**F) Supplemental Text and Figures**



Supplemental Material

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

Michael Witcher and Beverly M. Emerson

**Supplemental Experimental Procedures**

**Cell Culture**

U266, KMS12 and MDA-MB-435 cell lines were maintained in RPMI-1640 media supplemented with 10% FBS. HeLa S3, C33A, IMR90, MDA-MB-231 and T47D cells were grown in DMEM with 10% FBS. vHMEC cells were maintained in MCDB 170 with supplements as previously described (Hammond et al., 1984). T47D cells were treated with 5′-AZA-2′-deoxycytidine (Sigma) at a final concentration of 10μM. We thank Drs. H.P. Koeffler (U266), T. Otsuki (KMS12), C. Spruck (MDA-MB-435, T47D), K. Jones (C33A), M. Stampfer (vHMEC), and J. Karlseder (IMR90) for providing cell lines.

**Western Blotting and RT-PCR**

Nuclear extracts were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes, and blotted using antibodies against CTCF, Poly(ADP-Ribose) polymers (Upstate), Actin (Sigma), Topo IIβ, PARP-1, Nucleolin, Nucleophosmin (Santa Cruz) and phospho-serine (Zymed). For RT-PCR assays, cDNA was made from 500ng of total RNA using the Superscript II kit (Invitrogen).

**Chromatin Immunoprecipitations**

ChIPs were performed according to the Upstate Biotechnology protocol using 2 x $10^6$ cells per reaction with some modifications. Amplification of DNA was carried out within a linear range of all primers. Antibody sources for ChIP: bulk H3, H2A.Z, me1H3K79, me1H4K20 (Abcam), me2H3K27, me3H3K9, me3H3K4, CTCF, Poly(ADP-ribose) polymers (Millipore) and Topo IIβ, PARP-1 (Santa Cruz).

**CTCF and PARP-1 Knockdown**

pSHAG-MAGIC2 retroviral vectors encoding CTCF specific or scrambled shRNAs were purchased from OpenBiosystem. Plasmid vectors were transfected into Phoenix amphotropic packaging cells using calcium phosphate/chloroquine-mediated precipitation. Supernatant containing viral partides was collected 48 hr post-transfection. Cells were infected with retrovirus and polybrene on two sequential days. 72 hrs after viral exposure, successfully infected cells were selected using puromycin for a further 72 hrs. Protein and mRNA were collected and ChIP experiments performed within two passages after puromycin selection.

PARP-1 knockdown was achieved using the MIssion Lentiviral shRNA system from Sigma. Lentiviral particles were packaged in Hek293T cells, with virus collected 24 hours post-transfection. Cells infected with shRNA-containing virus and polybrene were selected using puromycin starting at 72 hours post infection.

**Supplemental Experimental Procedures continued**

**Inhibition of Transcription**
MDA-MB-435 cells were treated with Flavopiridol (Sigma) or Actinomycin D (Sigma) at 1μM and 2.5 μg/ml respectively for 24 hours. At this time cells were harvested and analyzed for gene expression and CTCF binding.

**Immunofluorescence**
Immunofluorescence was performed with a Zeiss Axioplan 2 microscope using software from Openlab and Improvision as previously described (Verdun et al., 2005) except that cells were fixed with a 90:10 mix of methanol-acetic acid on ice. CTCF antibody (Upstate) was used at a 1:200 dilution and secondary FITC-coupled anti-rabbit antibody (Jackson Laboratories) at a 1:300 dilution.

**Bisulphite Sequencing**
2.5 μg genomic DNA was digested with ECORV followed by repurification. DNA was denatured at 95°C for 15 minutes, cooled on ice and then denatured with 0.3M NaOH at 37°C for 20 minutes. After this hydroquinone was added to a final concentration of 1.3 mM and sodium metabisulphite to a final concentration of 3M. Reaction mixes were subjected to the following heating procedure: 4 times in thermal cycler at 55°C 4hr, 90°C 2 min, 20°C 10 min. Next, DNA was isolated from reation mix using DNA binding columns (Qiagen). Resupended DNA was treated with NaOH at a concentration of 0.3M for 20 minutes at room temperature. Sodium acetate (pH 5.4) was added to a concentration of 3M and DNA precipitated with etahanol. Recovered DNA was resuspended in water and amplified using primers specific for bisulphite modified DNA.

**Expression of CTCF in T47D Cells**
Full length CTCF cloned from IMR90 cells was inserted into an HA-tagged lentiviral packaging vector. Lentivirus was produced and delivered as described in Experimental Procedures of the main text. The parent vector was also used to infect cells as a control. Anti-HA Western blots were done using the F-7 antibody from Santa Cruz.

Table S1 Primer sets used for ChIP experiments

Figure 1.

Primer set A (p16 -6707 to -6420): #1 TATTCCTCCATTGCCTTTGC
#2 TGGGGTGATGCATTCTGATA

Primer set B (p16 -2871 to -2689): #1 ACTCTCCCACCCCCATTAAG
#2 ATGCTGCCATACCCAGCTAA

Primer set C (p16 -2360 to -2187): #1 GAGAGGTACCCCGAGGAAAA
#2 CCCTGGTTGACTTAAACCTTGT

Primer set D (p16 -1000 to -847): #1 TGGTCTTTGGATCACTGTGC
#2 TAATACGGACGGGGGAGAAT

Primer set E (p16 -635 to -243): #1 GGGCTCTCACAACTAGGAA
#2 CGGAGGAGGTGCTATTAACTC

Primer set F (p16 +3373 to +3524): #1 TCACAGTGCTCTCTGCCTGT
#2 ACACAAGCCCCAGGTGTCTA

Figure 2.

Primer sets for p16 as described for figure 1.

c-Myc CTCF insulator site (-2254 to - 2061): #1 GCCATTACCGGTTCTCCATA
#2 CAGGCGGTTCCTTAAAACAA

c-Myc CTCF negative (-5819 to - 5712): #1 GCCATTACCGGTTCTCCATA
#2 CAGGCGGTTCCTTAAAACAA

p21 gene (+1 to - +196): #1 CCGAAGTCAGTTCCTTGTGG
#2 CTGTGAACGCAGCACACAC

p21 gene (-4434 to - -4206): #1 TTTGCTTCTGGGCAGAACTT
#2 GGGGCTGCCTATGTAGTGAA

Figure 3. Primer sets as described above.

Figure 5. Primer sets as described above.

Figure 6.

RASSF1A gene (-1764 to -1533): #1 TATAGCCTGGGCAAGTCCTG
#2 GTACAGGGCGATCCACACTT

RASSF1A gene (-4218 to -4086 ): #1 ATTCAGGAGCTGCTGGTCAC
#2 TGTCCTTCAGGGAGAACAGG

RASSF1A gene (+9030 to -9290 ): #1 GCCTAGCCCCAAGTAGACTG
#2 TGGGCAGGTAAAAGGAAGTG

CDH1 gene (-186 to +30 ): #1 TAGAGGGTCACCGCGTCTAT
#2 TCACAGGTGCTTTGCAGTTC

Table S1 continued Primer sets used for ChIP experiments

| |
|---|
| Figure 6. continued |
| CDH1 gene (-5120 to -4913): #1 TCAGGAGCCTCTAGGAGCAG<br>#2 TCAGGCAGTCTTGTCCCTTT |
| CDH1 gene (+9187 to +9366): #1 TCAGGAGCCTCTAGGAGCAG<br>#2 TCAGGCAGTCTTGTCCCTTT |
| RARβ2 gene (-5104 to -4888): #1 ACAATTTTGTGCGTCCATCA<br>#2 ACCAATGGCCAGCTGTTAAG |
| RARβ2 gene (-1631 to - 1476): #1 ACTCTCCCTCCCTGCCTAAC<br>#2 CAATCTACCCTGCAGCCATT |
| RARβ2 gene (-423 to - 259): #1 AAGGCGCACAGAGGAATTTA<br>#2 TTAAAATGAGCAGGGGAGGA |

Table S1. Sequence and Genomic Location of Primers Used to Amplify Immunoprecipitated Material in ChIP Analyses.

**Figure S1**

A Bisulphite sequencing

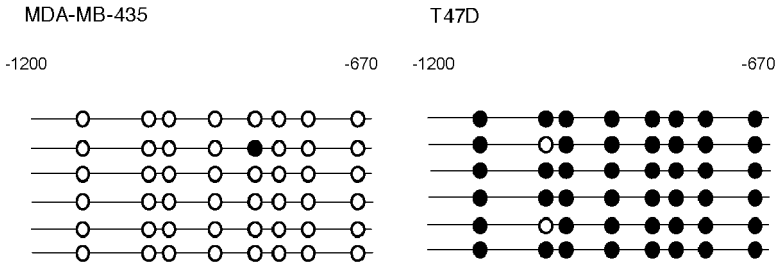


Figure S1. (A) Methylation Analysis of the *p16* Upstream Region. Schematic diagram showing results of bisulphite sequencing of the CTCF-associated region upstream of the p16 gene in MDA-MB-435 and T47D cells.

**Figure S2**

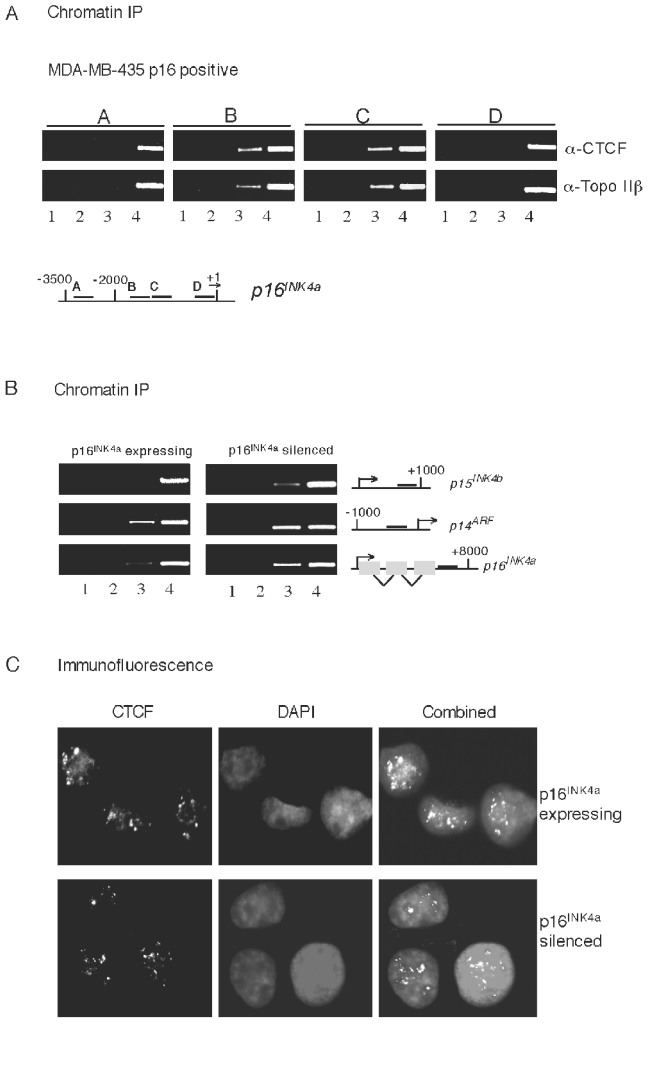
A
Chromatin IP
MDA-MB-435 p16 positive
A
B
C
D
α-CTCF
α-Topo IIβ
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
-3500 A -2000 B C D +1
p16INK4a
B
Chromatin IP
p16INK4a expressing
p16INK4a silenced
+1000
p15INK4b
-1000
p14ARF
+8000
p16INK4a
1 2 3 4 1 2 3 4
C
Immunofluorescence
CTCF
DAPI
Combined
p16INK4a expressing
p16INK4a silenced

Figure S2. Analyses of CTCF Binding and Cellular Localization.
(A) Chromatin IP using anti-CTCF antibody localizes CTCF binding to a region approximately 1kb upstream of the p16 start site. The CTCF binding partner Topo IIβ also binds this region. Lanes are as follows: 1. $H_2O$ control 2. No antibody control 3. IP using anti-CTCF antibody 4. 1.6% input
(B) ChIP using CTCF specific antibody. Amplification of known CTCF sites demonstrates a different binding pattern in T47D ($p16^{INK4a}$ silenced) and MDA-MB-435 cells ($p16^{INK4a}$ expressing) at these loci than is observed at the $p16^{INK4a}$ gene. Lanes are as described in (A).
(C) CTCF staining using immunofluorescent antibodies in MDA-435 (top panels) and T47D cells (bottom panels).

**Figure S3**

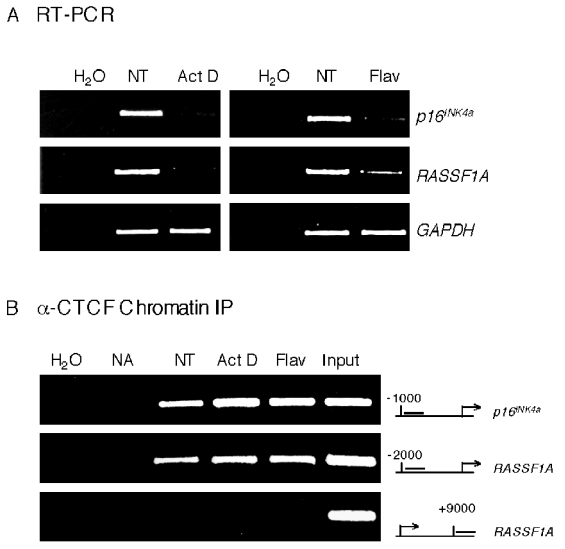


Figure S3. Inhibition of Transcription does not Impact CTCF Binding.
(A) RT-PCR showing inhibition of p16 and RASSF1A transcripts in response to 24 hour treatments of MDA-MB-435 cells with Actinomycin D or Flavopiridol.
(B) ChIP analyses of MDA-MB-435 and T47D cells treated with 2.5μg/ml Actinomycin D or 1μM Flavopiridol for 24 hours. CTCF was immunoprecipitated and analyzed for association with the *p16$^{INK4a}$* and *RASSF1A* gene. NA represents no antibody control.

**Figure S4**

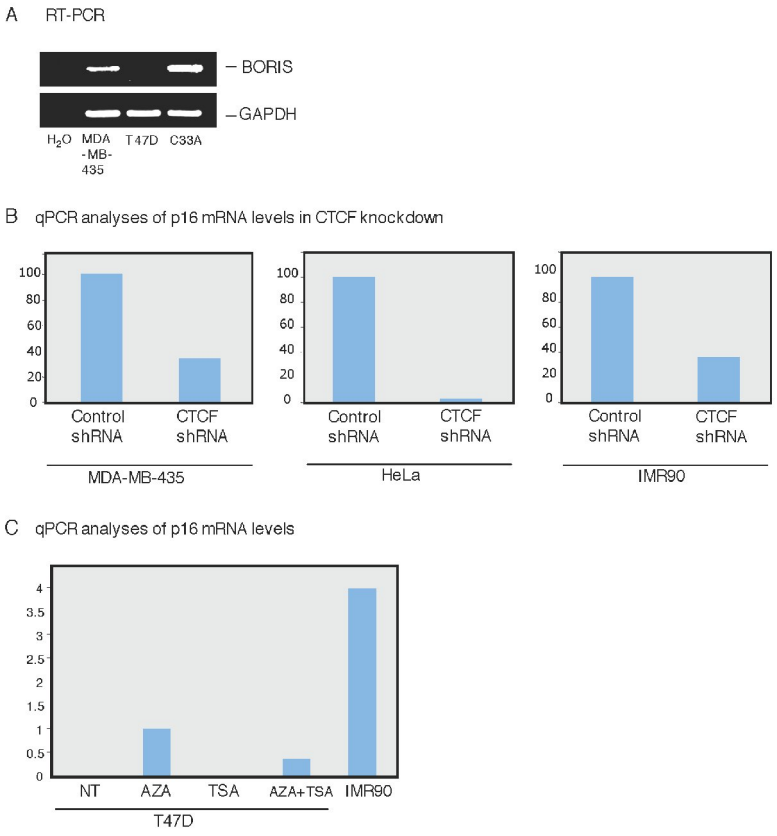


Figure S4. Quantification of p16 mRNA Levels by qPCR.
(A) RT-PCR analysis of BORIS expression in human cancer cells. BORIS expression does not correlate with p16 silencing in T47D cells.
(B) qPCR analyses of p16 mRNA levels in CTCF knockdown cells. All cells types studied show significant reduction of p16 transcripts with most pronounced reduction observed in Hela cells. mRNA levels are normalized to 18S mRNA.
(C) qPCR analyses of p16 mRNA levels in T47D cells treated with AZA and trichostatin A. Cellular p16 levels were not restored to physiological levels in response to AZA as demonstrated by comparison to IMR90 p16 levels.

**Figure S5**

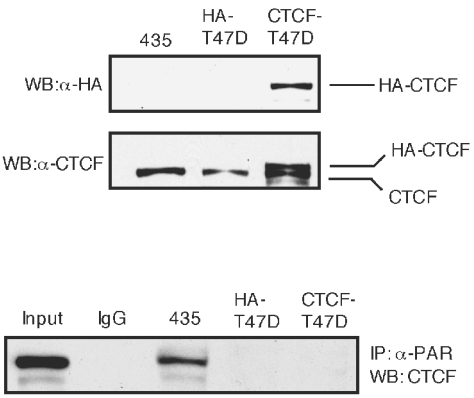


Figure S5. Analysis of Expression and PARlation of Full Length Recombinant CTCF in T47D Cells. CTCF was introduced using a lentiviral delivery system. Immunoprecipitations were done using an anti-PAR antibody on control cells infected with an empty vector and on CTCF expressing cells. MDA-MB-435 cells acted as a positive control.

**Supplemental References**

Verdun, R. E., Crabbe, L., Haggblom, C., and Karlseder, J. (2005). Functional human telomeres are recognized as DNA damage in G2 of the cell cycle. Mol Cell *20*, 551-561.

Hammond, S. L., Ham, R., G., and Stampfer, M., R., (1984). Serum-free growth of human mammary epithelial cells: rapid clonal growth in defined medium and extended serial passage with pituitary extract. Proc Natl Acad Sci 81(17), 5435 - 5439.

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