Genome Analysis Identifies the p15^{ink4b} Tumor Suppressor as a Direct Target of the ZNF217/CoREST Complex[†]

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The ZNF217 oncoprotein is a constituent of a core transcriptional complex that includes CoREST, histone deacetylase 1/2, lysine demethylase 1, and the C-terminal binding protein 1/2. We have combined genome-wide expression profiling and chromatin immunoprecipitation with directed selection and ligation (ChIP-DSL) to identify a subset of genes directly regulated by ZNF217. Our results establish p15^{ink4b} as a direct target of the ZNF217 complex. Downregulation of ZNF217 in MCF-7 breast cancer cells resulted in a dramatic increase in p15^{ink4b} expression and coincided with increases in dimethylation of H3-K4 and, surprisingly, a decrease in K9/K14-H3 acetylation. Stimulation of HaCaT cells with transforming growth factor β (TGF- β) resulted in a release of ZNF217 and a concomitant binding of SMAD2 to the proximal promoter, which preceded increases in ink4b protein expression. Furthermore, the changes in chromatin marks at the p15^{ink4b} promoter following TGF- β stimulation were similar to those observed following ZNF217 downregulation. Collectively, these results establish the ZNF217 complex as a novel negative regulator of the p15^{ink4b} gene and may constitute an important link between amplification of ZNF217 and the loss of TGF- β responsiveness in breast cancer.

ZNF217 is a candidate oncogene found at the core of the 20q13.2 amplicon (11). Amplification and overexpression of ZNF217 has been found in a significant proportion of tumors and transformed cell types of epithelial origin, including those of the breast, colorectum, ovary, and prostate, ranging in amplification frequency from 10 to 40% (47, 48, 50, 51). Several studies have also established correlations between amplification and overexpression of ZNF217 and clinical outcome, with increased ZNF217 expression correlating with a poor prognosis (2, 10, 26, 48, 51).

Insights into the oncogenic role of ZNF217 have come primarily from studies using human epithelial cells with a finite life span. Forced expression of ZNF217 by retroviral gene transfer in human mammary epithelial cells promotes loss of senescence, immortalization, and resistance to growth inhibition by transforming growth factor β (TGF- β) (38). In addition, prolonged growth of ZNF217-immortalized cells results in chromosomal instability, as well as telomere crisis and telomerase reactivation (9). More recently, transduction of finite life span ovarian cells with a ZNF217 retrovirus has also been shown to promote cellular immortalization, increased cellular proliferation, and telomerase activity, as well as anchorage-independent cell growth (32a). ZNF217 overexpression was also shown to suppress spontaneous and doxorubicin-induced apoptosis, suggesting that ZNF217 may promote oncogenic transformation by increasing cell survival (27).

The ZNF217 protein contains eight Kruppel-like zinc

fingers, suggesting that it most likely functions as a transcription factor. Molecular mapping studies using a transcription-based reporter assay and various regions of ZNF217 fused to the GAL4 DNA binding domain have identified two repression domains located within the carboxy terminus (12, 40). Biochemical purification studies in combination with mass spectrometry have identified ZNF217 as a constituent of several related transcriptional repressor complexes (12, 23, 32, 43, 44, 53). Comparative analysis of each of the purified complexes suggests that the ZNF217 complex is very similar to the CoREST complex previously implicated in neuronal differentiation (53) and consists of three core proteins: histone deacetylase 2, lysine demethylase 1 (LSD1), and the corepressor of REST (CoREST). In addition, the carboxy terminus of ZNF217 interacts directly with the C-terminal binding protein 1/2 (CtBP1/2) corepressor, and this interaction is, in part, essential for the repressor function of ZNF217 (12, 40).

CASTing (cyclic amplification and selection of targets) analysis, using degenerate oligonucleotides and the region of ZNF217 encompassing the sixth and seventh zinc fingers, has identified a core recognition sequence consisting of CAGAAAY (where Y is A, G, or T) (12). This sequence has been identified within the E-cadherin promoter, and chromatin immunoprecipitation (ChIP) assays have shown that the ZNF217 complex is present on the E-cadherin promoter in breast cancer cells (12). More recently, a bioinformatics approach in conjunction with ChIP analysis was used to identify a consensus ZNF217 binding site (ATTCNAC) in ZNF217 target genes. Interestingly, 65% of the genes identified in the ChIP screen also contained the CAGAAAY motif, suggesting that ZNF217 may use multiple zinc fingers to bind specific target genes (29).

In the present study, we have used a two-step approach to identify ZNF217 targets. First, we employed small interfering RNA (siRNA)-mediated gene silencing of ZNF217 coupled

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with microarray screening to identify genes with altered expression. Secondly, we used chromatin immunoprecipitation with directed selection and ligation (ChIP-DSL) to identify promoters directly bound by ZNF217. By comparative analysis of genes identified using both approaches, we have identified a subset of genes directly regulated by the ZNF217 complex. Our analysis has focused on the p15^{ink4b} tumor suppressor gene as a critical ZNF217 target. ChIP analysis in both MCF-7 and HaCaT cells confirmed that the ZNF217 complex occupies a region of the p15^{ink4b} promoter that is critically important for transcriptional activation. Stimulation of HaCaT cells with TGF- β resulted in a rapid release of ZNF217 and a concomitant recruitment of SMAD2 protein to the p15^{ink4b} gene promoter, which preceded increases in protein expression. Importantly, ZNF217 downregulation and TGF- β stimulation have similar effects on the chromatin modifications surrounding the p15^{ink4b} promoter, suggesting that ZNF217 and TGF- β are functioning through convergent mechanisms. Our results suggest that a coactivator/corepressor balance may constitute an important parameter regulating p15^{ink4b} expression and establish a possible link between overexpression of ZNF217 and the loss of TGF- β stimulation at selected targets.

MATERIALS AND METHODS

Plasmids, antibodies, and reagents. The affinity-purified anti-ZNF217 antibody was generated as previously described (12). A complete list of primers used can be found in Table S1 of the supplemental material. Antibodies used in this study are listed in Table S2 of the supplemental material. TGF- β was purchased from R&D Systems. The siRNAs were purchased from Dharmacon: ZNF217 siRNA1, GCAAUAACCUCAUCUGUAUU; ZNF217 siRNA2, GAACAGA ACCUCCAAGGAUU. LSD1 siRNA was from Qiagen, and the sequence was not available.

RNA isolation and real-time PCR. Total cellular RNA was isolated using an RNA EZ kit (Qiagen). The quality and quantity of RNA were evaluated by measuring the optical density at 260/280. In addition, RNA quality was evaluated by agarose gel electrophoresis. For real-time PCR analysis, 0.2 μ g of RNA was reverse transcribed with TaqMan reverse transcriptase (Applied Biosystems) using random hexamers to generate cDNA. All amplicons were detected using the 5' nuclease (Taqman) assay with 5'-6-carboxytetramethylrhodamine-labeled probes. Probes were already predesigned and quality tested (Applied Biosystems). Reactions were performed according to the manufacturer's recommendations (Applied Biosystems) and were run in replicates of two, in a 96-well format. Each reaction mixture included 18S RNA as a control for normalization, and reaction mixtures lacking cDNA served as negative controls. Two independent experiments were performed for each gene, and a mean value was obtained and compared to the mean expression level of each gene from cells transfected with control siRNA. Applied Biosystems 7500 real-time PCR system software was used to identify the cycle threshold (C_t) for each reaction.

RNA microarray analysis. Total RNA was extracted from MCF-7 cells transfected with ZNF217 siRNA or mock infected. Independent biological triplicates were performed for each siRNA and, including control transfections, brought the number of independent transfection experiments to nine. cDNA was prepared from control and each siRNA-transfected sample, labeled, and hybridized to an HgU133A+2 human Affymetrix DNA microarray, and a list of genes was then created for all nine experiments. The hybridization, washing, scanning, and analysis of gene chips were performed at the University of Western Ontario Genomic Centre (London, Ontario, Canada).

The average intensities of siRNA knockdowns (RNAi1 and -2) were compared to the control nontreated sample. Three biological replicates were done for each array, the data were transformed using Robust Multi-Array normalization (5), and values below 0.01 were set to 0.01. Each measurement was normalized by dividing all measurements in that sample by the 50th percentile. Ratios were then calculated for all nine samples against the median of the control samples (1, 4, and 9). A Student *t* test statistical analysis was conducted, and false positives were reduced using the Benjamini and Hochberg false discovery rate.

Western blot analysis. Cells were washed twice in phosphate-buffered saline (PBS), harvested, and lysed in lysis buffer (~300 μ l/10-cm plate) consisting of 20

mM Tris (pH 7.9), 300 mM KCl, 0.1% NP-40, 10% glycerol, 0.1 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM EGTA, and protease inhibitor cocktail. For experiments involving detection of p15^{ink4b}, radioimmunoprecipitation assay buffer was used to prepare the cell extracts and consisted of 50 mM Tris (pH 8.0), 150 mM NaCl, and 1% NP-40. Extracts were centrifuged for 10 min at 16,000 $\times$ g at 4°C, and the soluble extracts were retained. Samples were normalized for protein content and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose or a polyvinylidene difluoride membrane, and blocked overnight in PBS containing 0.1% Tween 20 and 5% nonfat dried milk. The appropriate antibodies were then diluted in blocking buffer, and the membrane was probed for 2 h at room temperature with rocking followed by the appropriate secondary antibody for 1 h. Proteins were detected using enhanced chemiluminescence according to the manufacturer's recommendations (Amersham).

Purification of the ZNF217 complex. ZNF217 was purified from MCF-7 cells essentially as previously described (13). Approximately 20 mg of nuclear extract was loaded onto a 10-ml phosphocellulose P₁₁ column. The column was then washed using buffer A (20 mM Tris [pH 7.9], 0.5 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, and 100 mM KCl), and ZNF217 was eluted with buffer A containing 0.3 M KCl. The ZNF217-containing fraction was assayed by Western blotting and then loaded and reloaded five times onto an anti-ZNF217 immunoaffinity column that was generated by cross-linking affinity-purified ZNF217 antibody to protein A-Sepharose according to standard procedures (24). The column was then washed with buffer A containing 0.3 M KCl and 0.1% NP-40. Bound proteins were eluted with 100 mM glycine (pH 2.8) containing 100 mM KCl and analyzed for various proteins by Western blotting.

Chromatin immunoprecipitation assay. MCF-7 cells were cross-linked with 1% formaldehyde at room temperature for 10 min. Cross-linking was quenched by immediately washing cells twice with ice-cold PBS. Cells were washed twice with ice-cold PBS containing 0.5 mM EDTA and harvested. Cell pellets were lysed in 0.3 ml of cell lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS, and protease inhibitors) and incubated on ice for 10 min. Cell lysates were sonicated to yield DNA fragments ranging in size from 300 to 1,000 bp. Approximately 450 μ g of the cross-linked, sheared chromatin solution was used for immunoprecipitation. A small portion of each IP mixture was saved as input DNA (5%). Supernatants were diluted 10-fold in dilution buffer (20 mM Tris-HCl [pH 8.1], 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, and protease inhibitors) and precleared with 60 μ l of a 50% slurry of protein A-Sepharose containing 2.5 μ g of sheared salmon sperm DNA for 2 h at 4°C. Immunoprecipitation was performed overnight at 4°C with 1.5 to 4 μ g of the antibodies. A 60- μ l volume of protein A-Sepharose containing 2.5 μ g of salmon sperm DNA per ml was added to the solution and incubated for 1 h at 4°C. The beads were washed one time with wash buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), wash buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), wash buffer III (0.25 M LiCl, 1% NP-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl), and twice with Tris-EDTA buffer. Immunocomplexes were extracted twice with 200 μ l elution buffer (1% SDS-0.1 M NaHCO₃). NaCl was added to a final concentration of 200 mM, and the cross-linking was reversed by heating at 65°C overnight. The DNA was purified using Qiagen PCR purification spin columns. For analysis by conventional PCR, conditions were as follows: an initial denaturing cycle at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, and a final elongation step of 72°C for 10 min. For experiments involving TGF- β treatment, HaCaT cells were plated to approximately 90% confluence and treated with 150 pM TGF- β for 90 min prior to ChIP analysis.

For some experiments, DNA isolated from ChIP experiments was subjected to quantitation by real-time PCR using Brilliant Sybr green master mix (600548; Stratagene). Primers were identified using the Primer Express program (Stratagene) and tested to establish optimum reaction conditions. Reactions were performed in a 25- μ l volume according to the manufacturer's recommendations. The reaction was carried out and measured using an Mx3000P real-time instrument. Standard curves were generated using total input DNA (copy number range, 8×10^5 to 8×10^1). The IP and immunoglobulin G (IgG)-based DNA copy numbers were calculated by extrapolating the respective C_t values from the standard curve. The nonimmune IgG copy number was subtracted from IP DNA copy number. The resulting IP copy number was normalized against the total input DNA by dividing the IP value by the input and expressing the IP value as a percentage of the input DNA. All measurements were done in duplicate, and an average C_t value was used to calculate copy number. Two independent real-time reactions were done for each experiment.

ChIP-DSL assay. ChIP-DSL was used to assess global promoter occupancy by ZNF217. MCF-7 cells were cross-linked with formaldehyde and subjected to a standard ChIP assay using affinity-purified anti-ZNF217 antibody. The proce-

dures for oligonucleotide annealing, solid-phase selection ligation, and PCR amplification were performed exactly as described by the manufacturer (H20K, catalog number AK-0504; Aviva Systems Biology). The antibody-enriched DNA and the total input were biotinylated followed by annealing to the 40-mer oligonucleotide pool. The DNA-oligonucleotide complexes were then selected by binding to streptavidin-conjugated magnetic beads, while the nonannealed oligonucleotides were washed away. Correctly paired 40-mers were then ligated to form the corresponding 80-mer, which was flanked by both universal primer annealing sites (T3 and T7), giving rise to a complete amplicon. PCR was then conducted on the amplicons using fluorescently labeled T7 and regular T3 primers. Total input DNA was PCR amplified using Cy5 (green)-labeled T7 primer, and the IP sample was amplified using Cy3 (red)-labeled T7 primer. The PCR products were cohybridized to the 40-mer array (Hu20K) to derive an enrichment ratio for each target. After hybridization and washing, array slides were scanned on a One View (Bio-Rad) chip reader, and the ArrayVision (v6.0) software package (Genomic Centre, London, Ontario, Canada) was used to quantify fluorescence intensity. The ChIP-on-chip intensity values were normalized using a Lowess curve, which was fit to the log intensity versus log ratio plot, and 20% of the data were used to calculate the Lowess fit at each point. Following normalization, a two-sided Student's *t* test was conducted with the standard deviation of the replicates used to calculate a *P* value. The fold change was calculated for each gene using a mean value that was calculated from all three biological replicates.

Ingenuity Pathways Systems analysis. Ingenuity Pathways Systems (<http://www.ingenuity.com>) analysis was employed to group statistically significant genes. Genes that had at least a single enrichment were imported into the Ingenuity systems. Only 1,215 genes were found in the system database, and only those genes were used for further analysis. The significance value associated with a function was expressed as a *P* value, which was calculated using the right-tailed Fisher exact test. This was done by comparing the number of genes from the gene expression profile that participated in a given function, relative to the total number of occurrences of those genes in all functional annotations stored in the Ingenuity Pathways database.

RESULTS

Purification of ZNF217 from MCF-7 breast cancer cells.

Using protein purification in combination with mass spectrometry, we and others have recently shown that ZNF217 is a component of a core complex consisting of CoREST, LSD1, CtBP1, and HDAC2 (12, 23, 32, 43, 44, 53). To assess the integrity of the ZNF217 complex in MCF-7 breast cancer cells, we purified ZNF217 by immunoaffinity chromatography using a similar approach (Fig. 1A). SDS-PAGE analysis of the immunopurified ZNF217 followed by silver staining indicated that the profile of proteins was similar to the ZNF217 complex we recently purified (12) and contained at least three additional polypeptides that were not present in the control immunopurification using rabbit IgG (Fig. 1B). Western blot analysis using selected antibodies indicated that CoREST, LSD1, HDAC2, and CtBP1 all copurified with ZNF217 (Fig. 1C). These results suggest that the ZNF217 complex found in MCF-7 cells is similar to the complex previously identified in HeLa cells. (12).

Identification of gene expression changes in ZNF217-depleted MCF-7 cells. To identify genes that are regulated by ZNF217 we initially performed expression array analysis on ZNF217-depleted cells. siRNA1 and siRNA2, which recognize distinct regions of the ZNF217 transcript, were transfected into MCF-7 cells. The use of multiple siRNAs minimizes potential off-target effects associated with individual siRNAs. Western blot analysis of cell lysates prepared 72 h after transfection confirmed that ZNF217 protein levels were significantly reduced (Fig. 2A). cDNA from three independent cultures of control and siRNA-treated MCF-7 cells was prepared, fluorescently labeled, and hybridized to Affymetrix arrays. In

FIG. 1. Purification of the ZNF217 complex from MCF-7 nuclear extracts. (A) Purification scheme used to purify the ZNF217 complex from MCF-7 cell nuclear extracts. ZNF217 was partially purified by passing MCF-7 cell nuclear extracts through a P_{11} phosphocellulose column prior to immunoaffinity chromatography using ZNF217 antibody. (B) Silver stained SDS-PAGE gel of the purified proteins. A 15- μ l aliquot of the purified ZNF217 complex was analyzed by SDS-PAGE followed by silver staining. IgG represents affinity purification using a rabbit IgG nonimmune affinity column. (C) Western blotting of various proteins found in the ZNF217 complex. A 15- μ l aliquot of the purified ZNF217 complex was analyzed by SDS-PAGE followed by Western blotting using various antibodies as indicated on the left of the figure.

our initial analysis, we identified genes displaying statistically significant changes in expression in three out of three replicates for each siRNA. From this preliminary list of genes we identified those genes which were common to both siRNAs. Using this approach, we identified 176 genes which were significantly upregulated and 875 genes which were downregulated following ZNF217 knockdown (Fig. 2B; see also Table S3 in the supplemental material). In our initial analysis, we focused on those genes which are upregulated by ZNF knockdown. This was based on the observation that ZNF217 is generally believed to function as a transcriptional repressor and depletion of ZNF217 should result in derepression of target genes. To confirm the results of the expression screen, quantitative real-time PCR analysis and Western blotting were performed on randomly selected genes (Fig. 2C and D), which indicated significant upregulation following ZNF217 depletion. Real-time PCR analysis and Western blotting were also performed on LSD1-depleted MCF-7 cells to examine whether gene expression was dependent on other components of the ZNF217 complex. For the majority of genes examined, upregu-

FIG. 2. Genome-wide expression screen to identify changes in gene expression associated with ZNF217 depletion. (A) Western blot of ZNF217-depleted MCF-7 cells. MCF-7 cells were transfected with siRNA recognizing two different regions of ZNF217 (siRNA1 and siRNA2). Cells were incubated for 72 h prior to analysis of whole-cell extracts by Western blotting using ZNF217 antibody. (B) Venn diagrams depicting the overlap in genes upregulated or downregulated from cells transfected with either siRNA1 or siRNA2. (C) Real-time PCR analysis of selected genes identified as significantly upregulated following ZNF217 or LSD1 knockdown using siRNA. Each bar represents the mean relative expression compared to the expression in cells transfected with control siRNA. (D) Western blot of selected genes significantly upregulated following ZNF217 or LSD1 gene knockdown using siRNA. MCF-7 cells were transfected with siRNA targeting either ZNF217 or LSD1. After 72 h, whole-cell extracts were prepared and proteins were analyzed by SDS-PAGE followed by Western blotting using the antibodies indicated on the left of each panel.

lation was observed in response to LSD1 knockdown, although the levels of upregulation were generally lower than those observed following ZNF217 downregulation, most likely resulting from our inability to fully downregulate LSD1.

Genome-wide identification of human promoters bound by ZNF217. To determine which of the genes identified in the expression analysis are directly bound by ZNF217, we used a genome-wide ChIP-DSL assay (18, 30). A standard ChIP assay was performed on MCF-7 cells using affinity-purified ZNF217 antibody. The resulting ZNF217-enriched and input DNA were then biotinylated and combined with 20,000 predesigned oligonucleotide pairs, each representing one-half of an 80-mer sequence, corresponding to promoter regions of target genes and flanked by a T3 and a T7 primer. After annealing, the biotinylated DNA was purified using streptavidin-conjugated magnetic beads. Adjacent oligonucleotides that anneal to the immunoprecipitated DNA were ligated, creating complete amplicons which were then amplified with fluorescently labeled primers and hybridized to a 20,000-gene promoter array. We first validated the functionality of the platform by demonstrating a normalized distribution of the intensity values (Fig. 3A). From three independent analyses we identified 1,431 promoters which are directly bound by ZNF217 (Fig. 3A and B; see also Table S4 in the supplemental material). To identify the nature of the target genes identified, the targets were analyzed using the Ingenuity Analysis program (<http://www.ingenuity.com/>). Based on this analysis we found that approximately 25% of the targets identified are transcription factors, suggesting that ZNF217 may play a prominent role in various differentiation processes (Fig. 3D). Interestingly, in terms of biological function, genes uniquely related to various aspects of cancer were ranked as the most significant, and approximately 73 genes were found to be consistently present in more than one cancer category (Fig. 3C; see Table S5 in the supplemental material). Additionally, a significant number of genes related to cell morphology and various aspects of the cell cycle were identified. Network analysis revealed two highly significantly networks of interacting genes (see Fig. S1 in the supplemental material), with many of the genes within each network linked to tumor suppressor activity.

By comparing the ZNF217 target genes with genes undergoing a significant change in expression following ZNF217 knockdown, we identified nine genes directly bound by ZNF217 which were found to be significantly upregulated (repressed by ZNF217) and 45 genes which were significantly downregulated (activated by ZNF217) (Table 1). The results of the ChIP-DSL screen were confirmed by standard ChIP using ZNF217 antibody and oligonucleotide primers corresponding to specific regions within selected promoters (Fig. 4A and B; see also Fig. S2 in the supplemental material). Surprisingly, for the vast majority of the target genes identified, no change in expression levels was observed in ZNF217-depleted MCF-7 cells. The reasons for this are not entirely clear, but our results are consistent with several recent ChIP-chip studies published for other human transcription factors and coregulators that suggest that loss of a single factor may not be sufficient to alter the expression of a gene, perhaps as a result of secondary repressive modifications which are not alleviated following ZNF217 knockdown (6–8, 41).

p15^{ink4b} is a direct target of the ZNF217 complex. Among the ZNF217 gene targets identified, we focused our initial investigation on the p15^{ink4b} tumor suppressor gene. p15^{ink4b} is a member of the INK4 family of CDK inhibitors which causes cell cycle arrest by directly inhibiting CDK4 and -6 (28). Inactivation of p15^{ink4b} has been found in a wide spectrum of cancers, suggesting that direct silencing of p15^{ink4b} by ZNF217 may contribute to its oncogenic properties (31).

As shown in Fig. 5, downregulation of ZNF217, or LSD1, using siRNA resulted in significant increases in p15^{ink4b} levels. To confirm binding of ZNF217 to the INK4b promoter and more accurately define its binding site, we performed ChIP analysis in MCF-7 cells using pairs of oligonucleotides encompassing approximately 150-bp intervals (Fig. 6A). The promoter arrays used in the ChIP-DSL screen consisted of unique 80-mer sequences located within 1 kb upstream from the transcription start site for each gene; therefore, we restricted our analysis to this region of the p15^{ink4b} promoter. ChIP analysis indicated that ZNF217 is highly enriched within a region of the promoter encompassing nucleotides (nt) –566 to –426 (Fig. 6B). In addition, both LSD1 and CoREST were also found predominantly within this region, confirming that the p15^{ink4b} gene is a target for the ZNF217 complex.

To determine whether the presence, or absence, of ZNF217 corresponded to specific chromatin marks at the p15^{ink4b} promoter, ChIP analysis was performed using antibodies corresponding to acetylated K9/14 on histone H3 (K9/K14-H3) and dimethyl K4 on histone H3 (dimetK4-H3), which are generally associated with transcriptionally active genes, as well as trimethyl K27 on histone H3 (trimet K27-H3), a marker for transcriptional repression (3, 4, 49). Real-time PCR analysis identified several significant changes following ZNF217 knockdown. First, a statistically significant increase in acetylation was observed at nt –566 to –426 (primer set 3), containing the ZNF217 binding region. However, the major changes were found downstream of the ZNF217 complex binding site, at nt –340 to –204 (primer set 4). These included a dramatic increase in dimetK4-H3, which would be consistent with the loss of LSD1. Surprisingly, in the presence of ZNF217, this region was found to be highly acetylated at K9/14 on histone H3, and following ZNF217 knockdown a complete loss of acetylation at K9/14-H3 was observed, despite the observation that p15^{ink4b} was highly expressed (Fig. 6C; see also Fig. S3 in the supplemental material).

Previous studies have shown that TGF- β stimulates rapid binding of SMAD, Fox0, and CEBP β to the p15^{ink4b} promoter, resulting in transcriptional activation of the p15^{ink4b} gene (21, 22). Interestingly, the ZNF217 binding region of the p15^{ink4b} promoter encompasses a SMAD binding site flanked by a forkhead binding element at nt –504 to –538. Based on the proximity of the ZNF217 binding region to the SMAD binding site, we speculated that binding of ZNF217 and SMADs may be mutually exclusive and that coregulator exchange, in response to TGF- β , is a prerequisite for transcriptional activation of the p15^{ink4b} gene. However, in preliminary experiments upregulation of the p15^{ink4b} gene in response to TGF- β was not observed in MCF-7 cells (data not shown). Therefore, to examine dynamic changes in ZNF217 complex assembly at the ink4b gene promoter, we used the HaCaT keratinocyte cell line, a well-established model for TGF- β -responsive events.

FIG. 3. ChIP-DSL analysis of ZNF217 target genes in MCF-7 cells. (A) Scatter plot of specific ChIP (y axis) versus total (x axis) averaged values from three independent biological replicates, demonstrating a normal cluster distribution. Data in red indicate genes significantly enriched. (B) Venn diagram depicting the overlap in genes enriched from three independent ChIP-DSL experiments (Rep 1 to 3). A onefold change and a *P* value of <0.05 (calculated using Student's *t* test) yielded 1,431 genes common to all three replicates. (C) Ingenuity functional analysis revealed the majority of genes whose promoter regions are bound by ZNF217 to be involved in various diseases. Genes were scored with a significance value which was a log *P* value calculated using the Fisher exact test measuring the uniqueness of a gene within a function. (D) Targets corresponding to molecular function were also categorized, revealing approximately 25% of ZNF217 target genes are transcription factors.

For these experiments, HaCaT cells were stimulated with TGF-β and promoter occupancy was assessed by ChIP assay using specific antibodies recognizing either ZNF217 or SMAD2. As shown in Fig. 7A, p15^{ink4b} protein levels were strongly upregulated upon TGF-β stimulation. Importantly, ChIP analysis indicated that stimulation with TGF-β for 90

min resulted in a rapid loss of ZNF217 and a concomitant increase in SMAD2 binding from the same region of the p15^{ink4b} promoter (Fig. 7B). To examine the effect of TGF-β on specific chromatin marks, HaCaT cells were stimulated with TGF-β for 90 min and ChIP assays were performed using antibodies recognizing

TABLE 1. Genes directly regulated by ZNF217^a

Direction of regulation and protein name	Accession no.	Description
Genes repressed by ZNF217		
MGC45400	AI743979	Transcription elongation factor 8
VAV3	AF118886	Cell motility
ABCA12	AL080207	Membrane transporter
p15 ^{INK4b}	AW444761	CDK inhibitor
FLJ39370	AI110850	Paired like homedomain 2
MAN1A1	BG287153	Glycosylase
EFNB2	BF001670	Transmembrane ligand
THY28	NM_014174	Thymocyte nuclear protein
FLJ11280	AL561943	Fam63A
Genes activated by ZNF217		
FAM35A	NM_019054	Hypothetical protein MGC5560
DAZAP1	BF512907	DAZ-associated protein 1
LOC55971	AA496034	Insulin receptor tyrosine kinase substrate
C18orf25	AI823360	mRNA sequence of wh53b02.x1 NCI_CGAP_Kid11 <i>Homo sapiens</i> cDNA
GAJ	AY028916	Meiotic nuclear division 1 homolog (<i>S. cerevisiae</i>)
C13orf3	AI829603	Chromosome 13 open reading frame 3
SF3A1	BF129339	Splicing factor 3a, subunit 1, 120 kDa
KLIP1	AA460299	KSHV ^b latent nuclear antigen-interacting protein 1
BRCA1	NM_007295	Breast cancer 1, early onset
ETF1	NM_004730	Eukaryotic translation termination factor 1
TCF3	M31523	Transcription factor 3
MCM8	BC005170	Minichromosome maintenance deficient 8
LBR	NM_002296	Lamin B receptor
CaMKIIN α	NM_018584	Calcium/calmodulin-dependent protein kinase II
PFKFB3	NM_004566	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
ZNF395	AK021850	Papillomavirus regulatory factor 1
SUSD2	Z92546	Sushi domain containing 2
XPO4	BF968638	Exportin 4
SF3A1	NM_005877	Splicing factor 3a, subunit 1, 120 kDa
AD-017	NM_018446	Glycosyltransferase AD-017
FLJ90022	AW264102	Hypothetical protein FLJ90022
PIR51	BE966146	mRNA sequence of wh53b02.x1 NCI_CGAP_Kid11
RAC3	NM_005052	rho family, small GTP binding protein
IRX2	AI928035	Iroquois homeobox protein 2
POP7	BC001430	Processing of precursor, <i>S. cerevisiae</i>
FLJ11029	BG165011	Hypothetical protein
CDC25C	NM_001790	Cell division cycle 25C
TUBB2	W72331	Tubulin, β , 2
CECR5	NM_017829	Cat eye syndrome chromosome region, candidate 5
ZBTB8	AW006067	wz92a02.x1 NCI_CGAP_Brn25 mRNA sequence
DCXR	NM_016286	Dicarbonyl/L-xylulose reductase
FLJ11127	NM_019018	Hypothetical protein
CDCA8	BC001651	Cell division cycle associated 8
FGFRL1	AF312678	Fibroblast growth factor receptor-like 1
TNRC5	NM_006586	Trinucleotide repeat containing 5
CALM1	AI653730	Calmodulin 1 (phosphorylase kinase δ)
FLJ10460	AW611729	Hypothetical protein FLJ10460
C4orf15	BC003648	Hypothetical protein MGC4701
KIAA1007	BC000779	KIAA1007 protein
SRP72	BE856385	Signal recognition particle, 72 kDa
PDXP	BC000320	Lectin, galactoside binding, soluble, 1 (galectin 1)
EIF4E	AI742789	Eukaryotic translation initiation factor 4E
NUP50	AF267865	Nucleoporin, 50 kDa
KCTD15	AI808448	Potassium channel tetramerization domain containing 15
LMNB1	NM_005573	Lamin B1

^a Comparative analysis of ChIP-chip data and expression analysis following ZNF217 depletion in MCF7 cells allowed for the identification of ZNF217 target genes.

^b KSHV, Kaposi's sarcoma-associated herpesvirus.

FIG. 4. ChIP analysis of selected ZNF217 targets. (A) Direct ZNF217 targets which were found significantly upregulated following ZNF217 depletion. Proliferating MCF-7 cells were cross-linked with 1% formaldehyde, and ChIP was performed with either control IgG or anti-ZNF217 antibody. The recovered DNA was then assayed by PCR using oligonucleotide primers corresponding to the promoter region of specific genes identified in the ChIP-DSL analysis. (B) Direct ZNF217 targets which were found to be significantly downregulated in ZNF217-depleted MCF-7 cells.

acetylated K9/K14-H3, dimethyl K4-H3, and trimethyl K27-H3 (Fig. 7C; see also Fig. S3 in the supplemental material). The major changes were again confined to the region from nt -340 to -204 of the p15^{ink4b} promoter and included increases in

FIG. 5. The p15^{ink4b} gene is regulated by the ZNF217 complex. (A) Western blot of p15^{ink4b} protein following ZNF217 knockdown in MCF-7 cells. MCF-7 cells were transfected with siRNA recognizing ZNF217 (siRNA1). Cells were incubated for 72 h prior to analysis of cell extracts by Western blotting using ZNF217 or p15^{ink4b} antibody. (B) Real-time PCR analysis of the p15^{ink4b} gene following knockdown of ZNF217 or LSD1 using siRNA. MCF-7 cells were transfected with siRNA recognizing ZNF217 (siRNA1) or LSD1. RNA was then prepared and reverse transcribed, and real-time analysis was performed. Each bar represents the mean comparative expression relative to cells transfected with control siRNA of two independent experiments.

FIG. 6. The p15^{ink4b} promoter is a direct target of the ZNF217 complex. (A) Schematic representation of the human p15^{ink4b} promoter showing the nucleotide sequence encompassing the SMAD binding element (SBE1) and the FoXO3 binding element (FoXO). The underlined sequences represent consensus ZNF217 binding sites identified in reference 29 (double underline) and reference 12 (single underline). (B) ChIP analysis of the p15^{ink4b} promoter. MCF-7 cells were cross-linked with 1% formaldehyde, and ChIP was performed with either control antibody (IgG) or the specific antibody (IP) indicated on the left. The recovered DNA was then assayed by PCR using pairs of oligonucleotides encompassing specific regions of the p15^{ink4b} promoter (primer set 1, 3, or 4). (C) ZNF217-dependent changes in histone marks across the p15^{ink4b} promoter. MCF-7 cells were transfected with siRNA recognizing ZNF217 (siRNA1). After 72 h, cells were cross-linked with 1% formaldehyde and ChIP was performed with either control antibody (IgG) or the histone modification-specific antibodies indicated at the bottom of the figure. The recovered DNA was then assayed by real-time PCR using pairs of oligonucleotides encompassing specific regions of the p15^{ink4b} promoter as indicated in panel A (primer sets 1 to 4).

dimethylation at H3-K4 and deacetylation at K9/K14-H3. Collectively, these results suggest that the loss of ZNF217 and the TGF- β stimulation result in similar changes in chromatin modifications at the p15^{ink4b} proximal promoter.

DISCUSSION

The ZNF217 gene codes for a Kruppel-like transcription factor and represents a strong candidate oncogene associated with the 20q13.2 amplification. Insights into the mechanism of ZNF217 have come from studies which identified ZNF217 as a major constituent of several related transcriptional repressor complexes containing HDAC2 and LSD1 (12, 23, 32, 43, 44, 53). Thus, ZNF217 may promote the development of cancer by inappropriately targeting histone-modifying enzymes to genes which are essential for normal cell growth and differentiation.

In the present study, we have used global genomic approaches to identify genes which are directly regulated by ZNF217. Using ChIP-DSL we were able to identify over 1,400 gene promoters bound by the ZNF217 transcription factor in a native chromatin context. Furthermore, in conjunction with siRNA knockdown and microarray analysis, we established that 54 genes are directly regulated by ZNF217. Thus, for the vast majority of the target genes identified, changes in gene expression were not correlated with ZNF217 promoter occupancy in MCF-7 cells. This was not entirely unexpected, as gene expression is dictated by the repertoire of transcription factors and coregulators that transiently occupy target promoters, and not by a single factor. While the manuscript was in preparation, a genome-wide ChIP-chip analysis identified approximately 1,045 genes, bound by ZNF217, in MCF-7 and Ntera2 cell lines, with ap-

FIG. 7. TGF- β -inducible release of ZNF217 from the p15^{ink4b} promoter. (A) Western blot of cells following stimulation with TGF- β . HaCaT cells were stimulated with TGF- β . After various intervals following stimulation, cell extracts were prepared and Western blotting was performed using antibodies against p15^{ink4b} or tubulin. Lane 1 (-) shows results for cells receiving no TGF- β that were grown for 48 h prior to lysis. (B) ChIP analysis of TGF- β -stimulated HaCaT cells. HaCaT cells were stimulated with TGF- β for 90 min. Cells were then cross-linked with 1% formaldehyde, and ChIP was performed with either control antibody (IgG), SMAD2, or ZNF217 antibodies. The recovered DNA was then assayed by standard PCR (right panel) or by real-time PCR using pairs of oligonucleotides encompassing the ZNF217 binding region of the p15^{ink4b} promoter (primer set 3). (C) TGF- β -dependent changes in histone marks. HaCaT cells were stimulated with TGF- β for 90 min. Cells were then cross-linked with 1% formaldehyde, and ChIP was performed with either control antibody (IgG) or the modification-specific antibodies indicated at the bottom of the figure. The recovered DNA was then assayed by real-time PCR using pairs of oligonucleotides encompassing specific regions of the p15^{ink4b} promoter as indicated (primer sets 1 to 4).

proximately 745 genes common to both cell types (30). However, only 54 of the target genes identified were found to be directly regulated by ZNF217. A comparison of target genes identified in our study and those from the study of Krig et al. indicated 7% overlap (73 genes), and of the 54 genes directly regulated by ZNF217, only 4 were found in our ChIP-DSL analysis. These differences are most likely attributable to variations in experimental design, the specific antibodies used which may recognize different epitopes, and more importantly, differences in the microarray platforms used. Unlike the more conventional ChIP-chip approach, which uses ligation-mediated PCR to amplify the immunoprecipitated DNA, in ChIP-DSL the DNA is not directly amplified but is used only as a

template for annealing of complementary oligonucleotides corresponding to specific promoter regions. Consequently, this greatly reduces the complexity of the hybridization mixture and may avoid certain biases inherent in the ligation-mediated PCR strategy. However, CHIP-DSL can only identify targets found within 1 kb of the transcription start site. Thus, ZNF217-dependent regulatory sites located many kilobases upstream would not be detectable by CHIP-DSL.

Surprisingly, 45 of the 54 target genes we identified are downregulated upon ZNF217 knockdown, suggesting that ZNF217 may also function in transcriptional activation. This finding is consistent with recent ChIP-on-chip studies of other transcriptional repressor proteins. For example, using ChIP-

FIG. 8. Model highlighting the role of the ZNF217 complex in $p15^{\text{ink4b}}$ expression. In normal proliferating epithelial cells, the ZNF217 complex is bound to the $p15^{\text{ink4b}}$ promoter, and expression of $p15^{\text{ink4b}}$ is repressed. Stimulation with TGF- β causes a release of the ZNF217 complex and a concomitant binding of activating transcription factors, which include SMADs (SM2/3 and SM4), CEBP β , FoxO3, and SP1. Additionally, associations between adjacent transcription factors, as well as downregulation of Myc following TGF- β stimulation, may result in full activation of the $p15^{\text{ink4b}}$ gene.

DSL, recent studies have demonstrated that LSD1 is recruited to target genes which are transcriptionally active as well as repressed (18, 30). LSD1 has been shown to function in both transcriptional repression by demethylating H3-K4 and in activation of specific genes by removing the dimethyl mark from H3-K9 rather than H3-K4. Although the underlying mechanism for defining LSD1 specificity is unclear, *in vitro* studies have suggested that LSD1 activity is allosterically regulated through interaction with other proteins, such as CoREST, BHC80, and the ligand-bound androgen receptor (32, 37). In addition, surrounding histone marks found at specific promoters may also play a role in substrate recognition by LSD1 (16, 17). Thus, the substrate specificity of LSD1 may be an important determinant for defining the transcriptional activity of ZNF217 at selected targets.

To obtain insight into the mechanism of repression of ZNF217, we focused our analysis on the $p15^{\text{ink4b}}$ gene. The $p15^{\text{ink4b}}$ gene is found within a 35-kb stretch of DNA, the INK4 locus, which also contains $p16^{\text{ink4A}}$ and $p14^{\text{ARF}}$, a splice variant of $p16^{\text{ink4A}}$. The entire locus has been found frequently to be deleted or mutated in many types of cancer (19, 45). Interestingly, we did not detect changes in expression levels of either $p16^{\text{ink4A}}$ or $p14^{\text{ARF}}$, based on the microarray expression analysis, indicating that the repressive effects of the ZNF217 complex are specific for $p15^{\text{ink4b}}$.

In many epithelial cell lines, $p15^{\text{ink4b}}$ is rapidly upregulated in response to TGF- β and, under normal growth conditions, contributes to the TGF- β -dependent cytostatic program. Previous studies using HaCaT cells have shown that the induction of $p15^{\text{ink4b}}$ occurs predominantly at the level of transcription through a dual mechanism involving downregulation of *c-myc* and the recruitment of activating transcription factors to the promoter region (20, 42, 46). *myc* acts as a negative regulator of the $p15^{\text{ink4b}}$ gene by preventing the transcription factor Miz1 from activating $p15^{\text{ink4b}}$ transcription (42, 46). The addition of TGF- β suppresses *myc* expression, depleting the cellular pools of *myc* available for binding to Miz1 which, in turn, relieves active repression of the $p15^{\text{ink4b}}$ gene. Concurrently, SMAD

proteins, as well as other transcription factors, bind to specific DNA elements in the promoter region and, in association with Miz1, elicit full activation of the $p15^{\text{ink4b}}$ gene (14, 15).

The identification of the ZNF217 complex as a negative regulator of the $p15^{\text{ink4b}}$ gene in breast cancer cells adds an additional layer of complexity in our understanding of the molecular events regulating $p15^{\text{ink4b}}$ gene transcription (Fig. 8). In MCF-7 cells the levels of $p15^{\text{ink4b}}$ are virtually undetectable, and downregulation of ZNF217 using siRNA resulted in a dramatic increase in $p15^{\text{ink4b}}$ protein levels, indicating that loss of ZNF217 alone was sufficient to relieve repression of the $p15^{\text{ink4b}}$ gene in this cell type. ChIP analysis indicated that ZNF217 binds a region of the promoter that has previously been shown to encompass a SMAD binding element flanked by a FoxO site (21, 22). Recent studies have shown that upregulation of $p15^{\text{ink4b}}$, in response to TGF- β , is dependent on rapid binding of both SMAD proteins and FoxO3 to their respective sites. Interestingly, a CAGAAA sequence and an ATTCAA motif directly overlap the SMAD and FoxO3 binding sites, respectively. Both of these elements have been identified as putative consensus ZNF217 binding sites in independent studies (12, 29). Based on these observations, we speculated that TGF- β -dependent activation of the $p15^{\text{ink4b}}$ gene is dependent on release of ZNF217. This was indeed confirmed in HaCaT cells, in which treatment with TGF- β resulted in a rapid loss in ZNF217 and a concomitant increase in SMAD2 binding at nt -566 to -426, which preceded increases in $p15^{\text{ink4b}}$ protein expression. Collectively, these findings establish the ZNF217 complex as a novel suppressor of the $p15^{\text{ink4b}}$ gene (Fig. 8).

We have also examined the relationship between promoter occupancy by ZNF217 and changes in chromatin modifications at the $p15^{\text{ink4b}}$ promoter. Following ZNF217 knockdown, a dramatic increase in dimethylation of K4-H3 was observed at nt -340 to -204. Dimethyl K4-H3 serves as a major substrate for LSD1, and this suggests that targeting of LSD1 may depend directly on ZNF217 recruitment. Unexpectedly, we have found that the region from nt -340 to -204 is also highly acetylated at K9/K14 of histone H3 when the $p15^{\text{ink4b}}$ gene is not ex-

pressed, and it is deacetylated upon ZNF217 knockdown. Although deacetylation of histones is generally associated with transcriptional silencing of genes, several reports have demonstrated a requirement for HDAC activity in transcriptional activation. For example, analysis of the beta interferon promoter has shown that deacetylation of specific lysine residues serves as a prerequisite for transcriptional activation (1). Similarly, removal of yeast repressor proteins RPD3 and SIN3 resulted in decreased transcription in a number of genes and increased silencing (13). One possibility is that hyperacetylation at K9/K14 is required for binding of repressor proteins containing specific recognition motifs, such as bromodomains, which bind to acetylated lysines with high affinity. For example, BRD4, a bromodomain-containing transcriptional adaptor protein, inhibits human papillomavirus transcription by binding to acetylated lysines on histone H3 and blocks recruitment of the core transcriptional machinery (52). Consequently, the loss of acetylation at K9/K14 and increases in dimethyl K4-H3, following ZNF217 release, may function cooperatively to target additional proteins to the p15^{ink4b} promoter which, in turn, may increase the accessibility of the chromatin to the transcriptional machinery and facilitate transcriptional activation of the p15^{ink4b} gene.

Similar changes in chromatin marks were also observed in HaCaT cells following stimulation with TGF- β . Importantly, the changes we have observed coincide with the release of ZNF217, indicating that loss of ZNF217 and stimulation with TGF- β are most likely part of a concerted signaling mechanism regulating the p15^{ink4b} gene. The differences in the magnitude of the changes in covalent modifications between MCF-7 and HaCaT cells may be attributable to differences in cell types as well as experimental conditions. Covalent modifications related to a specific transcriptional outcome often involve repetitive cycles of association and dissociation of transcription factors and a large number of coregulator proteins in a sequential manner (36). Consequently, 90 min after TGF- β stimulation may not be optimal for the establishment of specific changes in chromatin structure, even though increases in SMAD2 binding and loss of ZNF217 are clearly evident by this time point. Nevertheless, the changes in chromatin marks, following loss of ZNF217, or stimulation of cells with TGF- β are quite striking and suggest that the region from nt -340 to -204 is dynamically regulated and most likely plays a critical role in determining expression of the p15^{ink4b} gene.

Many tumors and cancer cell lines develop resistance to the growth-inhibitory effects of TGF- β , which in some cases result from loss-of-function mutations in TGF- β receptors or SMAD proteins (39). However, the majority of tumors which have lost TGF- β -dependent functional responses retain an intact TGF- β signaling mechanism, suggesting that specific downstream defects may be involved. This may include overexpression of specific proteins resulting in a deregulated transcriptional response at TGF- β -dependent target genes. The c-ski oncoprotein, which is overexpressed in a subset of leukemic patients, negatively regulates TGF- β signaling by interfering with the formation of SMAD complexes at target genes, resulting in abnormal silencing of transcription (25, 33, 34). More recently, it has been shown that excess levels of the C/EBP β isoform liver-enriched transcriptional inhibitor protein (LIP) may, in part, account for the loss of TGF- β responsiveness

(22). C/EBP β consists of multiple isoforms, liver-enriched transcriptional activator protein 1 and 2 (LAP1/2) and LIP, which lacks regulatory domains found in LAP and functions as a dominant negative for C/EBP β -dependent transcription. LAP binds directly to the p15^{ink4b} promoter and is required for induction of the p15^{ink4b} gene in response to TGF- β . A high LIP/LAP ratio has been reported in some metastatic breast cancers, and lowering of the LIP/LAP ratio by overexpression of LAP restores both TGF- β -dependent induction of p15^{ink4b} and the growth-inhibitory response to TGF- β . Finally, it has been shown that human mammary epithelial cells aberrantly expressing ZNF217 become immortalized and develop resistance to the growth-inhibitory properties of TGF- β (38).

The identification of the p15^{ink4b} gene as a direct target for the ZNF217 corepressor complex represents a potentially novel link between amplification of ZNF217 and the loss of TGF- β responsiveness in breast cancer. The proximity of the ZNF217 binding region to the SMAD binding region is consistent with this hypothesis and suggests that the balance between coactivators and corepressor proteins at the level of gene transcription represents a critical regulatory mechanism and an important determinant of cell growth.

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