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Epigenetic Silencing of the *p16^INK4a^* Tumor Suppressor is Associated with Loss of CTCF Binding and a Chromatin Boundary

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Summary

The *p16^INK4a^* tumor suppressor gene is a frequent target of epigenetic inactivation in human cancers, which is considered to be an early event in breast carcinogenesis. Here we describe the existence of a chromatin boundary upstream of the *p16* gene that is lost when this gene is aberrantly silenced. We show that the multifunctional protein CTCF associates in the vicinity of this boundary and that absence of CTCF binding strongly coincides with *p16* silencing in multiple types of cancer cells. CTCF binding also correlates with activation of the *RASSF1A* and *CDH1* genes and this interaction is absent when these genes are methylated and silenced. Interestingly, defective poly(ADP-ribosyl) ation of CTCF and dissociation from the molecular chaperone Nucleolin occurs in *p16*-silenced cells, abrogating its proper function. Thus, destabilization of specific chromosomal boundaries through aberrant crosstalk between CTCF, poly(ADP-ribosyl)ation, and DNA methylation may be a general mechanism to inactivate tumor suppressor genes and initiate tumorigenesis in numerous forms of human cancers.

Introduction

Aberrant transcriptional silencing of tumor suppressor genes by epigenetic deregulation is a common occurrence in human malignancies. This is characterized by altered patterns of DNA hypermethylation in specific promoter regions and acquisition of histone modifications that are characteristic of repressed chromatin (Feinberg, 2008; Jones and Baylin, 2007). Because of its importance in cell proliferation, the human *INK4* gene locus is a frequent target of inactivation by deletion or aberrant DNA methylation in a wide variety of human cancers (Kim and Sharpless, 2006). This locus encompasses approximately 42 kb on chromosome 9 and encodes three distinct tumor suppressor proteins, $p15^{INK4b}$, $p14^{ARF}$ and $p16^{INK4a}$ (referred to hereafter as p15, p14 and p16). p16 is a key regulator of G1 phase cell cycle arrest and senescence, which it achieves primarily through inhibiting the cyclin-dependent kinases CDK4 and CDK6. In fact, inactivation of the *p16* gene by promoter methylation or genetic change is one of the earliest losses of tumor suppressor function in numerous types of human cancers, such as breast, lung, colorectal cancers and multiple myeloma (Belinsky et al., 1998; Foster et al., 1998; Ng et al., 1997). Notably, *p16* promoter methylation and transcriptional silencing have been shown to exist in histologically normal mammary tissue of cancer-free women. This suggests that these aberrant epigenetic changes may represent a cancerous pre-condition and an early event in promoting genomic instability that leads to tumorigenesis (Holst et al., 2003).

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Although the precise mechanisms underlying epigenetic loss-of-function of the *p16* gene remain unresolved, an examination of proteins important for its regulation may provide insight into the cause of aberrant silencing. One study revealed that transcription of all three *INK4/ARF* genes is controlled by a common CDC6-binding regulatory element (Gonzalez et al., 2006). While this is intriguing, RNA expression and DNA methylation profiles in a variety of tumors and cancer cell lines show no obvious coupling of *p16* silencing with that of the *p15* and *p14* genes (Paz et al., 2003). *p16* silencing could also result from gain-of-function or aberrant targeting of repressor proteins that modulate epigenetic processes. For example, ID1 regulates *p16* expression during senescence through exchange of ID for ETS activators (Ohtani et al., 2001). However, it is unclear if this contributes to *p16* deregulation during tumorigenesis. Another repressor of *p16*, the polycomb protein BMI1, has oncogenic activity and controls cell proliferation and senescence through the *INK4a* locus (Jacobs et al., 1999). In primary breast tumors, however, no correlation between BMI1 and *p16* expression is observed (Silva et al., 2006). Other polycomb members such as EZH2 and SUZ12 also interact with p*16* in proliferating fibroblasts (Bracken et al., 2007; Kotake et al., 2007). Although data linking EZH2 and p16 silencing is lacking, recent evidence indicates that EZH2 can recruit DNA methyltransferases and maintain methylation patterns at silenced genes in cancer cells (Vire et al., 2006).

To further understand the mechanism(s) by which the *p16* gene becomes aberrantly silenced in human cancers, we examined epigenetic regulation at the level of the *INK4/ARF* chromosomal locus rather than solely at the *p16* promoter. Here we present evidence that a chromosomal boundary exists at approximately 2 kb upstream of the *p16* transcriptional start site. This boundary separates the *p16* gene locus into discrete domains characterized by the presence or absence of repressive epigenetic marks and the histone variant H2A.Z, a functionally diverse protein recently shown to confer memory of transcriptional status and facilitate re-activation of target promoters (Raisner and Madhani, 2006). By contrast, in breast cancer cells containing aberrantly silenced *p16* genes, the epigenetically defined domain at -2 kb disappears and regions 3′ of this boundary acquire characteristics of heterochromatin which is accompanied by loss of histone H2A.Z. Upon further examination, we noticed the presence of a recognition sequence for the zinc finger protein CTCF 3′ of the boundary. CTCF is a multi-functional transcription factor known to have a critical role in regulating chromosomal boundaries/insulators (Filippova, 2008; Wallace and Felsenfeld, 2007). Unexpectedly, we observed CTCF association with this region in numerous *p16*-expressing cell lines but complete absence in *p16* non-expressing breast cancer cells, even though CTCF binds to another target gene, *c-Myc*, in all cases. Moreover, ablation of CTCF protein from *p16*-expressing cells by shRNA results in epigenetic changes to the *p16* promoter and loss of transcription. In addition to breast cancer, aberrant *p16* gene silencing is widely documented in a variety of human malignancies. We examined multiple myeloma cell lines and found that inactivation of the *p16* gene is also correlated with absence of CTCF binding. Thus, our studies indicate that *p16* gene repression can result from destabilization of a chromosomal boundary through dissociation of CTCF.

A similar examination of other well characterized epigenetically silenced genes in human cancers, *RASSF1A* and *CDH1* (*E-cadherin*), also revealed a strong correlation between transcriptional inactivation and a loss of CTCF binding. The insulator function of CTCF has been shown to require its post-translational modification by poly(ADP-ribosyl)ation (PARlation) (Yu et al., 2004) and crosstalk between PARP-1 and CTCF strongly affects DNA methylation (Guastafierro et al., 2008). Strikingly, we found a defect in the poly(ADP-ribosylation) pathway in *p16*-silenced cells resulting in the absence of CTCF PARlation and dissociation from a new coregulator, Nucleolin. Furthermore, we demonstrated that chemical inhibition of PARlation or knockdown of PARP-1 directly impacts *p16* and *RASSF1A* expression. We propose that destabilization of specific chromosomal boundaries is caused by

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aberrant interactions between CTCF and the poly(ADP-ribosyl)ation enzymatic machinery and can be a general mechanism to initiate potentially reversible genomic instability and tumorigenesis in human cancers.

Results

Loss of a Chromosomal Boundary at the *p16* Gene Locus in Epigenetically Silenced Breast Cancer Cells

Aberrant transcriptional silencing of tumor suppressor genes is accompanied by dynamic changes in chromatin structure as revealed by the acquisition of histone modifications that are characteristic of repressed chromatin. To gain insight into chromatin structural alterations that may accompany *p16* gene inactivation, we analyzed histone modifications surrounding the gene in *p16*-expressing (MDA-MB-435) and non-expressing (T47D) human breast cancer cell lines (Figure 1). Initially, we examined the transcriptional status of the three genes within the *INK4/ARF* locus, *p15*, *p14*, and *p16* (diagrammed in Figure 1A). We found that each gene is active in MDA-MB-435 cells whereas *p16* alone is silenced in T47D cells (Figure 1B). This indicates that event(s) leading to *p16* deregulation in these cells specifically impacts this gene without affecting the entire *INK4/ARF* locus.

We next performed chromatin immunoprecipitations (ChIPs) to analyze a variety of histone modifications within the vicinity of the active *p16* gene in MDA-MB-435 cells. These modifications include those that are typically associated with repressed chromatin, like Me^{1}H4K20, Me^{2}H3K27, and Me^{3}H3K9, as well as marks that correlate with mammalian gene activation, such as Me^{3}H3K4 and the histone variant H2A.Z. We also examined the presence of Me^{1}H3K79 which is generally correlated with transcriptionally active genes in mammalian cells (Klose and Zhang, 2007). Localization of bulk histone 3 was measured to control for any large changes in nucleosomal placement and density. Surprisingly, in *p16*-expressing cells we found an enrichment of marks that are normally associated with silenced genes, as well as Me^{1}H3K79, between 2-7 kb upstream of the proximal promoter (Figure 1C; amplicons A-C, Table S1). This chromatin structural organization is lost in the vicinity of the p16 proximal promoter between -2 kb and +1 (amplicons D-E). As expected in this region of an expressed gene, Me^{3}H3K4 is enriched and H2A.Z is distributed in a similar pattern. The region of "active" chromatin between -2 kb and +1 is reversed downstream of the *p16* gene at +4 kb where chromatin again becomes repressed (amplicon F). These data indicate that the 11 kb region encompassing the *p16* gene is arranged into clearly demarcated domains of repressive versus active chromatin structures. Moreover, the data are consistent with the presence of a distinct chromosomal boundary/insulator within 2 kb upstream of the *p16* transcriptional start site.

A similar ChIP analysis was conducted in T47D breast cancer cells in which the *p16* gene is silenced and methylated (Figure S1) (Di Vinci et al., 2005). In these cells the chromatin structure of the aberrantly inactivated *p16* gene is quite different from that found in *p16*-expressing MDA-MB-435 cells and, most strikingly, the chromosomal domain organization is lost (Figure 1D). This is apparent from the spread of repressive histone marks, Me^{1}H4K20 and Me^{3}H3K9 as well as Me^{1}H3K79 from the upstream 2-7 kb domain through the -2 kb demarcation to encompass the entire 11 kb *p16* gene locus. A dramatic loss of H2A.Z and Me^{3}H3K4 from approximately -3 kb to +1 (amplicons B-E) is also evident. However, no significant change in the pattern of Me^{2}H3K27 is observed indicating that the enzymatic activity associated with this mark may function in an independent manner. Overall, these data substantiate the existence of a chromatin boundary upstream of the *p16* initiation site that functions to maintain the promoter in an active configuration by preventing the spread of repressive nucleosomal modifications from a neighboring domain. Interestingly, the disappearance of this boundary is correlated with aberrant epigenetic silencing of the *p16* gene in certain breast cancer cell lines.

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The Boundary/Insulator Protein CTCF Associates with the Transcriptionally Active but not Silenced *p16* Gene

CTCF is a ubiquitous, multifunctional protein that has a critical role in organizing distinct chromosomal domains through boundary/insulator formation and repressing or activating transcription. Because we saw a pronounced change in chromatin structure upstream of the *p16* gene when active or silenced (Figure 1), we explored the possibility that CTCF may associate within this region. To address this issue, a ChIP analysis was performed to identify sites of CTCF interaction within -7 to +4 kb of the *p16* gene locus in expressing and non-expressing cells. Our data revealed that in *p16*-expressing cells, CTCF clearly binds downstream (amplicon D) of the region enriched for marks of heterochromatin within -2 kb and +1 of the active *p16* gene (Figures 2A, S2A). However, no CTCF binding was observed at other distal regions in the locus near -7 kb (amplicon A) or +4 kb (amplicon F). Surprisingly, when we examined cells containing a silenced *p16* gene, CTCF interaction at the upstream promoter site was not apparent. Yet, we detected CTCF binding at a well-characterized target gene, *c-Myc*, in both *p16*-expressing and non-expressing cell types. This indicates that loss of CTCF binding from the *p16* gene in T47D cells is not due to a general defect in the ability of the endogenous protein to associate with its chromosomal targets. Moreover, CTCF dissociation from the *p16* gene is not mechanistically linked to the stability of other CTCF interactions that we examined in the *INK4/ARF* locus (Figure S2B). No significant reduction in bulk CTCF protein levels or in cellular localization was observed between T47D and MDA-MB-435 cells (Figure S2C). Importantly, loss of CTCF interaction is not a consequence of cessation of *p16* transcription since CTCF binding remains stable upon *p16* gene inactivation by pharmacological inhibitors (Figure S3).

p16 Gene Expression Correlates with CTCF Binding Near its Chromosomal Boundary in Multiple Types of Human Cancer Cells

Having established a strong correlation between CTCF interaction with the *p16* upstream promoter and *p16* expression in breast cancer cell lines, we asked whether our observations could be extended to other types of human cancer cells. For example, the *p16* gene is a frequent target of epigenetic inactivation in primary multiple myeloma cells (Ng et al., 1997). As shown in the ChIP analysis in Figure 2B, CTCF binding is highly correlated with *p16* expression in diverse cell types such as non-transformed fibroblasts (IMR90) and cervical cancer cell lines (HeLa, C33A). Conversely, in two multiple myeloma cell lines (U266, KMS12) and a primary breast epithelial-derived cell line (vHMEC), each of which harbors a silenced *p16* gene, CTCF binding was lost from the upstream promoter. Consistent with our findings in the MDA-MB-435 and TD47 breast cancer cell lines (Figure 2A), CTCF interaction at the *c-Myc* locus was constant in all cell types examined (Figure 2B). Thus, loss of CTCF binding from the *p16* upstream promoter near its chromosomal boundary is correlated with transcriptional silencing in both human breast cancer and multiple myeloma cell lines even though CTCF interaction with *c-Myc* remains unaffected. The loss of CTCF binding at *p16* could not be attributed to aberrant expression and recruitment of BORIS to replace CTCF, as we saw no correlation between *p16* silencing and BORIS expression (Figure S4A and data not shown).

Next we asked whether the striking relationship between CTCF binding and *p16* gene transcription could be extended to other factors that may regulate *p16* expression. To address this, we examined association of the ubiquitous nuclear factor Sp1 with the *p16* promoter in both *p16*-expressing and non-expressing cell lines because this protein has been implicated in *p16* gene transactivation (Wu et al., 2007). Unexpectedly, we found strong Sp1 binding to the *p16* promoter in multiple myeloma cells (U266) where the gene is silent (Figure 2C). The extent of Sp1 interaction with *p16* was comparable to its association with the *p21* gene. To extend this analysis, Sp1 binding to the *p16* promoter was examined using real-time PCR. Quantitative analysis of Sp1 binding revealed that, unlike CTCF, there is no clear correlation between Sp1

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interaction with the *p16* promoter and its transcriptional activity (Figure 2D). From these data we conclude that transcriptional silencing of the *p16* promoter is not due to occlusion of binding to regulatory factors in general. Instead, we hypothesize that silencing more likely results from the spread of heterochromatin caused by loss of the upstream chromatin boundary that is maintained by CTCF binding. Dissociation of another promoter-bound activator, Sp1, does not have this effect.

CTCF Epigenetically Regulates the *p16* Promoter and Gene Expression

To investigate the functional role of CTCF at the *p16* upstream region, we used shRNA to decrease expression of CTCF in several cell lines that contain an active *p16* gene. Reduction of CTCF in each cell line was confirmed at the level of mRNA by RT-PCR (Figure 3A, upper panels) and protein by Western analysis (lower panels). Near complete ablation of cellular CTCF resulted in considerably reduced *p16* mRNA levels in fibroblasts (IMR90), cervical cancer cells (HeLa), and breast cancer cells (MDA-MB-435) whereas no effect on *p16* expression was observed in cells infected with a scrambled shRNA (Figures 3A, S4B). In addition, mRNA abundance of the *H19* gene was significantly decreased in each CTCF knockdown cell line, consistent with the demonstrated involvement of CTCF in *H19* expression (Szabo et al., 2004). Expression of the *GAPDH* gene, which served as a control for total mRNA abundance, was also unchanged by the absence of CTCF. In contrast to a previous report (Qi et al., 2003), we observed no change of the cell cycle inhibitor p27 transcript levels upon CTCF knockdown, which may reflect tissue-specific consequences of CTCF depletion. Unexpectedly, the CTCF target gene *c-Myc* remained impervious to loss of CTCF. This suggests that CTCF may have distinct functional roles at the *p16*, *H19*, and *c-Myc* genes with different requirements for continuous, compared to transient, binding.

To explore the possibility that CTCF may influence chromatin organization at the *p16* locus, we analyzed histone modifications at the *p16* promoter in breast cancer cells (MDA-MB-435) whose CTCF levels were ablated by shRNA treatment (Figure 3B). Most strikingly, we observed a significant reduction of the histone variant H2A.Z at the *p16* promoter upon loss of cellular CTCF (Figure 3B, upper panels) and an increase in Me^1H4K20 in the same region (lower panels). The loss of H2A.Z and 3′ shift of the repressive histone mark to the region downstream of the -2 kb boundary corresponds to the epigenetic characteristics of the silenced *p16* gene (Figure 1D) which no longer interacts with CTCF (Figure 2A) and apparently undergoes heterochromatin “spreading” from upstream regions. Thus, our results are consistent with the idea that CTCF binding is required to maintain a chromosomal boundary near -2 kb which preserves the *p16* gene in a transcriptionally active chromatin domain.

Pharmacological Treatment of Cancer Cells Restores Temporary *p16* Gene Transcription but not CTCF Binding

The *p16* tumor suppressor gene is commonly silenced in numerous types of human cancers and remains a relevant therapeutic target of wide interest. One method that is extensively employed to restore *p16* expression, both clinically and *in vitro*, is treatment of cancer cells with hypomethylating-nucleoside analogues such as 5′-AZA-2′-deoxycytidine (AZA) (Otterson et al., 1995), which reverses DNA methylation. We reasoned that treatment of cells with AZA might also restore CTCF binding at the *p16* upstream promoter through one of two mechanisms. First, CTCF is known to bind DNA in a methylation-sensitive fashion (Hark et al., 2000) thus, demethylation of the *p16* locus might allow CTCF to reassociate. Second, demethylation of target promoters by AZA can change the surrounding chromatin structure (Fahrner et al., 2002) which may facilitate rebinding of regulatory proteins, as observed for Sp1 (Zhang et al., 2005). We conducted a time course of p16 mRNA induction after AZA treatment of T47D breast cancer cells, which contain a methylated and silenced *p16* gene. Significant reactivation of *p16* expression occurred by 72 hours (Figures 3C, S4C). No

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synergistic *p16* gene reactivation was observed in cells treated with both AZA and the HDAC inhibitor Trichostatin A (Figure S4C).

To examine whether any changes in histone modifications or potential reassociation of CTCF had occurred after reversal of *p16* transcriptional silencing, a ChIP analysis was performed at 96 hours post-AZA treatment. As shown in Figure 3D, several alterations in nucleosome modification at the *p16* promoter were apparent. Notably, Me^1H4K20 and Me^3H3K4 were reversed in accordance with gene activity. However, AZA treatment did not result in recruitment of CTCF or H2A.Z to the reactivated *p16* gene, or Sp1 (data not shown). While AZA can reactivate *p16* transcription, it does not entirely reverse alterations that occur during gene silencing. This is consistent with a recent study showing only partial reversal of the histone code to an active state at the *MLH1* promoter after AZA treatment (McGarvey et al., 2006). In fact, the general inability to sustain long-term *p16* gene expression after reversal of epigenetic silencing by AZA (Egger et al., 2007) may, in part, be explained by failure to reestablish the upstream chromatin domain boundary by CTCF.

Absence of CTCF PARlation in *p16*-Silenced Cells

As described above, CTCF may be important for maintaining an active *p16* gene. To further investigate possible CTCF defects that may impact its function in *p16*-silenced T47D breast cancer cells, we examined its post-translational modifications and association with several known protein interaction partners. CTCF is post-translationally modified by phosphorylation and poly(ADP-ribosyl)ation (PARlation) and interacts with multiple proteins, such as Topoisomerase IIβ, Nucleophosmin and PARP-1 (Yusufzai et al., 2004). As shown in Figure 4A, similar extents of CTCF phosphorylation at both serine and tyrosine residues were observed in normal fibroblasts, *p16*-expressing MDA-MB-435 and non-expressing TD47 breast cancer cells as determined by immunoprecipitation. In addition, cellular levels of CTCF and several known interaction partners, Topo IIα, Topo IIβ, Nucleophosmin and PARP-1, were comparable in MDA-MB-435 and TD47 cells as well as a new interactor, Nucleolin (Figure 4B). Co-immunoprecipitation of CTCF complexes indicated that CTCF interacts with Topo IIβ and Nucleophosmin similarly in both cell types but has opposite interaction characteristics with PARP-1 which, surprisingly, only associates with CTCF in *p16* silenced cells (Figure 4C). Moreover, PARP-1 and Nucleolin appear to associate with CTCF in a mutually exclusive manner, with Nucleolin being present in CTCF complexes only in *p16* expressing cells. To explore this further, we examined the PARlation status of CTCF in each cell type using an antibody that recognizes ADP-ribose polymers (PAR). Unexpectedly, we found PARlation associated with both CTCF and Nucleolin in *p16*-expressing MDA-MB-435 cells but not in *p16*-silenced TD47 cells (Figure 4D, upper panel). This PARlation is inhibited upon addition of the PARlation inhibitor 3-Aminobenzamide (3-ABA) demonstrating the specificity of this reaction (Figure 4D, lower panel). Initially, it appeared counterintuitive that CTCF could associate with PARP-1 but be unPARlated in TD47 cells whereas in MDA-MB-435 cells the opposite was true, CTCF was PARlated and dissociated from PARP-1. We surmised that differences in CTCF-PARP-1 interaction dynamics might reflect defects in the poly(ADP) ribosylation enzymatic pathway in TD47 cells. To substantiate this, we performed an *in vitro* binding assay using recombinant PARP-1 and CTCF proteins with or without the obligate poly (ADP)ribosylation substrate, β-NAD^+, under enzymatic reaction conditions. Interestingly, we found that in the absence of β-NAD^+ a stable complex formed between CTCF and PARP-1 when co-precipitated. Yet when β-NAD^+ was present and CTCF PARlated, the CTCF/PARP-1 complex dissociated (Figure 4E). This supports the notion that upon CTCF PARlation, it dissociates from the PARP-1 enzyme as is observed in MDA-MB-435 cells. If the enzymatic reaction is not productive and CTCF remains unPARlated, the enzyme-substrate complex fails to release as seen in T47D cells (Figures 4C, 4D). An examination of total cellular proteins that are PARlated in either MDA-MB-435 or T47D extracts revealed a very similar pattern

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with the primary exceptions being two proteins in the size range of CTCF and larger whose modification is clearly impaired in T47D cells (Figure 4F). This indicates that while there are no apparent gross defects in the poly(ADP)ribosylation enzymatic machinery, its ability to react with specific protein substrates, such as CTCF, is deregulated. Further supporting this hypothesis is our finding that reintroduction of exogenous CTCF into T47D cells does not reestablish CTCF PARlation (Figure S5), possibly reflecting altered dynamics between PARP-1 Nucleolin, and Nucleophosmin.

Differential Patterns of PARlation and CTCF Partner Binding at the *p16* Gene

To determine whether known interacting partners of CTCF associate with the *p16* gene when active or epigenetically silenced, we performed ChIP analyses on MDA-MB-435 or T47D extracts using antibodies to Topo IIα, Topo IIβ, and PARP-1. As shown in Figure 5A, in *p16*-positive cells CTCF, Topo IIβ, and PARP-1 each bind to the *p16* gene in the region around -1 kb whereas no Topo IIα was detected in the distal or proximal promoter. At the CTCF binding site upstream of the *c-Myc* promoter, weak PARP-1 binding was observed but no association of Topo IIα or Topo IIβ. By contrast, in *p16*-negative cells not only is CTCF lost from the silent *p16* gene but Topo IIβ is also dissociated. Interestingly, PARP-1 remains bound to the inactive *p16* gene, apparently interacting independently of CTCF. PARP-1 binding to the CTCF site proximal to the *c-Myc* gene is also highly enriched in T47D cells. The presence of PARP-1 at the *p16* and *c-Myc* genes led us to examine the distribution of chromatin-bound PARlated proteins using an anti-PAR antibody. As shown in Figure 5B, PARlation is enriched at the -1 kb region of the expressed *p16* gene, (possibly indicating the presence of a PARlated CTCF), with low level PARlation within the proximal promoter. However when the *p16* gene is silenced upon loss of CTCF, the pattern of PARlation shifts from -1 kb to being highly enriched at the proximal promoter even though PARP-1 is still bound near -1 kb. This redistribution may reflect PARlation of heterochromatin components that are enriched after CTCF dissociates, such as histone H1. Notably, modification of these components is unaffected by the aberration in the pathway that prevents poly(ADP)ribosylation of CTCF, underscoring the specific nature of this defect. In contrast to the *p16* locus, no PARlation was observed at the *c-Myc* insulator site even in the vicinity of bound CTCF and PARP-1. These results indicate that separate CTCF binding sites are distinct from one another in terms of cofactor interactions and PARlation, potentially allowing CTCF to exert specialized regulatory functions on different target genes.

Next, we examined whether PARlation of target proteins impacts the expression of CTCF-regulated tumor suppressor genes. To achieve this, we perturbed cellular PARlation activity in p16-expressing cells by two approaches: first, incubation with the broad-spectrum PARP inhibitor 3-ABA and second, shRNA-mediated ablation of PARP-1. In both cases, we observed a significant reduction of p16 mRNA levels as well as a dramatic decrease of the new CTCF target gene *RASSF1A* (Figures 5C,D, 6A). Collectively, these data show that normal PARP activity is required for full activation of these CTCF target genes and a disruption of this pathway may play a role in the long-term silencing of these tumor suppressors.

Epigenetic Silencing of the Tumor Suppressor Genes *RASSF1A* and *CDH1* also Correlates with Loss of CTCF Binding

Having established that CTCF interaction upstream of the *p16* promoter is abolished in several different types of human cancer cell lines in which the gene is hypermethylated and silenced, we speculated that CTCF binding sites might be present at other genes commonly inactivated in cancer. To this end, we identified potential CTCF recognition sequences in the promoters of the *RASSF1A, CDH1* and *RARβ32* genes and analyzed these regions for expression (Figure 6A) and CTCF binding in several breast cancer cell lines. Similar to p16, the RASSF1A protein is a tumor suppressor and aberrant methylation of its gene may represent an early event in

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breast tumorigenesis (Strunnikova et al., 2005). As shown in Figure 6B (upper panels), a ChIP analysis of *RASSF1A*-positive cells clearly demonstrates recruitment of CTCF to a region upstream (~1.8 kb) of the promoter. However, in breast cancer cells in which *RASSF1A* is silenced and methylated no such binding of CTCF was detected.

Hypermethylation of *CDH1* in breast cancer results in a loss of *E-cadherin* expression (Graff et al., 1995) and is highly associated with an invasive and infiltrating phenotype (Shinozaki et al., 2005). *CDH1*-positive breast cancer cells were examined to determine whether CTCF was bound to the *CDH1* promoter when transcriptionally active. Again, we found CTCF binding at the immediate upstream (~200 bp) region of a gene commonly methylated in cancer (Figure 6B, middle panels). As with the *p16* and *RASSF1A* genes, CTCF binding was not detectable in cells where *CDH1* is hypermethylated (Figure 6B, middle panels). Interestingly, although CTCF is absent from the *RASSF1A* and *CDH1* promoters in MDA-MB-231 cells, it is still bound to its *c-Myc* site (Figure 6B, lower panels). This is consistent with our observations in other cell lines and indicates that CTCF in these cells is still functional to bind a subset of its target promoters even if it can no longer interact with specific tumor suppressor genes.

The *RARβ32* gene is another common target of hypermethylation in breast cancer (Bovenzi et al., 1999) and, along with *RASSF1A*, may be a useful marker of increased breast cancer risk. However, upon examination of *RARβ2*-positive cells by ChIP we were unable to find CTCF association within the *RARβ2* promoter or regions upstream (Figure 6C). From these data we conclude that loss of CTCF binding from critical sites is a common feature of several genes that are frequently silenced in human cancers, however this correlation does not apparently exist for all targets of aberrant hypermethylation.

Discussion

Our studies reveal an epigenetic mechanism of *p16* transcriptional control that is deregulated when the gene is aberrantly silenced in human cancer cells (Figure 7). We observed that the chromatin structure surrounding the active *p16* gene is highly organized with a discrete partition of histone modifications at approximately 2 kb upstream of the transcription start site. We were surprised to find highly enriched marks of repressed chromatin so close to a transcriptionally active gene. In *p16*-silenced breast cancer cells, partitioning of the *p16* upstream region into distinct chromatin domains is lost and accompanied by disappearance of H2A.Z and Me^3H3K4 within 2 kb of the inactivated promoter. This demonstrates that deregulation of epigenetic processes at silenced genes is not limited to DNA methylation and histone modification but can include placement of variant histones like H2A.Z. Upon loss of the -2 kb boundary, repressive histone marks spread throughout the entire *p16* promoter region. Overall, our analyses indicate that a chromatin boundary exists upstream of the *p16* gene which is destabilized in certain human cancer cells leading to aberrant transcriptional inactivation.

CTCF is a multifunctional protein that has previously been associated with establishing transitions between distinct chromatin domains and acting as a shield against the spread of heterochromatin (Bell et al., 1999). These studies, coupled with the detection of a chromatin boundary in the *p16* upstream region led us to look for CTCF binding in the *p16* promoter. Our analyses demonstrated that CTCF interaction with this region is strongly correlated with *p16* transcription in a variety of human cell types. shRNA-knockdown of CTCF revealed that it plays an active role in maintaining *p16* gene expression when associated near the upstream boundary, perhaps through stabilization of chromatin in this region. A dramatic loss of H2A.Z and gain of Me^1H4K20 upon depletion of CTCF emphasizes an integral epigenetic organizational function for CTCF at this locus. It also suggests that CTCF facilitates the stabilization or deposition of this histone variant. Considering that both CTCF and H2A.Z may

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play important structural roles, we speculate that these two proteins may act cooperatively to organize nuclear chromatin in a spatial manner.

Intriguingly, we observed that CTCF is absent from the p16 upstream region in multiple types of human cancer cells where the *p16* gene is silenced and methylated. We extended this finding to two other genes that are commonly silenced in cancer, *RASSF1A* and *CDH1* (*E-cadherin*). Together, this indicates that loss of CTCF binding to critical regions may be a common event in epigenetic silencing of cancer-related genes. Indeed, CTCF has been suggested to regulate other tumor suppressor genes such as *BRCA1* (Butcher et al., 2004) and *RB* (De La Rosa-Velazquez et al., 2007). Because CTCF is involved in blocking the spread of heterochromatin and directing interactions between chromosomes (Ling et al., 2006), its dissociation from these tumor suppressors may have multiple consequences that are detrimental to transcription and localized genomic stability. Furthermore, silencing of the *p16* gene is an early step in breast carcinogenesis (Foster et al., 1998), which can lead to subsequent genomic instability (McDermott et al., 2006) and downstream methylation events (Reynolds et al., 2006). Thereby making CTCF a potentially critical target in tumor progression.

Surprisingly, we found that the probable cause of impaired CTCF binding to the *p16* upstream region is defective poly(ADP-ribosyl)ation, resulting in the absence of CTCF PARlation in *p16*-silenced cells. Poly(ADP-ribosyl)ation has been shown to regulate multiple biological processes including DNA methylation, DNA repair, genotoxic stress, and epigenetic programming by post-translational modification of critical regulatory proteins and chromatin components (Kraus, 2008). In fact, inhibition of CTCF PARlation is correlated with failure to maintain *IGF2* gene imprinting and insulator function in general (Yu et al., 2004).

We find that in *p16*-expressing cells, PARlated CTCF dissociates from PARP-1 and is complexed with cofactors Topo IIβ, Nucleophosmin, and a new interactor, Nucleolin. Nucleolin is a multifunctional protein with roles in cell membrane signaling, ribosomal RNA processing within the nucleolus, chromatin remodeling and transcription (Mongelard and Bouvet, 2007). The functional connection between PARP-1, Nucleolin and Nucleophosmin is very intriguing. These proteins have been isolated as a complex, and PARP-1 and Nucleolin have been shown to organize genomic DNA into topologically distinct domains through interaction with matrix/scaffold attachment regions that anchor chromatin onto the nuclear matrix (Galande, 2002). Strikingly, unPARlated CTCF fails to release from PARP-1 and loses its association with Nucleolin, but not Topo IIβ or Nucleophosmin. Such a complex is apparently insufficient to generate the *p16* gene boundary even in the presence of chromatin-bound PARP-1. This is supported by previous work that underscored the importance of PARlation for proper CTCF insulator function (Yu et al., 2004). Our data also reveal that functionally distinct CTCF complexes associate with the *p16* and *c-Myc* genes that differ in the requirement for specific cofactors. These include PARP-1 and Topo IIβ, which can coregulate transcription of some genes through transient DNA breakage and repair mechanisms (Ju et al., 2006). Thus, the absence of CTCF binding to *p16*, or other epigenetically silenced genes may result from defects in specific post-translational modifications, such as PARlation, or cofactor interactions without affecting the majority of CTCF genomic functions.

As expected, AZA treatment reactivates *p16* transcription in non-expressing breast cancer cells. This was associated with an increase in Me^3H3K4 and a decrease of Me^3H3K9 but neither CTCF binding nor H2A.Z deposition was restored. These data may have clinical applications as AZA or AZA and HDAC inhibitors are incapable of completely restoring the normal histone code (McGarvey et al., 2006) or post-translational modifications of CTCF or other proteins to reestablish long-term expression of tumor suppressors, thus limiting their usefulness as therapeutic agents.

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Overall, our results substantiate the critical role of CTCF in establishing and maintaining *p16* and other tumor suppressor genes in higher-order chromosomal domains through appropriate boundary formation. These data raise the possibility that dissociation of CTCF from *p16* during early tumorigenesis is not due to DNA methylation alone but may result from loss of PARlated CTCF that impairs the ability of CTCF to act as a functional component of a boundary or insulator element. This would result in secondary changes in chromatin structure that are incompatible with CTCF binding to DNA. When this integrity is breached by destabilized CTCF binding and loss of long-range epigenetic organization, aberrant gene silencing can ensue. Thus, the ability to restore CTCF interactions at vulnerable gene loci may have important therapeutic implications. Current efforts are now focused on targeted pharmacological intervention to restore CTCF PARlation and potentially reverse silencing of *p16* and other tumor suppressor genes in human cancer cells.

Experimental Procedures

Cell Culture

All cell lines were maintained as described in Supplemental Materials.

Western Blotting and RT-PCR

Nuclear extracts were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes, and blotted using various antibodies. For RT-PCR assays, cDNA was made from 500ng of total RNA using the Superscript II kit (Invitrogen). Antibody sources and primer sequences used to amplify specific genes and amplification conditions are available in Supplemental Materials.

Chromatin Immunoprecipitations

ChIPs were performed according to the Upstate Biotechnology protocol with some modifications as described in Supplemental Materials.

Co-Immunoprecipitations

2mg of whole cell lysates were diluted in IP buffer with 0.5% Triton X-100. Protein mixes were precleared for 1-2 hours with protein G Sepharose after which the beads were removed and CTCF (Upstate) or anto-phosphotyrosine (Upstate) antibody added overnight at 4°C to capture complexes. Complexes were recovered with protein G Sepharose, washed 4 times in IP buffer and subsequently analyzed by SDS-PAGE.

In Vitro Binding of PARP-1 and CTCF

Reactions were performed such that PARP-1 was catalytically active in presence of 1mM β-NAD^+. Reaction buffer contained 20mM Tris-HCl, pH 8.0, 1mM $MgCl_2$, 1mM DTT, 50ng salmon sperm DNA, 50ng BSA. 250ng of recombinant CTCF (isolated from overexpressing NIH3T3 cells) or PARP-1 (Alexis Biochemicals) protein was added where appropriate. Binding was carried out at 30°C for 1 hour. At this time reactions were diluted in 0.5% Triton IP buffer and CTCF was immunoprecipitated as described.

CTCF and PARP-1 Knockdown

CTCF knockdown was achieved using pSHAG-MAGIC2 retroviral vectors (OpenBiosystem) and PARP-1 knockdown using Mission Lentiviral shRNAs (Sigma) as described in Supplemental Materials.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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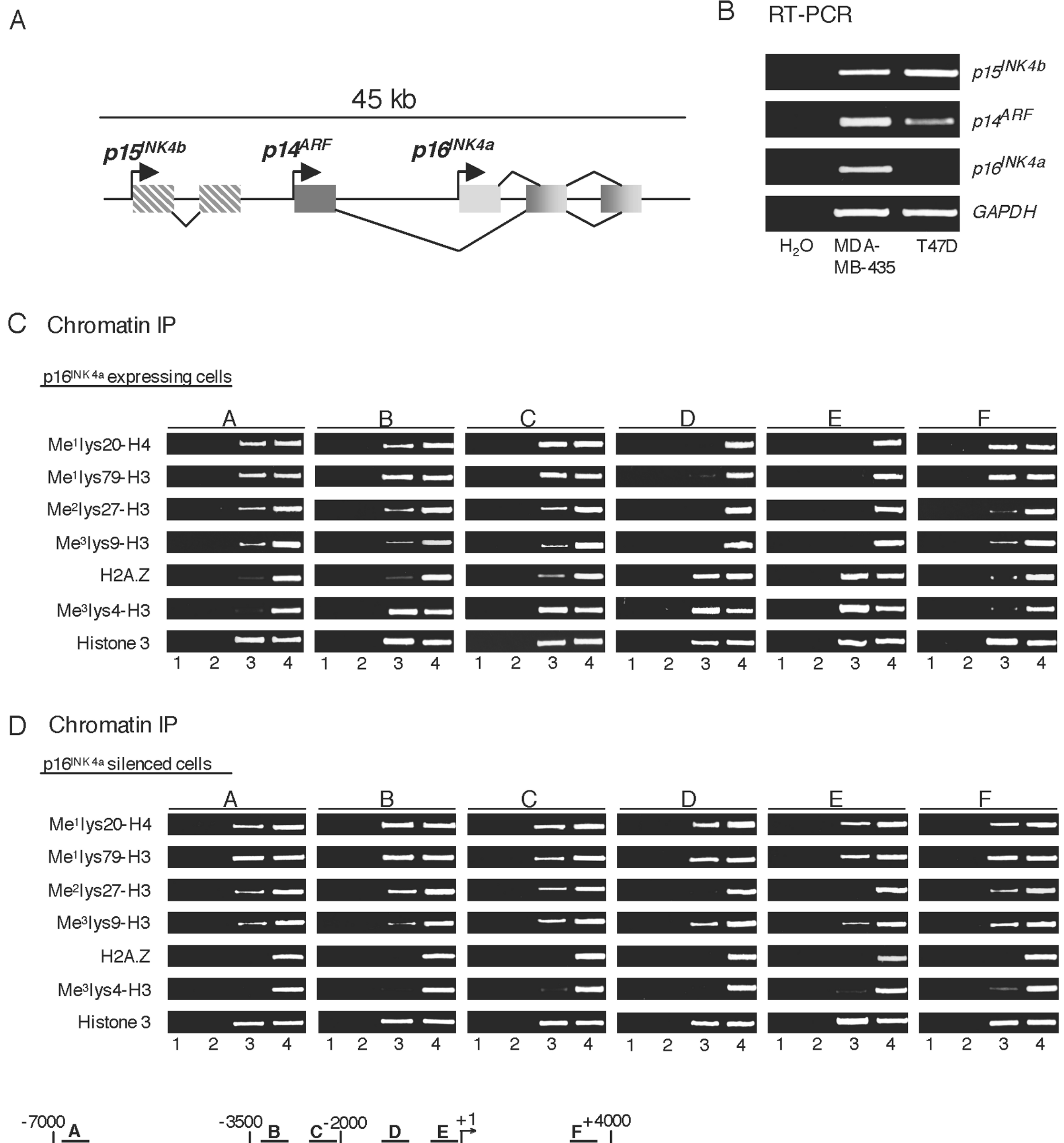

Figure 1.

Analyses of Histone Modifications at the *p16* Gene

(A) Diagram of gene organization at the *INK4/ARF* chromosomal locus.

(B) RT-PCR analysis of gene expression at the *INK4* locus in *p16*-expressing (MDA-MB-435) and non-expressing (T47D) breast cancer cells.

(C) ChIP analyses of histone modifications surrounding the *p16* gene in *p16*-expressing cells. Lanes: 1, H_2O; 2, no antibody; 3, antibodies to various histone modifications; 4, 1.6% total input DNA. ChIP-enriched DNA was PCR-amplified using specific amplicons (A-F) distributed throughout the *p16* gene locus.

(D) A similar ChIP analysis in *p16* non-expressing cells.

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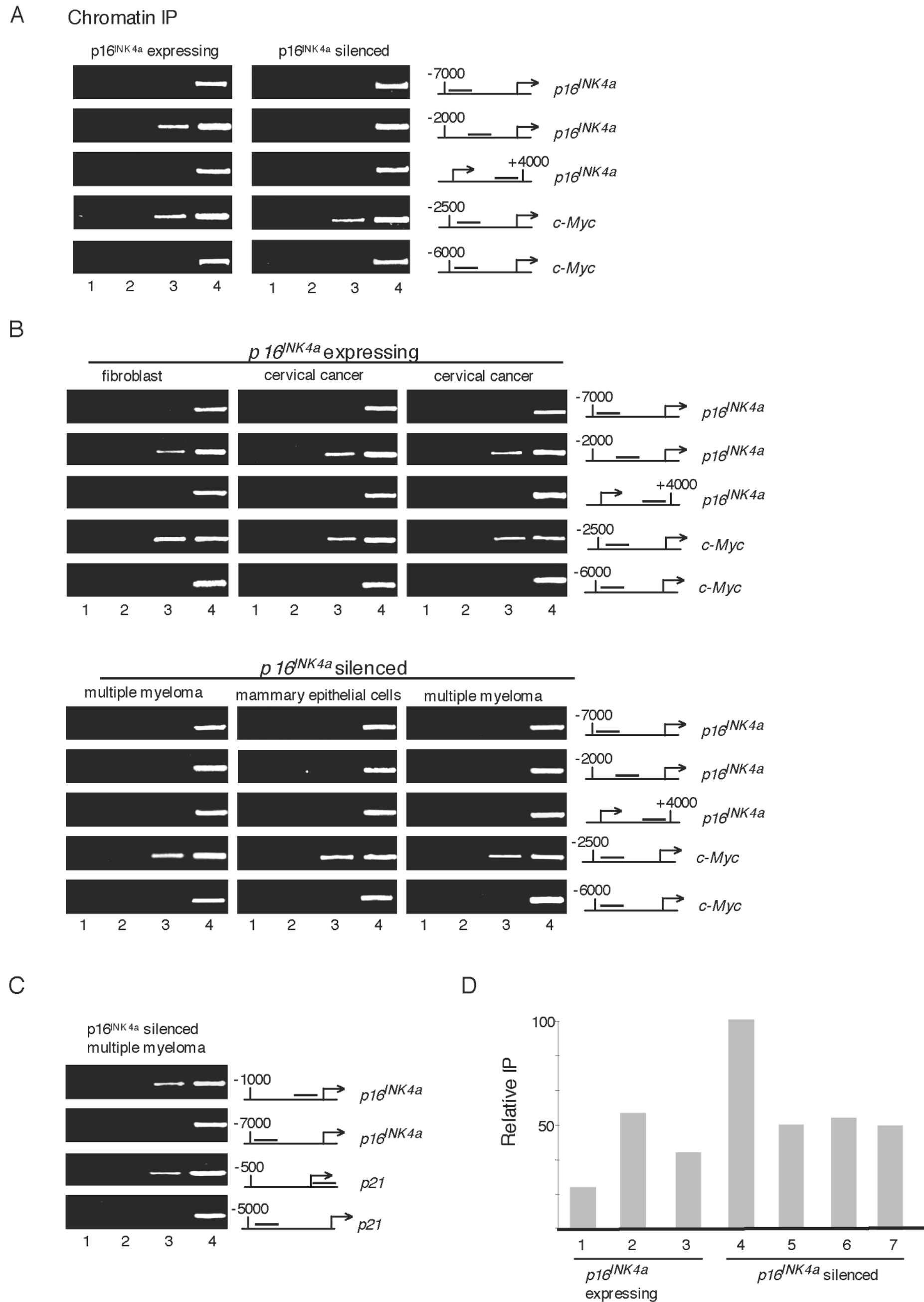

Figure 2.

CTCF Associates with the Active but not Silent *p16* Gene

(A) ChIP analysis of CTCF binding in p16-expressing and non-expressing breast cancer cells. CTCF binding was also measured at the *c-Myc* gene in both cell lines. Lanes: 1, H_2O; 2, no antibody; 3, anti-CTCF antibody; 4, 1.6% input DNA.

(B) CTCF Binding Correlates with *p16* Expression in Multiple Types of Human Cancer Cells. ChIP analyses showing binding to the *p16* gene in human primary fibroblasts (IMR90) and human cervical cancer cell lines (HeLa, C33A), but reduced association in *p16* non-expressing cells: human multiple myeloma (KMS12, U266) and a primary breast epithelial-derived cell line (vHMEC). Lanes 1-4 are described as in (A).

(C) ChIP analyses of Sp1 binding to the *p16* promoter in multiple myeloma (U266) cells. *p21* is a positive control. Lane order is as described in (A).

(D) qPCR ChIP analyses of Sp1 binding reveal no clear correlation between Sp1 interaction and *p16* expression. Lanes correspond to Sp1 IPs from the following cells: 1, cervical cancer (HeLa); 2, human primary fibroblasts (IMR90); 3, breast cancer (MDA-MB-435); 4, multiple myeloma (U266); 5, multiple myeloma (KMS12); 6, breast cancer (T47D); 7, primary breast epithelial-derived (vHMEC).

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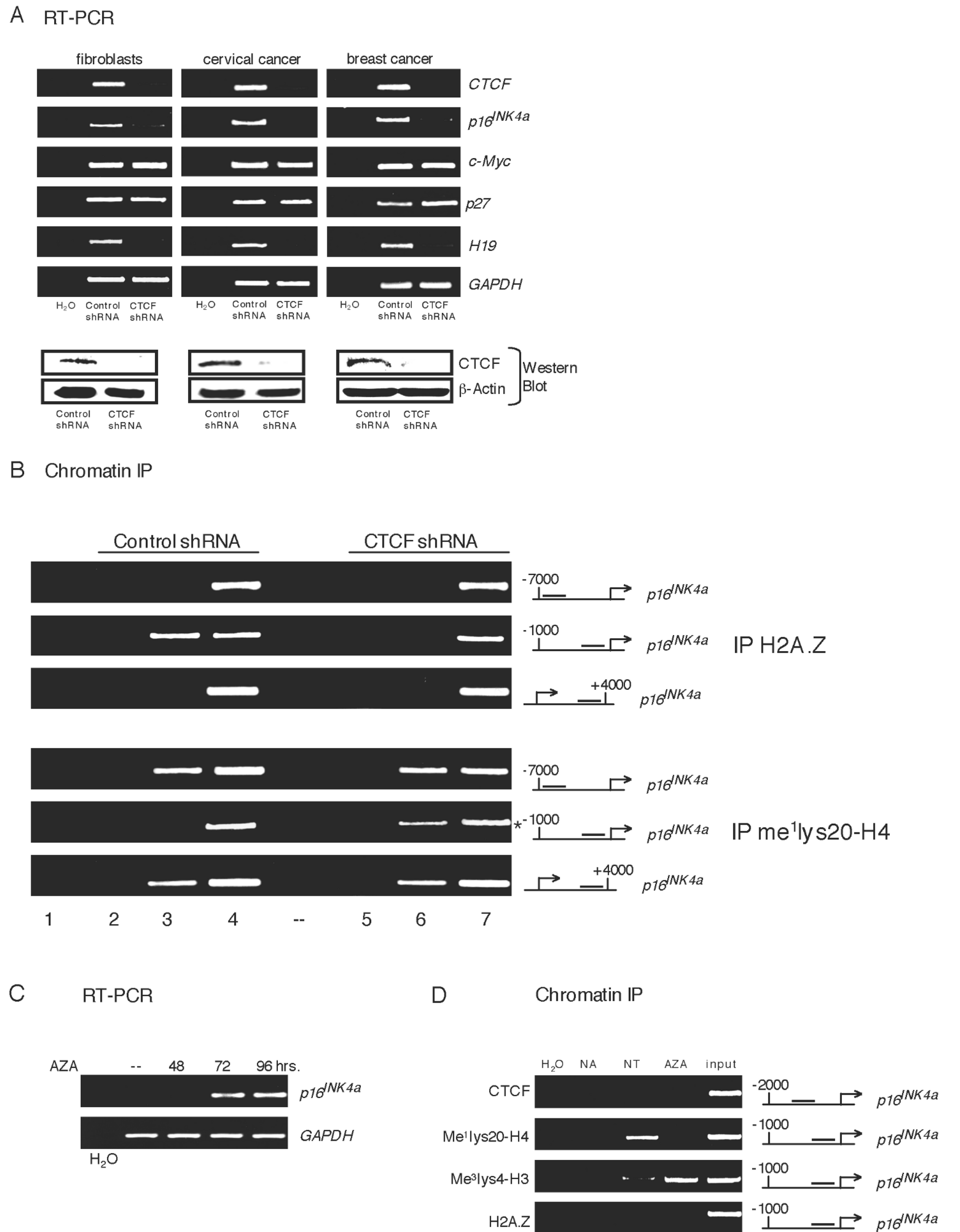

Figure 3.

CTCF Knockdown Results in Transcriptional Silencing of the *p16* Gene and Acquisition of Repressive Chromatin Modifications

(A) RT-PCR analysis of mRNA from *p16*-expressing cells infected with either control (scrambled) or CTCF-specific shRNA.

Lower panel: Western blot of CTCF protein expression in these shRNA-infected cells.

(B) ChIP analysis of the *p16* gene after CTCF knockdown in *p16*-expressing cells (MDA-MB-435) using antibodies to the histone variant H2A.Z and Me^1H4K20. Reactions from cells infected with scrambled shRNA (lanes 2-4) and CTCF-specific shRNA (lanes 5-7) are designated.

Lanes: 1, H_2O; 2, no antibody; 3, anti-H2A.Z antibody; 4, 1.6% input DNA; 5, no antibody; 6, anti-Me^1H4K20 antibody; 7, 1.6% input DNA; * denotes 0.25% input DNA in lanes 4 and 7 for this panel *(p16* proximal promoter) only.

(C) RT-PCR analysis showing time course of AZA induction of *p16* mRNA in breast cancer cells (T47D) containing a silenced *p16* gene.

(D) ChIP analysis of the *p16* promoter in T47D cells treated with AZA for 96 hours using the indicated antibodies. NA = no antibody, NT = no AZA treatment, AZA = AZA treatment, input = 1.6% of total DNA.

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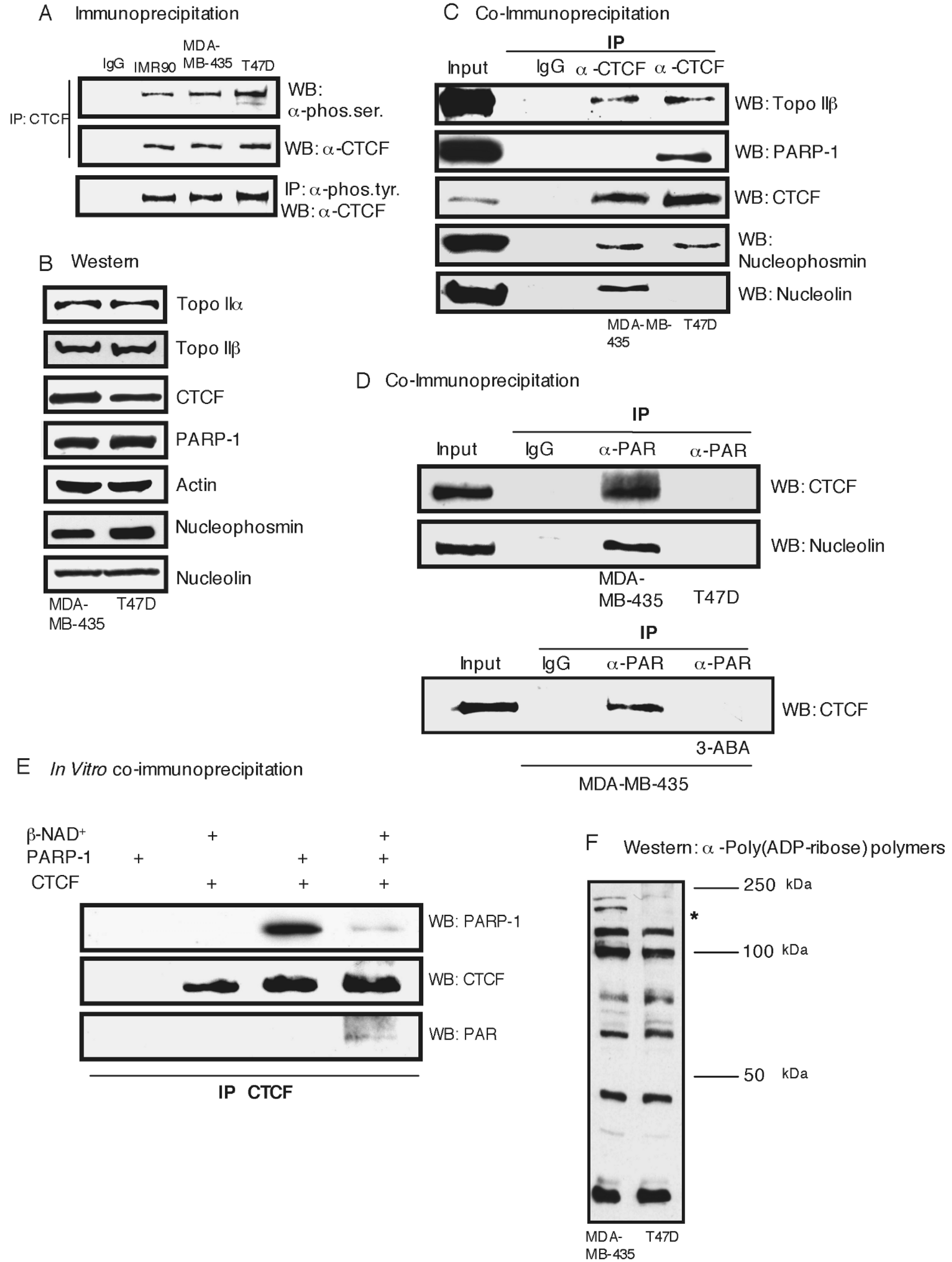

Figure 4.

CTCF is Differentially Poly(ADP)ribosylated in *p16* Expressing and Non-Expressing Breast Cancer Cells.

(A) Immunoprecipitations show similar levels of CTCF phosphorylation on serine (left panel) and tyrosine (right panel) residues in multiple cell lines.

(B) Western blot showing protein levels of CTCF and putative interacting partners in MDA-MB-435 and T47D cells.

(C) Co-IP of CTCF with Topo IIβ, PARP-1, Nucleophosmin and Nucleolin.

(D) Anti-PAR antibody co-IPs CTCF and Nucleolin in MDA-MB-435 cells. Bottom panel shows similar IP using material treated with 3-ABA for 24 hours. Inputs are equal to 2.5% starting material.

(E) *In vitro* binding of recombinant CTCF and PARP-1. IP of CTCF from the reactions following protein binding assays.

(F) Western blot of poly(ADP)ribosylated proteins in MDA-MB-435 and T47D cells. Asterisk denotes protein with similar molecular weight as CTCF that is differentially PARlated in MDA-MB-435 and T47D cells.

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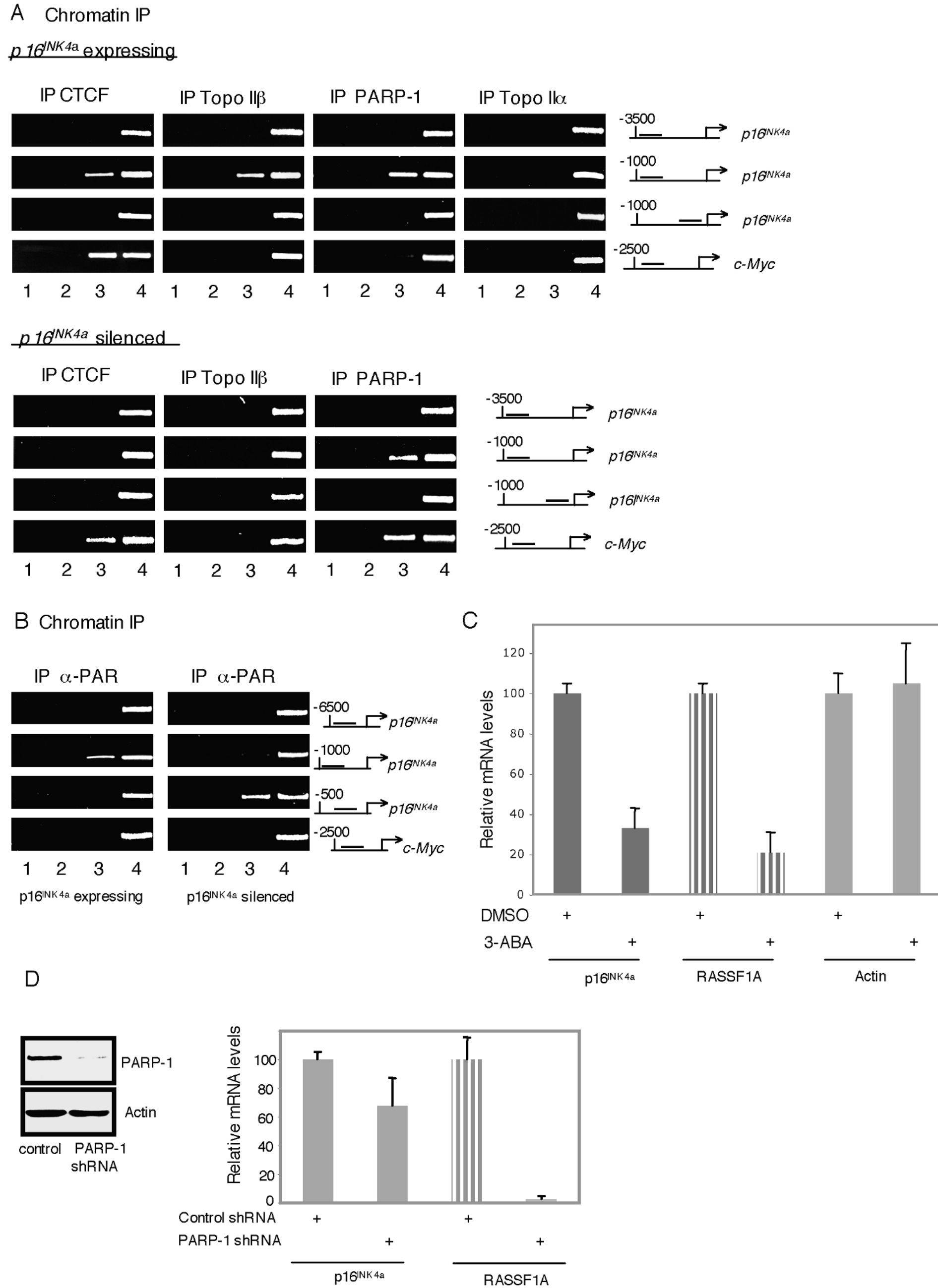

Figure 5.

Pattern of PARlation at the *p16* Promoter Region Changes in *p16* Silenced Cells.

(A) ChIP analyses of CTCF and putative interacting partners at the *p16* and *c-Myc* genes in *p16* positive MDA-MB-435 and *p16* silenced T47D cells. Lanes: 1, H_2O; 2, no antibody; 3, anti-H2A.Z antibody; 4, 1.6% input DNA;

(B) ChIP analyses of PARlation pattern at the *p16* and *c-Myc* genes in the same cells as described in (A). Lane order is as in (A) but showing amplification of 0.25% input material.

(C-D) qPCR expression analysis of the CTCF target genes *p16* and *RASSF1A* upon inhibition of PARP activity. Results are normalized to c-Myc levels. Error bars represent ± STDEV (C) Amplification of mRNA from MDA-MB-435 cells treated for 24 hours with 5 mM 3-ABA.

(D) Amplification of mRNA from MDA-MB-435 cells infected with shRNA directed towards PARP-1.

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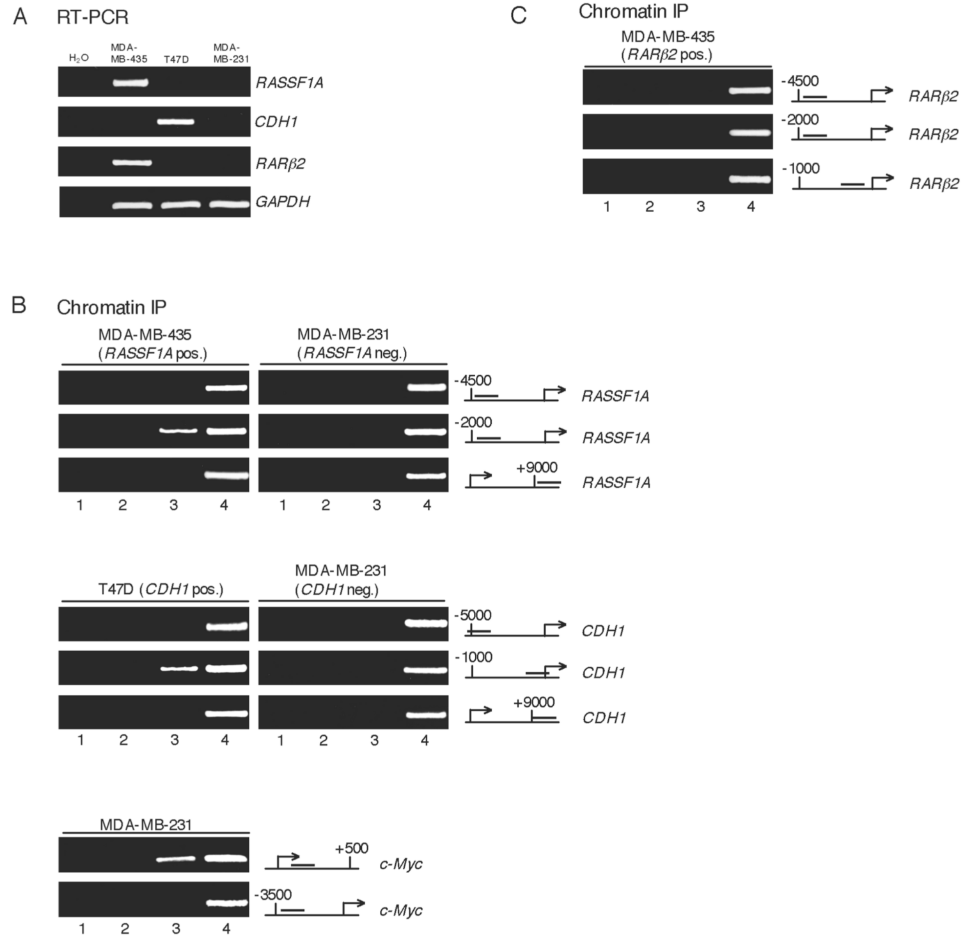

Figure 6.

Loss of CTCF Binding from Genes that are Commonly Silenced in Human Cancers

(A) RT-PCR of *CDH1, RASSF1A* and *RARβ2* expression in the indicated breast cancer cells.

(B) ChIP analyses of CTCF binding to the *RASSF1A* and *CDH1* genes in expressing cells but not in cells where the genes are silenced. Lanes: 1, H_2O; 2, no antibody; 3, anti-CTCF antibody; 4, 1.6% input DNA.

(C) CTCF does not bind to all genes commonly hypermethylated in cancer such as *RARβ2*. Lane order is as described in (B).

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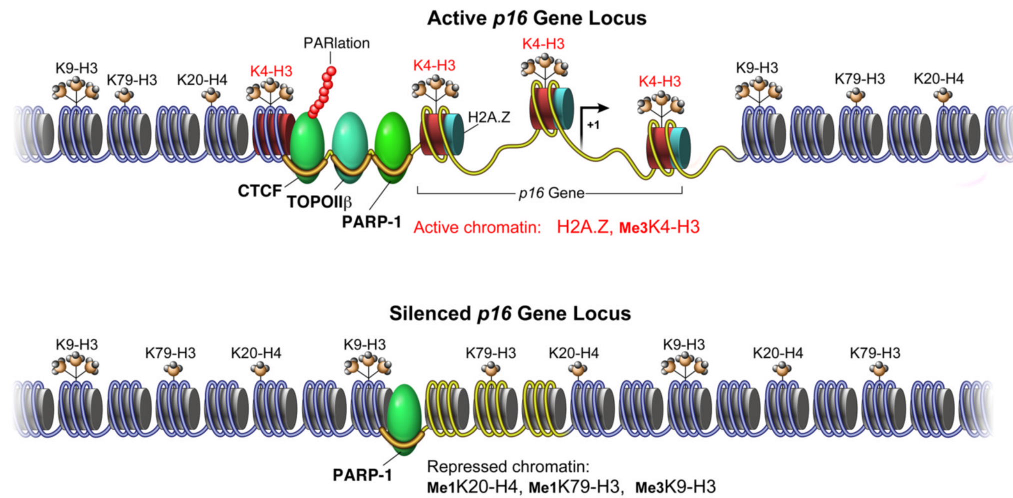

Figure 7.

Model of CTCF Function in Aberrant Tumor Suppressor Gene Silencing in Human Cancers. See text for discussion.