalyze the addition of methyl groups to H3-K27 that serve as signals for the recruitment of PRC1. Binding of PRC1 is then proposed to either block the recruitment of transcriptional activating factors, such as SWI/SNF (a Trithorax complex), facilitating the establishment of a stable, repressive chromatin structure or to prevent transcription initiation by prebound factors (Simon 2003; Dellino et al. 2004).

Although an attractive model, it has not yet been demonstrated that the recruitment of HKMT-containing PRC complexes results in histone modification in mammalian cells owing to the fact that no target genes for these complexes have been identified. In contrast, target genes for PcG complexes have been identified in *Drosophila*. In the fruit fly, PcG target genes are regulated by specific DNA regions called Polycomb response elements (PREs; Simon et al. 1993). PREs are ~2–3 kb long and are composed of multiple different DNA motifs (Bloyer et al. 2003). Usually PREs contain binding sites for GAGA and PHO, two site-specific DNA-binding proteins that interact with PcG proteins (Poux et al. 2001) and function to recruit *Drosophila* PcG complexes to DNA (for review, see Pirrotta et al. 2003). Mammalian PREs have not yet been identified, and therefore, it remains unclear how mammalian PcG complexes are recruited to chromatin to regulate expression of specific target genes. Previous reports have demonstrated an interaction between PcG proteins and members of the general transcriptional machinery, suggesting that binding of PcG complexes to promoters may be stabilized by these interactions (Breiling et al. 2001; Saurin et al. 2001). However, it is likely that a HKMT-containing PRC complex must first be recruited by a sequence-specific DNA-binding protein.

Because none of the HKMT-containing PRC complexes contain a site-specific DNA-binding protein, target genes cannot be identified using a bioinformatics approach to search for consensus binding sites in the genome. Therefore, we have used two global screening methods to identify genes that are regulated by mammalian PRC2/3 complexes. First, using oligonucleotide cDNA arrays, we identified genes whose expression changes upon siRNA-mediated depletion of Suz12. Second, we used a combination of chromatin immunoprecipitation and genomic microarrays to identify promoters bound by Suz12. Interestingly, we show that multiple components of the PRC2/3 complexes colocalize on these newly identified target genes and that binding of these proteins to target genes correlates with methylation of H3-K27.

Results

Suz12 protein levels are increased in human colon tumors

Although *Drosophila* PcG proteins have been mostly implicated in transcriptional control during development, human PcG proteins have been linked to cancer development. For example, previous studies have demonstrated that Ezh2 is overexpressed in various human cancers (Varambally et al. 2002; Bracken et al. 2003; Kleer et al. 2003). Additionally, in endometrial stromal tumors, a fragment of the *Suz12* gene is frequently fused to a second gene encoding a zinc finger protein (Koontz et al. 2001). In our previous studies, we have observed up-regulation of Suz12 mRNA in tumors of the colon, breast, and liver (Kirmizis et al. 2003). Specifically, we found that eight of nine colon cancer patients tested had high levels of Suz12 mRNA in the tumor tissue as compared with the normal tissue. Furthermore, we showed that the up-regulation of Suz12 mRNA is mediated by the TCF/β-catenin transcription complex (Kirmizis et al. 2003), whose increased activity is responsible for the development of the majority of human colon cancers (for review, see Bienz and Clevers 2000). The fact that Suz12 mRNA is present at high levels in colon tumors suggests that colon cancer provides a good model system to identify PRC target genes. However, it was first necessary to determine if the increased Suz12 mRNA expression in colon tumors correlates with increased Suz12 protein levels. We developed an antibody against the Suz12 protein and demonstrated that it recognizes the correct protein, using cells transfected with a plasmid expressing an HA-tagged Suz12. Western analysis showed that the HA antibody recognized a 93-kDa protein in cells transfected with HA-Suz12 but not in cells transfected with the empty vector (Fig. 1A, left panel). The purified Suz12 antibody recognized the same protein in both transfected cell populations (Fig. 1A, right panel). This result is expected because the endogenous protein runs the same size as the HA-tagged protein. We next analyzed whole-tissue extracts from five different colon cancer patients for Suz12 protein levels. As shown in Figure 1B, four out of five samples showed increased levels of Suz12 in the

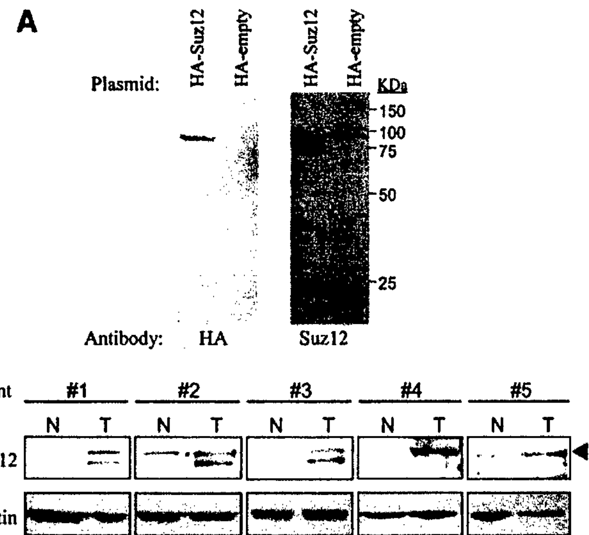

Figure 1. Suz12 protein expression is elevated in human colon tumors. (*A*) Western blot analysis to demonstrate the specificity of the Suz12 antibody. SW480 cells were transfected with an HA-Suz12 or a control (HA-empty) construct and subjected to immunoblot analysis. Two separate blots were prepared and probed with either an HA antibody (*left* panel) or a Suz12 antibody (*right* panel). The HA antibody recognizes only the HA-Suz12 protein and the Suz12 antibody detects both the HA-Suz12 and endogenous Suz12 proteins. (*B*) Western blot analysis of whole-tissue extracts prepared from normal (N) and tumor (T) tissues of five different colon cancer patients. The blot was first probed with an anti-Suz12 antibody and then reprobed with an anti-actin antibody as a loading control. The arrowhead points to the band with the correct size of Suz12 (93 kDa). A second lower band, detected with the Suz12 antibody in patients 1–3, probably represents a protein degradation product.

tumor tissue as compared with adjacent normal tissue. One patient had high Suz12 levels in both the normal and tumor tissues, and, therefore, it is possible that the normal sample was contaminated with tumor tissue. These findings are in agreement with our previous results showing up-regulation of Suz12 mRNA in colon tumors. In three of our extract preparations, the antibody recognized the 93-kDa band plus a lower band of ~85 kDa. This lower band has also been detected by others using a different Suz12 antibody (Tie et al. 2003). It is possible that the lower band represents a Suz12 degradation product that is produced during extraction of the tumor tissue because we have not detected it in nuclear extracts prepared from cell lines (data not shown).

Depletion of Suz12 in colon cancer cells results in alteration of gene expression

Recent studies have shown that Suz12 is a component of the PRC2/3 complexes (Cao et al. 2002; Czermin et al. 2002; Kuzmichev et al. 2002, 2004; Muller et al. 2002), suggesting that up-regulation of Suz12 in colon cancers may modulate the tumor phenotype by modifying histones and thus changing gene expression profiles. To examine this hypothesis, we used RNA interference (RNAi) to decrease Suz12 expression in colon cancer cells. We designed small interfering RNAs (siRNAs) to target the Suz12 transcript and tested them using two human colon cancer cell lines (Fig. 2; data not shown). We observed a decrease in both Suz12 mRNA and protein levels by 24 h after transfecting cells with the Suz12 siRNAs (Fig. 2A,B, cf. lanes 1 and 4). The down-regulation of Suz12 was more prominent after 72 h of transfection (Fig. 2A,B, cf. lanes 3 and 6). Lamin A/C and green fluorescent protein (GFP) siRNA duplexes did not affect the amounts of Suz12 verifying the specificity of the RNAi approach. We next examined the phenotype of the colon cancer cells having reduced Suz12 expression. We did not detect any changes in the morphology or the cell cycle profile of the cells after treatment with Suz12 siRNAs for 72 h (data not shown). However, RNAi does not eliminate Suz12 expression entirely from the cells, and the remaining Suz12 may be sufficient to mask any obvious phenotypes, especially in a short-term assay. It is also possible that certain functions of Suz12 in these cells are redundant with another protein, and, therefore, removing only Suz12 is not enough to cause changes in cell proliferation.

To determine whether short-term removal of Suz12 from cells results in altered transcription profiles, we

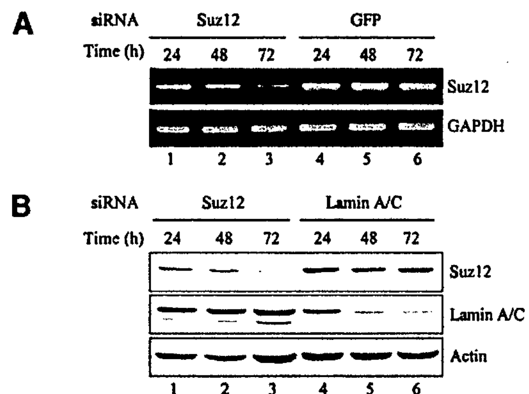

Figure 2. Suz12 expression in colon cancer cells is disrupted by RNA interference. (*A*) RT–PCR analysis on total RNA extracted from SW480 cells at various time points after transfection with Suz12 or GFP siRNA duplexes. Primers specific to the GAPDH mRNA were used in RT–PCR to ensure that the RNA was correctly quantitated. (*B*) Western blot analysis on whole-cell extracts prepared from SW480 cells at various time points after transfection with Suz12 or Lamin A/C siRNA duplexes. Antibodies against Suz12 and Lamin A/C were used to show specific depletion of the respective proteins. The blot was also probed with the actin antibody to demonstrate equal loading of protein samples.

performed RNAi experiments and monitored global gene expression using oligonucleotide microarrays. We transfected SW480 colon cancer cells with either Suz12 or GFP siRNAs and harvested total RNA after 48 h. Although depletion of Suz12 was best at 72 h (Fig. 2), we chose to monitor expression at the 48-h time point to minimize indirect effects of the Suz12 down-regulation. Three independent experiments were performed with the Suz12 siRNAs or the GFP siRNA, and the three RNA preparations from each treatment were pooled into one sample to reduce the experimental variation. The pooled Suz12-siRNA and GFP-siRNA samples were then labeled with a fluorescent dye and hybridized onto four separate Affymetrix U133A gene chips (Fig. 3A). Using the default settings on the microarray analysis software, we selected the genes whose expression was significantly altered by loss of Suz12. We found 46 up-regulated transcripts (representing 35 unique genes) and 23 down-regulated transcripts (representing 15 unique genes) in cells having reduced Suz12 levels (Fig. 3B). A list of the UniGene IDs and fold changes of the 46 up-regulated and 23 down-regulated mRNAs is provided as Supplemental Material (Supplementary Table S1). Two of the transcripts that were down-regulated correspond to Suz12 and thus verify the RNAi results obtained using RT–PCR (Fig. 2B). To confirm the results obtained in the microarray analyses, we selected 14 genes that represented the entire range of the fold changes (both up and down) observed. RNA was prepared from cells treated with siRNAs either to Suz12 or to GFP, using samples independent from those used for the microarray analyses. RT–PCR analysis demonstrated that the genes identified on the arrays show the expected response to the Suz12 siRNA treatment in the independent experiment (Fig. 3C).

Suz12 binds directly to promoters

The experiments described above show that down-regulation of Suz12 alters the expression level of multiple

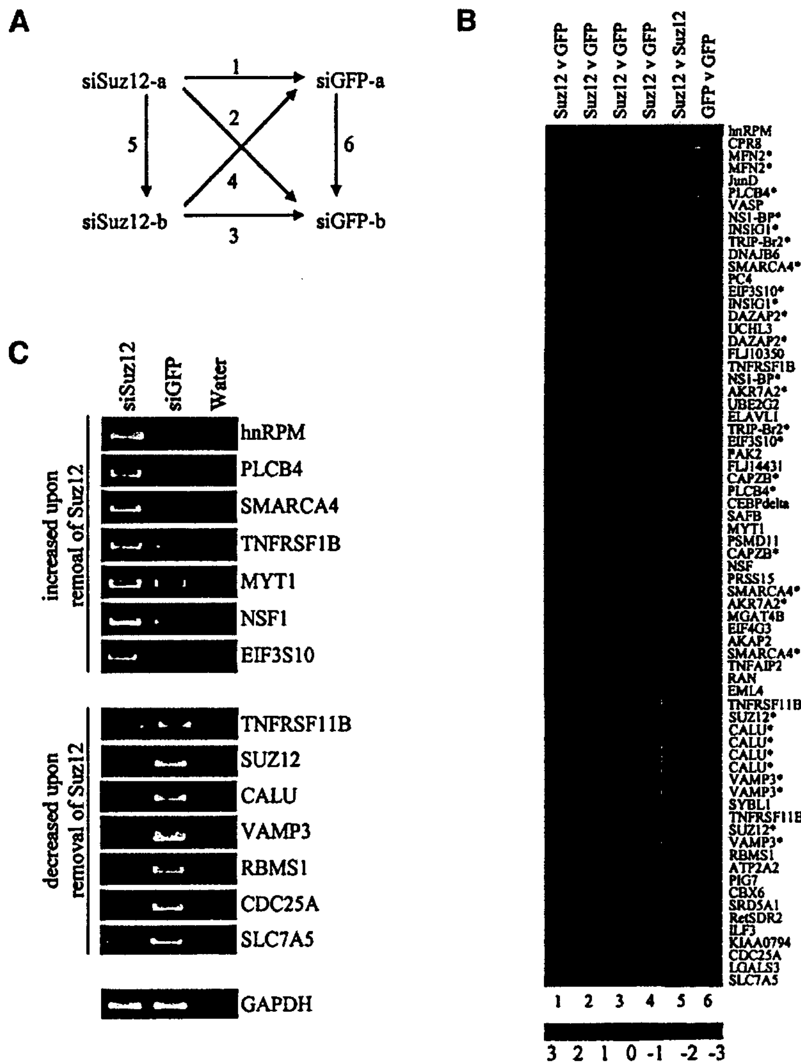

Figure 3. Removal of Suz12 from SW480 cells results in alteration of gene expression. (*A*) Schematic showing the comparisons among the four Affymetrix U133A arrays that were used to analyze gene expression in cells incubated with either Suz12 or GFP siRNAs. The numbers on the arrows correspond to the columns in *B*. Columns 1, 2, 3, and 4 represent siSuz12 versus siGFP comparisons and thus allow identification of up- and down-regulated genes. Columns 5 and 6 are comparisons of identical samples; these should show no variation and were used to identify false positives. (*B*) Tree-view diagram depicting the genes that were significantly deregulated upon Suz12 depletion. Red represents up-regulated genes, and green represents down-regulated genes in the Suz12 RNAi samples in reference to the GFP RNAi samples. The numbers on the color scheme represent the fold changes in gene expression. Genes denoted with an asterisk (*) were identified more than once on the microarrays. (*C*) RT–PCR analysis of independent RNAi experiments confirming the results obtained using the microarrays. Primers specific to GAPDH mRNA were used in RT–PCR to ensure that the RNA was correctly quantitated.

genes. However, these findings do not demonstrate if Suz12 controls the expression of these genes directly by binding to their promoters. It is possible that removal of Suz12 from the cells changes the expression of these genes through an indirect mechanism. Therefore, we used chromatin immunoprecipitation (ChIP) to investigate whether Suz12 is recruited to the promoters of the deregulated genes. Because Suz12 is a component of transcriptional repressive complexes, we focused our initial investigation on the genes whose expression increased upon depletion of Suz12 from the cells. We performed ChIP experiments in SW480 cells using antibodies to Suz12, RNA polymerase II (Pol II), and a nonspecific IgG control. Using PCR primers that spanned the transcriptional start site, we monitored binding of Suz12 and Pol II to six promoters that drive expression of mRNAs that were up-regulated upon loss of Suz12. Two of the genes, Myelin transcription factor 1 (*MYT1*) and *N-ethylmaleimide-sensitive factor* (*NSF*), are expressed from two different transcriptional start sites separated by several kilobases. Therefore, we monitored binding of Suz12 to both the proximal and distal promoter regions of these two genes. Suz12 showed robust binding to the distal promoter region of *MYT1* but not to the proximal promoter region (Fig. 4, lane 3). We did not detect strong binding of Suz12 to the distal or the proximal promoter region of the *NSF* gene or at the other four tested promoters. Studying specific protein/DNA interactions through ChIP assays requires the use of a highly specific antibody. The specificity of the Suz12 antibody was first

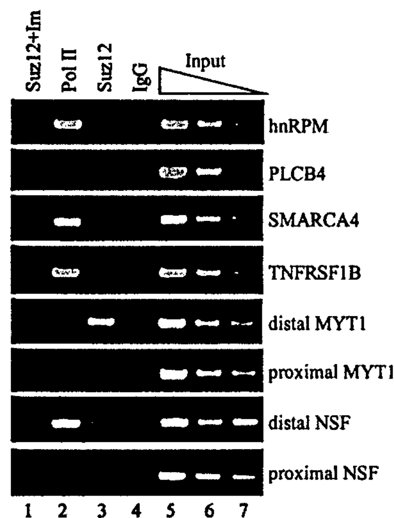

Figure 4. The *MYT1* promoter is directly regulated by Suz12. Chromatin immunoprecipitation experiments were performed in SW480 cells using antibodies to RNA Polymerase II (lane *2*), Suz12 (lane *3*), and a control IgG (lane *4*). A control immunoprecipitation was also performed with a Suz12 antibody that was preincubated with the Suz12 immunogen at a 10-fold excess by weight (lane *1*). Three dilutions of the total input are also shown (lanes *5*–*7*). The precipitated chromatin was analyzed with PCR using primers specific to the promoters of the indicated genes.

illustrated by the fact that it recognized a single protein band by immunoblot analysis in nuclear extract preparations of cultured cells (data not shown) and by the fact that the protein detected on the Western blot is diminished after introduction of Suz12 siRNAs to the cells (Fig. 2B). To further demonstrate the specificity of the Suz12 antibody, we performed a ChIP assay in which the antibody was preincubated with the Suz12 immunogen (a fragment of Suz12 protein used to develop the antibody) at a 10-fold excess by weight. The immunogen abrogated the ability of the Suz12 antibody to enrich the *MYT1* distal promoter (Fig. 4, lane 1). Collectively, these results demonstrate that the Suz12 antibody used in the ChIP experiments specifically enriches selected chromatin fragments. Therefore, the ChIP analysis demonstrated that *MYT1* expression is directly regulated by Suz12, whereas expression of the other five tested genes may be indirectly regulated by Suz12.

The above ChIP experiments allowed the identification of *MYT1* as the first direct target gene of Suz12. However, similar analysis for detecting Suz12 binding to the remaining genes identified in the gene expression microarrays would require preparation of numerous PCR primers. Additionally, the lack of any known *cis* element responsible for recruiting Suz12 to chromatin does not allow us, by sequence examination, to determine where to place the primers for optimal detection of Suz12 on these loci. To circumvent these caveats, we combined the ChIP analysis with custom oligonucleotide promoter microarrays. The custom arrays contained probes spaced at a high density that represented 5-kb promoter regions (4 kb upstream to 1 kb downstream relative to the transcriptional start site) of all the up-regulated and down-regulated genes that were identified in the gene expression study described above. This combination of ChIP with custom promoter arrays will identify the genes that are directly regulated by Suz12 and precisely localize the binding of Suz12 to these promoters in a high-throughput manner. We first performed a standard ChIP assay in SW480 cells using the Suz12 antibody and a nonspecific IgG as a control. The immunoprecipitated chromatin was examined using *MYT1* promoter primers to ensure specific enrichment of Suz12-bound DNA fragments (data not shown). We next prepared amplicons of ~500 bp from the Suz12- and IgG-immunoprecipitated chromatin samples as well as from an input chromatin reference sample (see Fig. 7C, right panel [below]). The amplicons were labeled with a fluorescent dye and hybridized onto separate custom promoter arrays. To identify the genes that are directly regulated by Suz12, we compared the hybridization intensities between the Suz12 array and the input array and calculated a Suz12/Input ratio for each oligonucleotide probe. Regions with a Suz12/Input ratio higher than 2 were considered positive for Suz12 binding. To eliminate from consideration promoter regions that are enriched nonspecifically during the ChIP procedure, we also calculated an IgG/Input ratio for each oligonucleotide probe. We performed three independent analyses and found eight promoters that were repeatedly enriched by the Suz12 antibody suggesting that those

genes are directly regulated by Suz12 (Table 1). Profiles from a representative experiment of four of the eight positive promoters are shown in Figure 5. Use of the high-density oligonucleotide arrays allowed for more precise localization of Suz12 recruitment to its target loci than did the standard ChIP experiments. The small size of the amplicons in combination with the high density of the custom oligonucleotide arrays mapped the binding site of Suz12 within 500-bp regions. The results from these ChIP-chip experiments were validated in independent ChIP experiments using PCR primers that spanned the regions showing the highest enrichment by the Suz12 antibody (Fig. 5, inserts). As expected, analysis of the *MYT1* promoter showed specific recruitment of Suz12. Interestingly, Suz12 binding to *MYT1*, as well as to some of the other promoters, occurred at two distinct promoter regions; one near the transcriptional start site and one farther upstream (e.g., MYT1 and SYBL1). The two occupied regions on these promoters were separated by several hundred bases, suggesting that Suz12 may associate with the transcriptional start site via a DNA looping mechanism mediated by protein–protein interactions that is initiated by specific binding of Suz12 to an upstream region. Such a looping mechanism has been proposed to mediate the interaction of *Drosophila* PcG proteins with their target promoters (Orlando et al. 1998). However, it remains possible that Suz12 is recruited specifically to the region near the start site via a site-specific DNA-binding protein. Interestingly, we observed direct recruitment of Suz12 to genes that were

Table 1. *Confirmed Suz12 target genes*

No.	Gene name		Position(s) of enrichment
Targets identified by oligonucleotide expression arrays			
Genes repressed by SUZ12			
1	EIF3S10	Eukaryotic translation initiation factor 3	–500
2	*MYT1*	Myelin transcription factor	–1700 and +1
3	PSMD11	Proteasome 26S subunit non-ATPase 11	–1000 and +500
4	MGAT4B	Mannosyl glycoprotein acetylglucosaminyltransferase 4B	–3500 and –1000
5	EML4	Echinoderm microtubule-associated-protein-like 4	–500
Genes activated by SUZ12			
6	SYBL1	Synaptobrevin-like 1	–2000 and +1
7	SRD6A1	Steroid-5-α-reductase, α polypeptide 1	+1
8	RBMS1	RNA-binding motif single-stranded interacting protein 1	–2000
Targets identified by CpG island arrays			
1	DMRTA2	Doublesex-mab-3 (DM) domain-like family A2	–4000 and –2000
2	KCNA1	Potassium voltage-gated channel	–2000
3	LOC119392	Hypothetical protein	+1
4	MFAP1	Microfibrillar-associated protein	+1
5	LOC115811	Similar to RIKEN cDNA 4933433C09	+200
6	CNTFR	Ciliary neurotrophic factor receptor	–2000, –1000, and +1
7	KIAA0254	Sorting nexin 19	–1000
8	TRIM52	Tripartite motif-containing 52	+1
9	RDC1	G-protein-coupled receptor	+1
10	WNT1	Wingless-type MMTV integration site member 1	–2000 and +500
11	KIF2C	Kinesin family member 2C	+1
12	HSPC228	Chromosome 6 open reading frame 55	+1
13	KIS	Kinase interacting with stathmin	+1
14	SLC25A3	Solute carrier family 25 member 3	+300
15	NKX2C	NK2 transcription factor related lcous 3	+600
16	AK056349	Cholinergic receptor, muscarinic 3	–200
17	SRP9	Signal recognition particle 9KDa	+1
18	NUDT2	Nudix-type motif 2	–3000 and +1
19	CNR1*	Cannabinoid receptor 1	+1
20	RBMS1 3′-end	RNA-binding motif single-strand interacting protein	+95000

The position of enrichment indicates the location of Suz12 binding in the promoter relative to the transcriptional start site, which is represented by +1. The top part indicates the genes that were identified by the gene expression analysis (Fig. 3), and the bottom part indicates the genes that were identified by the ChIP-CpG-island arrays (Fig. 6). For the genes identified using the gene expression analyses (Fig. 3), the region spanning from –4 kb to +1 kb relative to the start site was represented on the custom oligonucleotide arrays. Most of the CpG islands detected by the ChIP-CpG analyses (Fig. 6) corresponded to promoter regions. For these cases, –4 kb to +1 kb relative to the start site was analyzed on the custom arrays. However, in some cases the CpG island was located 3′ of a gene (e.g., *RBMS1*). In these cases, a 5-kb region surrounding the CpG clone was represented on the arrays. The *CNR* promoter, indicated with an asterisk (*), was not included in the custom oligonucleotide arrays, but Suz12 binding to that promoter was confirmed by PCR analysis.

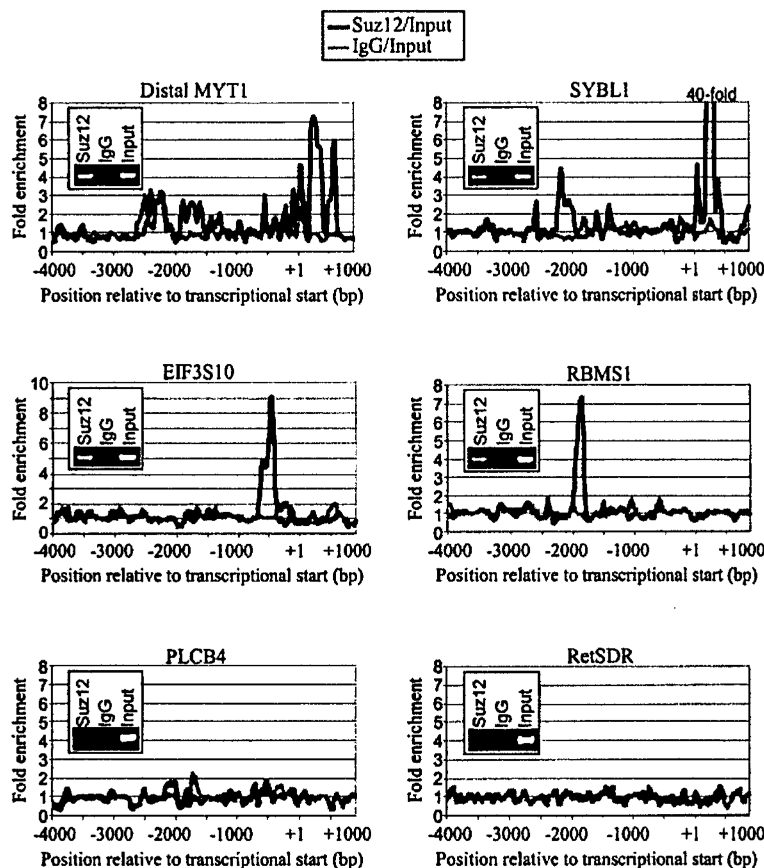

Figure 5. Mapping of Suz12 binding to its target promoters. Oligonucleotide microarrays containing probes that represent a 5-kb region from each promoter of the identified Suz12-regulated genes (Fig. 3B) were prepared. The arrays were hybridized with amplicons prepared from ChIP experiments using a Suz12 antibody, a nonspecific IgG antibody, and an input control. Shown are six promoter regions: three that were up-regulated (*MYT1, EIF3S10, PLCB4*) upon removal of Suz12 from cells and three that were down-regulated (*SYBL1, RBMS1, RetSDR*) upon removal of Suz12 from cells. The fold enrichment was calculated by dividing the Suz12 or IgG hybridization intensity signal by the input control signal for each oligonucleotide probe. The inserts within each graph show independent ChIP confirmation using PCR analysis. The primers used in the PCR analysis were designed to span the region showing the highest peak of enrichment for each promoter. A complete list of the Suz12-bound promoters and the position of binding is shown in Table 1.

up-regulated (MYT1 and EIF3S10) as well as down-regulated (SYBL1 and RBMS1) after depletion of Suz12 from SW480 cells, indicating that Suz12 might have a role in both transcriptional repression and activation. The majority of the genes tested did not show Suz12 binding (e.g., *PLCB4* and *RetSDR*), suggesting that expression of those genes is indirectly regulated by Suz12. However, it remains possible that Suz12 binds to a region farther upstream than −4 kb or farther downstream than +1 kb in these promoters.

Identification of additional Suz12 target promoters using CpG-island microarrays

Although the experiments described above identified a small set of direct target genes of Suz12, they did not identify numerous high-affinity binding sites. As noted above, this could be caused by the fact that most of the genes identified in the siRNA experiments are indirectly regulated by Suz12. Therefore, to identify additional Suz12 targets, we used a ChIP-CpG-island microarray technique that has previously been used in our laboratory to identify genomic loci bound by E2F family members (Weinmann et al. 2002; Oberley et al. 2003; Wells et al. 2003). This technique will identify direct Suz12 targets that are within several kilobases of the CpG islands present on the microarray. Using the Suz12 antibody and preimmune (PI) serum as a control, we performed a standard ChIP assay in SW480 cells. We next prepared amplicons from the Suz12- and PI-immunoprecipitated chromatin samples as well as from an input chromatin reference sample. The amplicons from the immunoprecipitated samples were labeled with one fluorescent dye, whereas the reference sample (input) was labeled with a different fluorescent dye. To identify Suz12-bound loci, we applied the Suz12 and input amplicons to an array containing ~12,000 CpG islands. We selected all the CpG clones that had intensity signals from the Suz12 chromatin at least threefold higher than the input control (Fig. 6A). To control for CpG clones that were enriched nonspecifically by the ChIP procedure, we hybridized a second array with the preimmune versus input amplicons. CpG islands that were enriched by the preimmune serum were discarded from further analysis. This procedure was repeated twice, and 52 CpG clones were selected as positives. Next, we used the University of California-Santa Cruz Genome Browser (http://genome.ucsc.edu) to determine the genomic location of all the CpG clones. Out of 52 clones, 41 contained sequences that matched to locations near promoters or 3′-ends of known and predicted genes (Supplementary Table S2). Because there is some degree of redundancy of the CpG islands printed on the arrays, three gene pro-

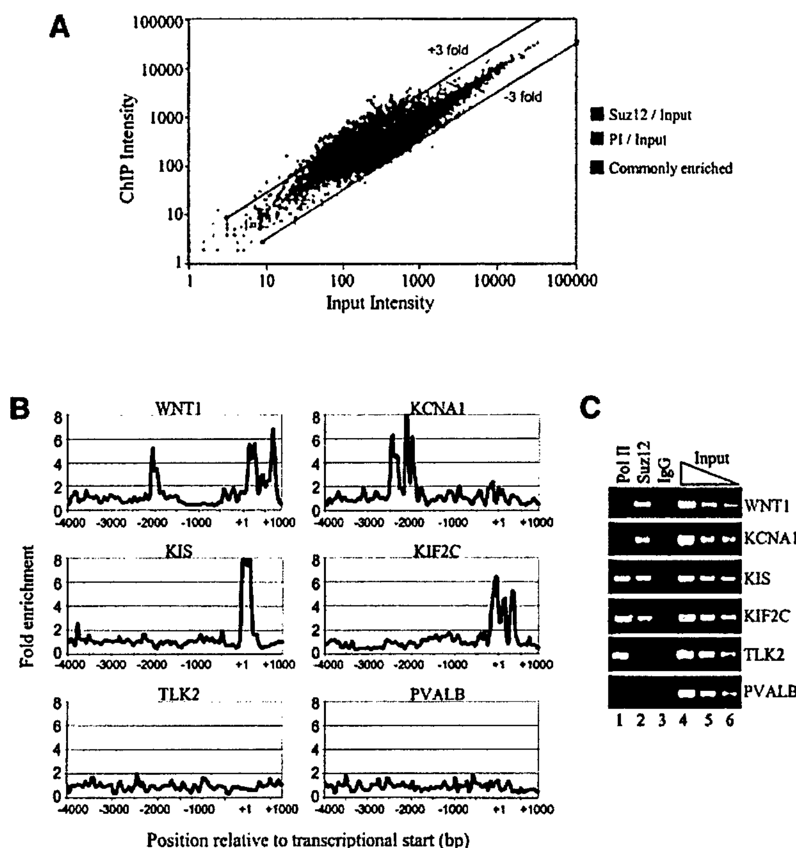

Figure 6. Identification of additional direct Suz12 target genes. (*A*) Scatter plot showing the intensities of the spots on the CpG-island microarrays. Two independent experiments were performed to identify Suz12-bound loci in SW480 cells. In red are the spot intensities from a representative array that was hybridized with Suz12 and input amplicons. Shown in green are the spot intensities of a representative array that was hybridized with IgG and input amplicons. Red spots that enriched at least threefold were selected for further analysis. The spots shown in blue were enriched at least threefold in both the Suz12 and IgG control immunoprecipitations and represent DNA fragments that precipitate nonspecifically in the ChIP procedure. (*B*) Confirmation and mapping of Suz12 binding to a 5-kb region of each promoter identified in *A* was performed using custom oligonucleotide promoter arrays. The arrays were prepared and hybridized as in Figure 5. Shown are the fold enrichments from the Suz12 and input comparisons of six promoters: four true positive targets and two (*TLK2* and *PVALB*) false positives. (*C*) Confirmation of the results shown in *B* using PCR analysis. Independent ChIP experiments were performed in SW480 cells using antibodies to RNA Polymerase II (lane *1*), Suz12 (lane *2*), and a control IgG (lane *3*). Three dilutions of the input chromatin are also shown (lanes *4–6*). The precipitated chromatin was analyzed with PCR using primers specific to the promoters of the indicated genes.

moters were identified twice as unique clones. Also, three clones contained sequences that matched to two different gene promoters. Probably during the construction of the CpG-island library, DNA fragments representing different CpG islands were introduced in tandem within one clone. Additionally, two CpG clones did not match to any genomic loci, three matched to sequences that were not near annotated genes, and six matched to repeat elements; these 11 clones are not listed in Supplementary Table S2.

To determine if the identified CpG islands are real targets of Suz12 and to precisely map the binding of Suz12 to the CpG islands, we again coupled the ChIP assay with custom oligonucleotide promoter arrays. These custom arrays represented 5-kb regions of the promoters of all the genes that were associated with the identified CpG islands. Using the same triplicate preparations of Suz12, IgG, and Input amplicons used in the previous experiments, we hybridized the custom promoter arrays; the results are summarized in Table 1. Of the 41 promoters represented on the arrays, 20 showed robust binding of Suz12 (Table 1). As shown in Figure 6B, some promoters showed enrichment at multiple loci (such as *WNT1*), whereas others were bound by Suz12 only at one locus (e.g., *KCNA1*, *KIS*, and *KIF2C*). The remaining 21 promoters that did not bind to Suz12, such as *TLK2* and *PVALB*, either represent false-positive clones selected in the CpG-island array experiments or are bound by Suz12 outside the 5-kb region that was printed on the custom promoter arrays (e.g., at +2 kb). The results obtained using the custom promoter arrays were further validated in independent ChIP experiments using PCR analysis (Fig. 6C). ChIP analysis demonstrates that two of the promoters show robust binding of Suz12 and almost no binding of Pol II, a pattern similar to that seen on the *MYT1* distal promoter. Interestingly, two other promoters show moderate binding of Suz12 and robust binding of Pol II. Finally, two of the genes show no binding of Suz12.

PRC-mediated repression of Suz12 target genes is associated with methylation of H3-K27

Recent studies have shown that Suz12 is a component of two different PRC complexes, each of which contains Ezh2, an HKMT that is associated with transcriptional silencing, and at least one form of Eed (Kuzmichev et al. 2004). Therefore, we examined binding of Suz12, Ezh2, and Eed along a 5-kb region around the distal *MYT1* promoter (Fig. 7A). For these experiments, we generated am-

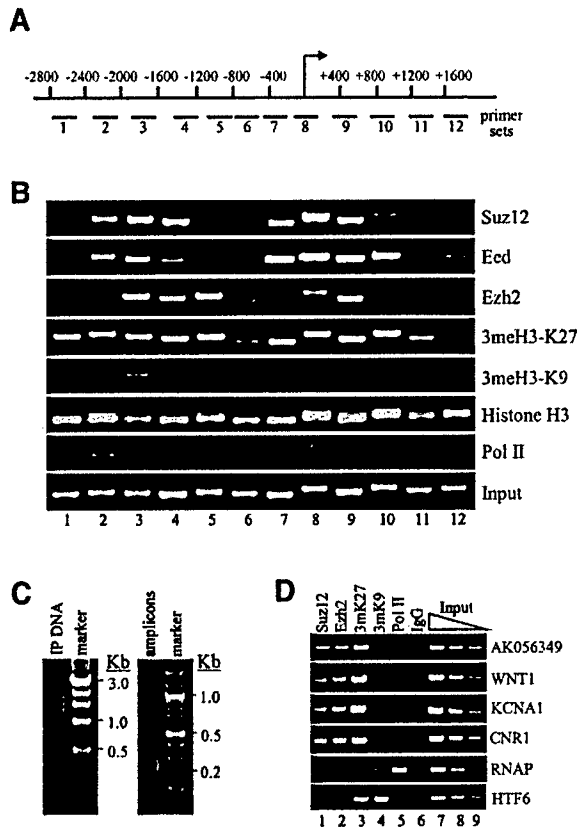

Figure 7. The Suz12 target promoters are bound by other PRC2/3 components and are methylated at Lys 27 of histone H3. (*A*) A schematic of the distal *MYT1* promoter locus is shown; the positive and negative numbers indicate the position in the promoter relative to the transcriptional start site represented by the arrow. The lines *below* the *MYT1* locus represent the promoter fragments amplified by the different PCR primer pairs. (*B*) ChIP experiments were performed in SW480 cells using antibodies to Suz12, Eed, Ezh2, tri-methyl H3-K27, tri-methyl H3-K9, histone H3, and RNA Polymerase II. The immunoprecipitated chromatin from each ChIP sample was used to prepare amplicons by LM–PCR as described in Materials and Methods. The amplicons were then analyzed by PCR. The number of each lane corresponds to the primer pair indicated in *A*. Amplicons prepared from input chromatin were used as a positive control. (*C*) Shown are examples of the size of DNA from a ChIP experiment (*left* panel) and amplicons prepared from a typical LM–PCR reaction (*right* panel). (*D*) ChIP experiments in SW480 cells using the same antibodies as in *B*. A nonspecific IgG antibody (lane 6) was used as a negative control, and three dilutions of the input chromatin (lanes 7–9) were used as positive controls to demonstrate linear amplification. The immunoprecipitated chromatin was analyzed by PCR using primers specific to the promoters of the genes indicated.

plicons from ChIP samples by LM–PCR and used them in conventional PCR using promoter-specific primers. This type of analysis allows precise localization of the binding of a factor to a promoter region because, as shown in Figure 7C, amplicons have a smaller range in size (200–500 bp) as compared with immunoprecipitated DNA fragments (1–3 kb) isolated from a standard ChIP assay. Using this analysis, we showed that Suz12 binds to two regions in the 5-kb sequence; one near the distal transcriptional start site and one several kilobases upstream (Fig. 7B). These results are in complete agreement with our findings from the experiments using the custom oligonucleotide arrays (cf. Figs. 7B and 5). Using this high-resolution PCR analysis, we also found that Ezh2 and Eed colocalize with Suz12 on the *MYT1* promoter, confirming that *MYT1* is a target gene of the PRC complexes (Fig. 7B). To determine if the recruitment of the PRC complexes correlates with the HKMT activity of Ezh2, we next examined the methylation of H3-K27 at the 5-kb region of the *MYT1* promoter. We show that binding of Suz12, Ezh2, and Eed to the *MYT1* promoter correlates with, but does not precisely overlap, trimethylation of H3-K27. This result suggests that the PRC complexes can methylate histones located at the PRC-binding sites and in the surrounding region. Interestingly, no methylation of H3-K9 was detected at the repressed *MYT1* locus, indicating that in vivo PRC-mediated silencing is associated with trimethylation of Lys 27 but not Lys 9 of histone H3 (Fig. 7B), as previously suggested (Kuzmichev et al. 2002). The results of the siRNA experiments (demonstrating increased MYT1 mRNA in the absence of Suz12) and ChIP experiments (demonstrating methylation of H3-K27) suggest that the *MYT1* promoter is kept in a repressed state by its association with the PRC2/3 complexes. Interestingly, we could not detect binding of Pol II to any location within the 5-kb sequence surrounding the *MYT1* distal promoter. This lack of Pol II suggests a model for the PRC-mediated regulation of the *MYT1* promoter; first, recruitment of PRC2/3 causes methylation of H3-K27, which then results in the formation of a chromatin structure that is incompatible with binding of Pol II, and thus the formation of a transcription initiation complex is prevented.

The robust Suz12 and weak Pol II signals seen on four of the promoters identified on the CpG-island arrays (Fig. 6C; data not shown) suggest that they may be regulated by a PRC complex in a manner similar to that of the *MYT1* promoter. To test this hypothesis, we examined binding of Ezh2 to the *AK056349*, *Wnt1*, *KCNA1*, and *CNR1* promoters. As shown in Figure 7D, Ezh2 is recruited to all of these Suz12 target promoters. Interestingly, binding of Suz12 and Ezh2 correlates with methylation of H3-K27, but not of H3-K9, at these four promoters. To demonstrate the specificity of our antibodies, we analyzed the methylation status of two other promoters, one active (the largest subunit of *Pol II*) and one inactive (the pericentromeric gene *HTF6*), in SW480 cells. As expected, the *Pol II* promoter was bound by Pol II but not by Suz12 or Ezh2 and showed no methylation of H3-K27 or H3-K9. In contrast, the silenced pericentromeric *HTF6* promoter was characterized by methylation of H3-K27 and H3-K9. Methylation of H3-K27 at the *HTF6* promoter may be mediated by a methyltransferase activity other than that of the PRC complexes because Suz12 and Ezh2 were not bound to the promoter (Fig. 7D). Alternatively, the kinetics of binding of an

Ezh2-containing complex to this promoter may be different from the other promoters studied.

Our RNAi experiments described above showed that removal of Suz12 from colon cancer cells can result in increased expression of some target genes. Because of the correlation between Suz12 binding and H3-K27 methylation, we hypothesized that the increased expression is caused by the loss of the histone methyltransferase activity after depletion of Suz12 from cells. To test this hypothesis, we examined the binding pattern of Ezh2 and the status of H3-K27 methylation on five target promoters after siRNA-mediated removal of Suz12 from SW480 cells. We incubated cells with Suz12 siRNAs for 6 d, and then we performed ChIP assays using antibodies to Suz12, Ezh2, trimethyl H3-K27, and IgG as a control. A 6-d treatment was performed to allow for a greater loss of the methylated histones from the target promoters. As expected, we found that depletion of Suz12 from cells decreases the binding of Suz12 to the tested promoters (Fig. 8, cf. lanes 1 and 7). Interestingly, depletion of Suz12 from cells also resulted in a dramatic decrease of Ezh2 binding and H3-K27 methylation at the Suz12 target promoters. (Fig. 8, cf. lanes 2 and 8, and lanes 3 and 9). These results demonstrate that repression of the identified Suz12 target promoters is associated with methylation of H3-K27. Also, these data show that the decreased expression of Suz12 in cultured cells disrupts the function of the PRC2/3 complexes by preventing their recruitment to target promoters.

Discussion

Identification of mammalian PcG target genes has remained elusive for two main reasons. First, the majority of the previous PcG studies focused mostly on the biochemical purification and in vitro characterization of the activities of the PcG complexes and second, the lack of DNA-binding domains within PcG proteins makes the search for their target loci difficult. In this present study, we have identified the first known direct target genes of mammalian PcG complexes. To do so, we first used RNAi to identify genes deregulated by the loss of Suz12 protein in colon cancer cells. Next, we showed that Suz12 binds directly to the promoter of one of these genes (*MYT1*). We also showed that other members of the PRC2/3 complexes colocalize with Suz12 at the *MYT1* promoter. Most importantly, we demonstrated that recruitment of Suz12, Ezh2, and Eed to the *MYT1* promoter correlates with methylation of H3-K27. To demonstrate that this silencing mechanism is not unique to *MYT1*, we identified other Suz12 target genes using a ChIP assay coupled to a CpG island microarray. Similarly to *MYT1*, the other target promoters of Suz12 are bound by the PRC2/3 components and are characterized by H3-K27 methylation. Thus, the first identified human PcG target genes all appear to be regulated by the histone methylase activity of the PRC complexes.

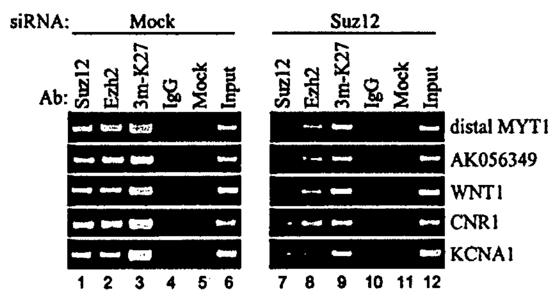

Figure 8. Methylation of H3-K27 at the Suz12 target promoters is due to Suz12-mediated recruitment of the methyltransferase Ezh2. ChIP experiments were performed using antibodies to Suz12 (lanes *1,6*), Ezh2 (lanes *2,7*), trimethyl H3-K27 (lanes *3,8*), and a control IgG (lanes *4,9*) in SW480 cells that were incubated with siSuz12 smart-pool or no siRNA (mock). For the siRNA transfections, cells were incubated with the indicated siRNA for 72 h, then replated and transfected for another 72 h. The immunoprecipitated chromatin was analyzed by PCR using primers specific to the promoters of the genes indicated. An equal amount of DNA from both treatments was monitored using PCR as shown by the similar intensity of the two input signals (cf. lanes *6* and *12*).

The Suz12 target gene *MYT1* was originally cloned from a human brain cDNA library on the basis of its ability to bind *cis*-regulatory elements of the glia-specific *myelin proteolipid protein* (*PLP*) gene and is suggested to be the prototype of the C2HC-type zinc finger protein family (Kim and Hudson 1992). More recently, the *Xenopus* ortholog of *MYT1* (*X-MYT1*) was identified as a transcriptional activator because it could induce expression of an *N-tubulin* promoter reporter construct in transient transfection assays (Bellefroid et al. 1996). In the same study, the authors demonstrated that dominant-negative forms of X-MYT1 inhibited normal neurogenesis, suggesting that X-MYT1 is essential for inducing neuronal differentiation. Intriguingly, a recent report showed that the *Xenopus* ortholog of Ezh2 (*XEZ*) is expressed exclusively in the anterior neural plate during early *Xenopus* embryogenesis and postulated that *XEZ* might be involved in delaying anterior neuronal differentiation (Barnett et al. 2001). Based on our findings, it is possible that Ezh2 delays neuronal differentiation, via the PRC2/3 complexes, by repressing the activity of the *MYT1* gene. Besides *MYT1*, we have identified four additional promoters as being robustly bound by components of the PRC2/3 complexes; each of these promoters is also characterized by high levels of H3-K27. Although a link between components of the PRC2/3 complexes and Wnt1, the cannabinoid receptor (CNR1), or the potassium channel KCNA1 have not been previously reported, it is intriguing to note that these mRNAs are expressed at very low levels in most human tissues (see http://expression.gnf.org), suggesting that they may be generally silenced by the PRC complexes. In support of this hypothesis, we have shown that some of these target genes are bound by PRC2/3 components in other cell lines, such as the human MCF7 and mouse F9 (data not shown).

Previous studies have used mRNA expression profiling to identify Ezh2 target genes. In one study, gene ex-

pression was monitored in cells that lacked Ezh2 (Bracken et al. 2003), whereas in the other study (Varambally et al. 2002) the analysis was performed in cells that overexpressed Ezh2. The genes identified in these previous studies do not overlap with the PRC2/3 target genes that we have now identified. It is possible that many, if not all, of the previously identified genes are indirectly regulated by Ezh2. It is also possible that the distinct sets of identified genes might be the result of using different cell types and/or different DNA microarrays. Therefore, our future studies will aim at the identification of a large set of mammalian PRC target genes, using various different normal and cancer cell types.

Although we have described the first known direct target genes of a human PRC complex, identifying a larger set of targets is essential to better understand the mechanisms that mediate the functions of Polycomb complexes. For example, it is unclear if all Suz12 and/or Ezh2 target genes will be regulated by the PRC2/3 complexes or if different Eed isoforms (and thus different PRC complexes) will predominate in different cell or tumor types. Therefore, it remains possible that methylation of histone H1, but not histone H3, will occur at certain PRC target genes. Also, it is possible that binding of components of the PRC2/3 complexes may sometimes lead to activation, rather than repression of gene expression. A PcG protein that has both activation and repression functions is now classified in the Enhancer of Trithorax and Polycomb (ETP) group (for review, see Brock and van Lohuizen 2001). Previous studies have shown that loss of expression of Ezh2 in *Drosophila* results in both increased and decreased gene expression (LaJeunesse and Shearn 1996). Similar findings were presented in another study in which siRNA-mediated depletion of Ezh2 or Eed in mammalian cells resulted in decreased expression of several genes (Bracken et al. 2003). Unfortunately, the previous studies did not distinguish between direct and indirect regulation by mammalian Ezh2 or Eed. However, our findings provide support for the hypothesis that components of the PRC2/3 complex can directly function as activators. Using ChIP, we showed that Suz12 and Ezh2 are recruited to the promoters of some genes (i.e., *SYBL1*) that are down-regulated upon Suz12 depletion from cells (Fig. 6; data not shown), suggesting that the PRC complexes normally activate the expression of these genes. Future studies should determine the mechanism of transcriptional activation mediated by the PRC complexes.

Finally, it is still unclear how the PRC complexes are directed to their target loci. Because no site-specific DNA-binding factor has been purified in the PRC complexes, it is not yet possible to identify a PRC target promoter by sequence inspection. In fact, even identifying a gene as being regulated by a PRC complex does not provide information as to exactly where the complex is recruited because the PRC-specific element (i.e., the PRE) might be located at a distance from the start of transcription. For example, we found that Suz12 was bound at −4000 in some promoters and at +1 in others. Interestingly, we also showed that many Suz12 target genes had multiple binding sites. In summary, this current study, which has identified the first set of human PRC2/3 target genes and demonstrated the utility of custom oligonucleotide arrays in presenting a detailed profiling of the binding of PRC components to large regions of target promoters, provides a significant step toward the goal of defining a mammalian PRE. Once we have identified a large number of direct target genes, we will use the information derived from the analysis of the custom arrays to search for common sequence elements in small, defined regions of the PRC target promoters.

Materials and methods

Antibody production

Using PCR, a 366-bp fragment from the N terminus and a 318-bp fragment from the C terminus of Suz12 ORF were generated and cloned into the Pet15b expression vector (catalog no. 69661, Novagen). The sequence of the primers can be found in Supplementary Table S3. The KIAA0160 (alternative name for Suz12) human cDNA clone, a gift from Takahiro Nagase (Kazusa DNA Research Institute, Osaka, Japan) was used as a template. Protein expression and purification were performed using the Bugbuster His-bind Purification Kit (catalog no. 70793, Novagen) according to the manufacturer's instructions. To prepare the Suz12 immunogen for rabbit injections, the N- and C-terminal protein fragments were combined in equal amounts to a final concentration of 1 μg/μL. Rabbit immunizations and antibody purifications were performed at Covance Inc. The Suz12 antibody is now available at Abcam, Inc. (catalog no. ab12201).

Plasmid constructs and transient transfections

To prepare a construct expressing HA-tagged Suz12, primers containing EcoRI sites on their 5′-ends were used in PCR to generate full-length cDNA of Suz12 (2219 bp). The KIAA0160 human cDNA clone was used as a template. The sequence of the primers can be found in Supplementary Table S3. The PCR product was digested with EcoRI and ligated into the EcoRI site of the pCMV-HA vector (Clontech). Bacterial colonies were screened for inserts and sequenced at the University of Wisconsin Biotechnology Sequencing Facility to ensure proper sequence and orientation. For transient transfections, SW480 human colon cancer cells were cultured in McCoy's 5A medium (Invitrogen) supplemented with 10% fetal bovine serum (NovaTech) and 1% Penicillin/Streptomycin (Invitrogen), and incubated at 37°C in a humidified 5% CO_2 incubator. One day before the transfection, SW480 cells were plated at 2×10^5 cells per 60-mm dish. The cells were transfected with 2 μg of the HA-Suz12-expressing construct (pCMV-HA-Suz12) or 2 μg of the control construct (pCMV-HA-empty) using the FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol. Cells were harvested 48 h after transfection and were subjected to immunoblot analysis.

Immunoblot analysis

Normal and diseased human colon tissues obtained from the Cooperative Human Tissue Network (CHTN) were minced into small pieces using a razor blade. About 300 mg of minced tissue was resuspended in 3 mL of 1× PBS buffer and disaggregated using a Medimachine (catalog no. 340587, Becton Dickinson) to achieve single cells. The cells were then diluted in a 2× volume

of whole-cell lysis buffer (0.1 M HEPES, 0.5 M KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 35% glycerol, 5 mM NaF, 1 mM DTT, 1 mM PMSF) and were alternately frozen and thawed three times in liquid nitrogen to rupture the cell membranes. The samples were incubated for 30 min on ice to lyse the nuclei and then centrifuged at 4000 × g for 5 min to pellet the cell membranes. The protein content of the resulting supernatant was quantitated using a standard Bradford assay. Equal amounts of protein (20–40 µg) from the tumor and normal tissue samples of each patient were subjected to standard Western analysis. In the case of the RNAi experiments (see below) and the transient transfections (see above), 5×10^5 cells were harvested from each treatment and examined by a standard Western analysis. Antibodies used for probing included anti-Suz12 (described above), anti-Lamin A/C (sc-7292, Santa Cruz Biotechnology), anti-HA (catalog no. MMS-101R, Covance Research Products), and anti-actin (sc-1615, Santa Cruz Biotechnology).

RNA interference

Two different 21-nt duplex siRNAs for Suz12 (target sequences of 5′-CCCGGAAATTTCCCGTCCC-3′ and 5′-GAGATGACCT GCATTGCCC-3′, custom orders), Suz12 smart-pool (M0069570050) and control siRNAs for Lamin A/C (target sequence 5′-CTG GACTTCCAGAAGAACA-3′, D0010000105) or GFP (target sequence 5′-GGCTACGTCCAGGAGCGCACC-3′, D0013000120) were synthesized by Dharmacon. For the immunoblot analysis (see above) and RNA extraction (see below), SW480 and HT29 human colon cancer cells were plated at 2×10^5 cells per well in a 12-well plate. Twenty-four hours after plating, the cells were transfected either with both Suz12 siRNA duplexes together (100 nmoles each) or with each control siRNA (200 nmoles each) using oligofectamine (Invitrogen) or trans-IT TKO (Mirus) according to the manufacturers' instructions. At various time points after transfection, the cells were treated with trypsin and harvested for immunoblot analysis, or RNA preparation. For the RNAi-ChIP experiments, SW480 cells were plated at a density of 1.2×10^6 cells per 100-mm dish, and after 24 h they were transfected with 100 nm of Suz12 smart-pool using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were incubated with the Suz12 smart-pool for 72 h and then were harvested and replated for another 72 h of transfection with 100 nm of Suz12 smart-pool. At the end of the second 72-h incubation, the cells were harvested and used in ChIP assays as described below.

RT–PCR analysis

Total RNA was prepared from 1×10^6 SW480 cells of each indicated treatment using the RNeasy kit (QIAGEN) as described by the manufacturer. For each RT–PCR reaction, 100 ng of RNA was used, and the amplifications were performed as described previously (Graveel et al. 2001). A list of all the primer sequences used in RT–PCR is available as Supplementary Table S3. All the primers used in this study were obtained from the University of Wisconsin Biotechnology Center.

RNA microarray analysis

Total RNA was extracted from cells transfected with either Suz12 siRNAs or control GFP siRNA as described above. For each treatment, RNA was prepared from three independent experiments and was combined into one sample to reduce the experimental variation. Targets for microarray hybridization were generated from the RNA according to the supplier's instructions (Affymetrix). The human U133A gene chip (Affymetrix) was used for gene expression profiling, which represents ~33,000 transcripts. Four U133A arrays were used for this analysis; two arrays were hybridized with cRNA obtained from cells treated with siRNAs to Suz12, and two arrays were hybridized with cRNA obtained from cells treated with siRNA to GFP. Hybridization, washing, scanning, and analysis of gene chips were performed at the University of Wisconsin Gene Expression Center according to the manufacturer's instructions. Expression levels were analyzed by the statistical algorithm in the Microarray Analysis Suite (MAS) 5.0 software (Affymetrix) using the default parameters. The data from the GFP siRNA treatment were used as a baseline expression for comparison with the Suz12 siRNA-treated sample.

ChIPs

Formaldehyde cross-linking and ChIP in SW480 cells were performed as described before (Weinmann et al. 2001) with the following exceptions. Precleared chromatin from 2×10^6 cells was used for each ChIP sample. After reversing the cross-links, each individual ChIP sample was purified using the Qiaquick PCR purification kit (QIAGEN), and samples were eluted with 30 µL of elution buffer. The antibodies used in the ChIP assays included purified Suz12 antibody (see above), Ezh2, Eed-M26, trimethyl H3-K27, trimethyl H3-K9 (ab8898, Abcam Inc.), RNA Polymerase II (sc-899, Santa Cruz Biotechnology), histone H3 (ab1791, Abcam Inc.), rabbit anti-goat (catalog no. 55335, ICN Pharmaceuticals; used as an IgG control), and rabbit preimmune serum (Covance; used as a control in CpG-island array experiments). All antibodies were used at 1 µg per IP except the Suz12 antibody, which was used at 3 µg per IP. For the immunogen IP control (experiments shown in Fig. 4), Suz12 antibody was mixed with a 10-fold excess by weight of immunogen (bacterially expressed Suz12 protein fragments) and incubated with rotation at 4°C overnight prior to use in the ChIP assay. A detailed protocol of the ChIP procedure and the sequences of all the primers used in PCR can be found in Supplementary Table S3.

LM–PCR

The generation of amplicons from individual ChIP samples was adapted from Ren et al. (2000). A detailed protocol can be found in Oberley et al. (2004) or at http://mcardle.oncology.wisc.edu/farnham.

Oligonucleotide promoter array analysis

Oligonucleotide promoter array analysis was performed by NimbleGen Systems as part of a Chromatin Immunoprecipitation (ChIP) Array Service. The custom arrays, each having 362,890 60-nt oligonucleotides, contained probes that tiled through 5 kb of the promoter regions of the selected genes. The probes represented both the forward and reverse strands, were spaced every 15 bases, and were printed at five (reverse strand) or six (forward strand) random locations on the array. For labeling, hybridization, and data analysis, refer to the Supplemental Material.

CpG-island microarray analysis

For detailed protocols on these steps, see Oberley et al. (2004) or http://mcardle.oncology.wisc.edu/farnham. The Cy5 dye was coupled to the Suz12 (or the preimmune) amplicon, and Cy3 dye was coupled to the input reference amplicon, using standard methods. Labeled chromatin was hybridized with human CpG-island microarrays obtained from the Ontario University Health

Network (http://www.microarrays.ca). Hybridizations were performed as described previously (Oberley et al. 2003). The hybridized microarrays were analyzed using the Genepix Pro 4.1 (Axon Instruments) software package. This provided a set of raw values for each feature on each array. To identify clones that are selectively enriched during ChIP relative to the starting population, the Cy5 and Cy3 channels were normalized across the entire array, by taking the ratio of the medians for all features and normalizing them to unity. After normalization, features of poor intensity (<500) and those that had obvious blemishes were manually flagged and removed from the putative positive list. For all quality features, a ratio was generated for each feature that was the intensity in the Cy5 channel minus background divided by the intensity in the Cy3 channel minus background. Features with Suz12/Input intensity ratios above 3 and PI/Input intensity ratios below 1.3 were selected for further analysis.

Acknowledgments

We thank Takahiro Nagase for providing the KIAA0160 cDNA clone and Arie Otte for providing the anti-EED (M26) antibody; Sandra Splinter, Wayne Davis, Mike Singer, and Todd Richmond for assistance with the microarray analyses; and Matthew J. Oberley for helpful discussions. We also thank the Cooperative Human Tissue Network (CHTN) for providing samples of human colon tissues. This work was supported by a DOD grant (BC020760) to P.J.F., and by an NIH grant (GM37120) and the Howard Hughes Medical Institute to D.R.

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