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**Characterization of the Role of JJAZ1 in Human Breast Cancer**

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**Abstract:**
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.

**Subject Terms:**
Chromatin, Polycomb, Transcription, Microarray, Suz12
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**Attached appendices:**
- Kirmizis et al. 2003
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:

2. Abstracts presented at meetings:

3. Oral Presentations by Dr. Farnham:
   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
Peggy J. Farnham

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

References:

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

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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 upregulated 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 Rb³ 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 PPARα 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

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1 Supported in part by USPHS Grants CA445240, 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; PpG, Polycomb group; ESC, extra sex comb; CNP, chromatin immunoprecipitation; EST, expressed sequence tag; DEN, diethylnitrosamine; TCF, T cell factor; ICU, 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. Over-expression 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 PPARα (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°C 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-dimen-sional 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°C 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. 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 Med-1 machine to achieve single cells. Antibodies used were to RNA polymerase II (Santa Cruz Biotechnology; sc-899), TGF-β (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 HindIII site at the 5' end to facilitate cloning of the PCR fragments into the HindIII 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-32P]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,

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8 The several modifications required by ChIP protocol are described in detail at http://mcardle.oncology.wisc.edu/famham/.
Table 1  Primer sequences

All of the primers are listed in the 5’ to 3’ orientation. The consensus TCF site (a/t,a/t,caaaag) is underlined in each oligonucleotide used in the gel shift experiments.

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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; 3650X) 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, 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/.
The majority of the mRNA. We found that the downstream fragment, which corresponds to position -617 to +26 relative to the upstream start site; 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.

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. 2B). Interestingly, this transcription start site maps to the same position as one of the longer ESTs (GenBank accession no. BG655867). Using primer B, we detected a slightly weaker signal (Fig. 2B) 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 promoter and/or inefficient hybridization of that particular primer, we prepared another primer complementary to the promoter region shown. Indicated are the consensus Spl, E2F, and TCF sites.

Upstream promoter fragments were cloned upstream of the luciferase cDNA (Fig. 2B). Interestingly, this transcription start site does mediate promoter activity and that this fragment functionalizes bidirectionally. Interestingly, the first mammalian promoter shown to be regulated by E2F was the bidirectional dfrf/msh3 locus (37). Other bidirectional E2F-regulated promoters include thymidylate synthase and RanBP1/Hif9 (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...
is driven from two promoters and that the bidirectional upstream promoter is the major promoter for SU(Z)12 mRNA.

**TCF and $\beta$-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 transcription factors, located at −206 and −776, relative to the upstream transcription start site. TCF family members do not contain transactivation domains. Rather, the coactivator $\beta$-catenin interacts with TCF family members and the TCF/$\beta$-catenin complex activates transcription. $\beta$-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 $\beta$-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/$\beta$-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/$\beta$-catenin complex (Lanes 7 and 8); the other bands are likely to be other TCF family members alone or in complex with $\beta$-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/$\beta$-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. 4A). Antibodies to E2F4, TCF4, and $\beta$-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/$\beta$-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 $\beta$-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 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 $\beta$-catenin (Fig. 4B). Unlike the Myc promoter, the SU(Z)12 and cyclin D1 promoters were only bound by $\beta$-catenin in the colon tumor sample. These results support

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**Fig. 3.** Identification of TCF/$\beta$-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 $\beta$-catenin (Lane 7) or TCF (Lane 8) were incubated with the probe and whole cell extracts to identify the bands containing the TCF/$\beta$-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.

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**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 and c-Myc 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.
SU(Z)12 is Up-Regulated in Human Tumors

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. 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).

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/famham/.

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).
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 tumor types will also be observed.

Other previously identified β-catenin target genes include fra-1, c-jun, Myc, matrilysin, cyclin D1, PGHS-2, and PPAR8 (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 PPAR8 promoters are bound by β-catenin in colon tumors (Fig. 5 and unpublished data), 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 (43). 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 EGT, 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

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

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References


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]

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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 Tri thorax 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 (Ese), Enhancer of zeste [E(z)], and Suppressor of zeste 12 [Su(z)12] and the histone-binding protein NURF55 (Czernin 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...
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 nucleosomes 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
A

Figure 1. Suzl2 protein expression is elevated in human colon tumors. (A) Western blot analysis to demonstrate the specificity of the Suzl2 antibody. SW480 cells were transfected with an HA-Suzl2 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 Suzl2 antibody (right panel). The HA antibody recognizes only the HA-Suzl2 protein and the Suzl2 antibody detects both the HA-Suzl2 and endogenous Suzl2 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-Suzl2 antibody and then reprobed with an anti-actin antibody as a loading control. The arrowhead points to the band with the correct size of Suzl2 (93 kDa). A second lower band, detected with the Suzl2 antibody in patients 1–3, probably represents a protein degradation product.

B

Depletion of Suzl2 in colon cancer cells results in alteration of gene expression

Recent studies have shown that Suzl2 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 Suzl2 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 Suzl2 expression in colon cancer cells. We designed small interfering RNAs (siRNAs) to target the Suzl2 transcript and tested them using two human colon cancer cell lines (Fig. 2; data not shown). We observed a decrease in both Suzl2 mRNA and protein levels by 24 h after transfecting cells with the Suzl2 siRNAs (Fig. 2A,B, cf. lanes 1 and 4). The down-regulation of Suzl2 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 Suzl2 verifying the specificity of the RNAi approach. We next examined the phenotype of the colon cancer cells having reduced Suzl2 expression. We did not detect any changes in the morphology or the cell cycle profile of the cells after treatment with Suzl2 siRNAs for 72 h (data not shown). However, RNAi does not eliminate Suzl2 expression entirely from the cells, and the remaining Suzl2 may be sufficient to mask any obvious phenotypes, especially in a short-term assay. It is also possible that certain functions of Suzl2 in these cells are redundant with another protein, and, therefore, removing only Suzl2 is not enough to cause changes in cell proliferation.

To determine whether short-term removal of Suzl2 from cells results in altered transcription profiles, we
performed RNAi experiments and monitored global gene expression using oligonucleotide microarrays. We transfected SW480 colon cancer cells with either Suzl2 or GFP siRNAs and harvested total RNA after 48 h. Although depletion of Suzl2 was best at 72 h (Fig. 2), we chose to monitor expression at the 48-h time point to minimize indirect effects of the Suzl2 down-regulation. Three independent experiments were performed with the Suzl2 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 Suzl2-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 Suzl2. We found 46 up-regulated transcripts (representing 35 unique genes) and 23 down-regulated transcripts (representing 15 unique genes) in cells having reduced Suzl2 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 Suzl2 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 Suzl2 siRNA treatment in the independent experiment (Fig. 3C).

Suzl2 binds directly to promoters

The experiments described above show that down-regulation of Suzl2 alters the expression level of multiple

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**Figure 3.** Removal of Suzl2 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 Suzl2 or GFP siRNAs. The numbers on the arrows correspond to the columns in B. Columns 1, 2, 3, and 4 represent siSuzl2 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 Suzl2 depletion. Red represents up-regulated genes, and green represents down-regulated genes in the Suzl2 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.

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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, Myeloid 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 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

![Figure 4](image-url)
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 PRC 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

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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 (MYTI, 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.

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-
Identified 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-
A schematic of the distal MYT1 promoter locus is shown; the positive and negative numbers indicate the position 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. (A) 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. Amplicons prepared from a typical LM–PCR reaction were then analyzed by high-resolution PCR analysis, we also found that Ezh2, Eed, and Ezh2 were not bound to the promoter of the genes indicated, cells. As expected, the activated Suz12 and weak Pol II signals seen on four motifs in the promoter region because, 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.

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 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. Amplicons prepared from a typical LM–PCR reaction were then analyzed by high-resolution PCR analysis, we also found that Ezh2, Eed, and Ezh2 were not bound to the promoter of the genes indicated, cells. As expected, the activated Suz12 and weak Pol II signals seen on four motifs in the promoter region because, 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.

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 bio-

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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].

chemical 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.

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-
Interestingly, we also showed that many Suzl2 target genes achieve single cell resolution. The cells were then diluted in a 2x volume of lysis buffer and disaggregated by ultrasonic vibrations. For example, we found that Suz12 was overexpressed in human colon cancer cells cultured in McCoy's 5A medium. One day before the transfection, SW480 cells were plated at 2 x 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 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 µg of minced tissue was resuspended in 3 mL of 1x PBS buffer and disaggregated using a Medimachine (catalog no. 340587, Becton Dickinson) to achieve single cells. The cell suspension was then diluted in a 2x volume of 1x PBS buffer.
of whole-cell lysis buffer (0.1 M HEPES, 0.5 M KCl, 5 mM MgCl₂, 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 × 10⁶ 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-7299, Santa Cruz Biotechnology), anti-HA [catalog no. MMS-101R, Covance Research Products], and anti-β-actin (sc-1615, Santa Cruz Biotechnology).

RNA interference

Two different 21-nt duplex siRNAs for Suz12 (target sequences of 5'-CCCGGAAATTTCCCTGCCC-3' and 5'-GAGATGACCTGCCAGGCCACC-3', custom orders), Suz12 smart-pool (M0069570051) and control siRNAs for Lamin A/C [target sequence 5'-CTC GACTTCCAGGAAGAACA-3', D010000105] or GFP [target sequence 5'-GGCTACGTCCAGGAGCGCACC-3', D013000120] 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 × 10⁶ 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 manufactures' 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 × 10⁶ 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 manufac- turers' instructions. The cells were incubated with the Suz12 smart-pool for 72 h and then were harvested and replaced 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 × 10⁶ SW480 cells of each indicated treatment condition 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 above [Weinmann et al. 2001] with the following exceptions. Precleared chromatin from 2 × 10⁶ cells was used for each ChIP sample. After reversing the cross-links, each individual ChIP sample was purified using the Qiagen PCR purification kit (Qiagen), and control siRNAs for Lamin A/C (sc-7299, Santa Cruz Biotechnology), anti-HA [catalog no. MMS-101R, Covance Research Products], and anti-β-actin (sc-1615, Santa Cruz Biotechnology).

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Total RNA was prepared from 1 × 10⁶ SW480 cells of each indicated treatment condition 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 above [Weinmann et al. 2001] with the following exceptions. Precleared chromatin from 2 × 10⁶ cells was used for each ChIP sample. After reversing the cross-links, each individual ChIP sample was purified using the Qiagen 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] ampiclon, and Cy3 dye was coupled to the input reference ampiclon, using standard methods. Labeled chromatin was hybridized with human CpG-island microarrays obtained from the Ontario University Health
For all quality features, a ratio was generated for each feature's transcriptional state by Polycomb-group proteins. Features with Suz12/Input intensity ratios above 3 and P1/Input intensity ratios below 1.3 were selected for further analysis.

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