Loss of Imprinting of Insulin-like Growth Factor-II in Wilms' Tumor Commonly Involves Altered Methylation but not Mutations of CTCF or Its Binding Site

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Advances in Brief

Loss of Imprinting of *Insulin-like Growth Factor-II* in Wilms' Tumor Commonly Involves Altered Methylation but not Mutations of *CTCF* or Its Binding Site[1]

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Abstract

Loss of imprinting (LOI) is the most common molecular abnormality in Wilms' tumor (WT), other embryonal cancers, and most other tumor types. LOI in WT involves activation of the normally silent maternal allele of the *insulin-like growth factor-II* (*IGF2*) gene, silencing of the normally active maternal allele of the *H19* gene, and aberrant methylation of a differentially methylated region (DMR) upstream of the maternal copy of *H19*. Recently, the transcription factor CTCF, which binds to the *H19* DMR, has been implicated in the maintenance of *H19* and *IGF2* imprinting. Here, we show that mutations in the *CTCF* gene or in the *H19* DMR do not occur at significant frequency in WT, nor is there transcriptional silencing of *CTCF*. We also confirm that methylation of the *H19* DMR in WT with LOI includes the CTCF core consensus site. However, some WTs with normal imprinting of *IGF2* also show aberrant methylation of CTCF binding sites, indicating that methylation of these sites is necessary but not sufficient for LOI in WT.

Introduction

Genomic imprinting is an epigenetic modification of a specific parental chromosome in the gamete or zygote that leads to preferential expression of genes on that chromosome in somatic cells of the offspring. Several genes important in cancer are imprinted, including *IGF2*,[3] $p57^{KIP2}$, and *ARH1* (1–5). We and others have shown previously that LOI occurs commonly in cancer and can lead to activation of the silent copy of growth promoting genes such as *IGF2* (2, 3) or silencing of the active copy of growth inhibitory genes such as $p57^{KIP2}$ (4). We and others have also shown that LOI of *IGF2* in WT is linked to aberrant methylation of a DMR upstream of the *H19* gene (6, 7). One of the effects of methylation of this DMR that has been shown in normal cells is the abrogation of binding of the transcription factor CTCF (8–11), and CTCF can discriminate differentially methylated DMRs on the paternal *versus* maternal alleles *in vivo* (8).

The *CTCF* gene product was originally identified as a transcription factor for *myc* and other genes (12) and later also was found to be an insulator protein that isolates enhancers from promoters, leading to transcriptional repression (13). Recently, four groups simultaneously reported that CTCF is also involved in the regulation of the *Igf2*/*H19* imprinting cluster (8–11). Binding of CTCF to the *H19* DMR prevents the access of one or more enhancers telomeric to the *H19* gene, preventing their interaction with the *Igf2* promoter (8–11). Insulator activity is abolished by methylation of the *H19* DMR in mouse, leading to activation of *Igf2* in reporter constructs (8–11). Interestingly, both CTCF and its binding sequences in the *H19* DMR are conserved between human and mouse, suggesting that a similar mechanism may apply in humans.

Because of the association of *CTCF* with the regulation of normal imprinting, we examined this gene in WT with LOI, comparing to normal fetal kidney the tissue from which WT are derived. The complete coding sequence of *CTCF* and flanking intronic sequence was examined for mutations in 25 samples. In addition, CTCF binding sites were examined for mutations, and levels of *CTCF* mRNA was assayed by RTQ-PCR. Finally, genomic bisulfite sequencing was performed to examine DNA methylation. Here we show that CTCF disruption does not commonly involve genetic alterations in the sequence of the *CTCF* gene or its binding site within the *H19* DMR. We also confirm by bisulfite sequencing that the methylation we previously observed in the *H19* DMR (6) includes site-specific methylation of the CpGs within the CTCF binding sites that is known to abrogate CTCF binding. Thus, functional disruption of *CTCF* in WT arises most commonly by an epigenetic rather than a genetic mechanism.

Materials and Methods

DNA and RNA Preparation. DNA and RNA were isolated from tissues snap-frozen in liquid nitrogen. DNA was extracted as described (14). RNA was prepared using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. All specimens were obtained from Johns Hopkins Hospital, the Cooperative Human Tissue Network, or the University of Washington Fetal Tissue Bank.

Detection of Mutation. To detect mutation of both the *CTCF* gene and CTCF binding sites upstream of *H19*, direct PCR sequencing of genomic DNA was carried out. For the *CTCF* gene, the entirety of all coding exons as well as flanking intronic sequence were screened. About 200 ng of genomic DNA were amplified using the primers listed in Table 1 under the following conditions: 94°C for 1 min; 36 PCR cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. Rather than analyze only CTCF binding sites within the *H19* DMR, we performed sequence analysis of the entire DMR, corresponding to GenBank nucleotides 2057 to 8070 (accession no. AF087017). The primers used are provided in Table 1 and the same conditions described above were used. All of the PCR products were purified using the QIAEX II gel extraction kit (Qiagen) and directly sequenced with an ABI Prism 377 DNA sequencer using the BigDye Terminator Cycle Sequencing kit and following the manufacturer's protocol (PE Applied Biosystems, Foster City, CA).

Detection of IGF2 LOI. Total RNA was treated with RNase-free DNase I (Ambion, Austin, TX) in a reaction containing 10 μg of RNA, 4 μl of 10× DNase I buffer, 1 μl (40 units) of RNasin (N211; Promega, Madison, WI), and 4 μl of DNase I and incubated at 37°C for 25 min with subsequent heat inactivation at 65°C for 15 min. The treated RNA was then extracted with

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[3] The abbreviations used are: IGF2, insulin-like growth factor-II; LOI, loss of imprinting; WT, Wilms' tumor; DMR, differentially methylated region; LOH, loss of heterozygosity; SNP, single nucleotide polymorphism; RTQ-PCR, real-time quantitative polymerase chain reaction.

Table 1 *PCR primers used for sequence analysis of CTCF and H19 DMR*

Sequence[a]	Forward primer	Reverse primer
C1	5′-CATCAAGAGCACATGTCTGTTGTG-3′	5′-TGCACTGTGTTGTATGCTTATCC-3′
C2	5′-GGTCGTTATGTGGGTACCGTTC-3′	5′-ACCAGGCATCTATTGCCTGAGAC-3′
C3	5′-TCCAGTCTCATAGCAGTTCTGTGC-3′	5′-ATCTTAAGTCCGTTTGGGTAGTAG-3′
C4	5′-GCTTTTGTGCCTAACCTACTGTGC-3′	5′-CTGAACAACGAATTCAGAGGATATGC-3′
C5	5′-TCTCTGTGGTGTAGCTATTCTG-3′	5′-TGTTATGAGAGTCAGAAGGTGAAGT-3′
C6	5′-GAATCGAGAAATGTATTAGTAACTTG-3′	5′-GGTGACATTCCTCATAATCCACAG-3′
C7	5′-CGTGTGGAGTCTAGACCTAGCTTGG-3′	5′-CCATGCTCTGCAGAGGAAGAC-3′
C8	5′-TCAGGACACACTTAGCAGATACTAG-3′	5′-GCTCCAAAGCCAGCCATAGTAAGC-3′
C9	5′-TTCATCTTCCACCACCCTTCTC-3′	5′-GACTTCCTCAGATGTTCCTCAGT-3′
H1	5′-ATCTTGCTGACCTCACCAAGG-3′	5′-CGATACGAAGACGTGGTGTGG-3′
H2	5′-CCGACTAAGGACAGCCCCCAAA-3′	5′-TGGAAGTCTCTGCTCTCCTGTC-3′
H3	5′-ACAGTGTTCCTGGAGTCTCGCT-3′	5′-CACTTCCGATTCCACAGCTACA-3′
H4	5′-ACAGGGTCTCTGGCAGGCTCAA-3′	5′-ATGAGTGTCCTATTCCCAGATG-3′
H5	5′-AACTGGGGTTCGCCCGTGGAA-3′	5′-CAAATTCACCTCTCCACGTGC-3′
H6	5′-GATCCTGATGGGGTTAGGATGT-3′	5′-GGAATTTCCATGGCATGAAAAT-3′
H7	5′-GGTCTGCCTTGGTCTCCTAACT-3′	5′-GGCCACTTTCCTGTCTGAAGAC-3′
H8	5′-CAGTCTCCACTCCACTCCCAAC-3′	5′-GACCTCTCCCTCCCAGACCACT-3′

[a] C, *CTCF* exon number; H, arbitrarily divided segments within the *H19* DMR.

Table 2 *SNPs identified within the H19 DMR*

Position[a]	−2089	−2131	−2550	−2574	−2660	−2740	−2755	−2794	−2905	−2948
SNP	A/C	C/T	A/G	A/G	A/C	A/G	A/G	A/C	A/G	A/C
Frequency (%)	29	37	19	12	25	30	14	30	22	39

[a] bp upstream of *H19* transcriptional start site.

phenol/chloroform. RT-PCR and IGF2 imprinting analysis were done as described previously (14).

Identification of LOH. LOH on chromosome 16 was identified using microsatellite marker D16S3095. PCR was carried out using 1 μl of genomic DNA (~0.1 μg) in a final volume of 10 μl containing 0.1 μM of each primer, 0.15 mM dNTP, 1.5 mM $MgCl_2$, 1× PCR buffer, and 0.06 units Taq polymerase (LTI, Bethesda, MD). In each reaction, one primer was 5′-end-labeled. The PCR products were analyzed on 6% denatured polyacrylamide gels.[4]

RTQ-PCR. RTQ-PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems) in a 25-μl reaction containing 12.5 μl of 2× Taqman Master Mix, 900 nm of forward and reverse primers, and 200 nm of Taqman probe, according to the manufacturer's recommendation. Primers to detect *CTCF* mRNA were designed to span an intron-exon boundary (exons 9–10; GenBank accession nos. AF145476 and AF145477): 5′-CAGAACCAACCAGCCCAAA-3′ and 5′-AACTATAATGTTCTCAATTGCACCTGTATT-3′. The TaqMan probe VIC-AACCAGCCAACAGCTATCATTCAGGTTGAA-TAMRA also spanned the exon-intron boundary. The input amount of cDNA was normalized using a Taqman primer-probe set for *β-actin* (Applied Biosystems).

Analysis of DNA Methylation. To confirm that the previously reported methylation of the *H19* DMR (6, 7) included the CTCF binding region, we performed bisulfite genomic sequencing. Bisulfite treatment was carried out using the CpG Genome DNA Modification kit (Intergen, Purchase, NY) with the following modifications of the manufacturer's protocol: denatured genomic DNA (~4 μg) was incubated at 55°C in the dark overnight in 1100 μl of freshly prepared Reagent I, with subsequent column purification with the QIAquick PCR purification kit (Qiagen). Purified DNA was treated at 37°C for 15 min with freshly prepared 3 M NaOH to a final concentration of 0.3 M NaOH. Then the DNA was precipitated with ethanol and dissolved in 40 μl of 10 mM Tris (pH 8)-1 mM EDTA for nested PCR. PCR products were purified on 2% agarose gels for direct sequencing as described above. The annealing temperature was 55°C. The first round of PCR primers were: 5′-GTATAGGTATTTTTGGAGGTTTTTTA-3′ and 5′-CCTAAAATAAATCAAACACATAACCC-3′. The second PCR primers were: 5′-GAGGTTTTTTATTTTAGTTTTGG-3′ and 5′-ACTATAATATATAAACCTACAC-3′. For sequencing individual clones, the PCR products were subcloned into a TA Cloning vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and 10–15 clones were selected for sequencing.

[4] These primer sequences were obtained at Internet address: http://www.ncbi.nlm.nih.gov.

Results

No Mutations of the *CTCF* Gene in WT. To test the hypothesis that the *CTCF* gene might be mutated in WT, we first screened all of the coding sequence (10 exons) and flanking intronic sequence of *CTCF* in 15 WT patients with LOI. Although one polymorphism in the 3′-UTR was identified, none of these patients' tumors exhibited any change in the coding sequence (Table 3). Thus, mutation of *CTCF* is not a common mechanism of LOI in WT.

We and others previously reported chromosome 16 as an infrequent locus for LOH in WT (15, 16). Loss of *16q* is a marker for poor prognosis (16), suggesting that it harbors an important tumor progression gene for WT. Because *CTCF* maps to *16q* (17), we thought that if *CTCF* is that gene, sequence analysis of tumors with LOH of *16q* might enrich for those malignancies with mutations of *CTCF*. We therefore analyzed 98 WTs for LOH of *16q* using a microsatellite marker near and telomeric to *CTCF*. Ten of these tumors (10%) exhibited LOH of *16q*, which is consistent with previous studies (15, 16).

DNA samples from all ten of these tumors were sequenced over all 10 coding exons of *CTCF*, including the flanking intronic sequence. As with the nonselected set of WTs, none of these tumors showed mutations in *CTCF*. Therefore, *CTCF* is not mutated at appreciable frequency in WT, and *CTCF* does not appear to be the WT tumor suppressor gene on *16q*.

CTCF mRNA Levels Are Comparable in WT with and without LOI. Because no *CTCF* mutations were observed in WT, we compared levels of *CTCF* mRNA quantitatively between WTs with normal imprinting and those with LOI of *IGF2*. There was no significant difference in the expression level of *CTCF* between these two groups. The average normalized expression level for WTs with normal imprinting ($n = 24$) was 2.62 ± 2.66 (relative units normalized to *β-actin*), compared with 2.72 ± 2.26 for WTs with LOI ($n = 24$; not statistically significant; two-tailed *t* test). However, tumors overall showed a 2.2-fold higher level of expression compared with fetal kidney (2.68 ± 2.44, $n = 48$ compared with 1.22 ± 0.35, $n = 26$; $P = 0.0035$), although no significant difference was seen comparing

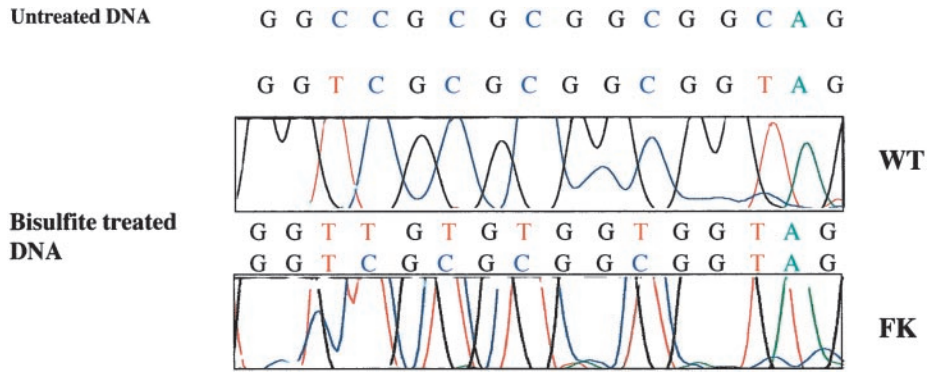

Fig. 1. Complete methylation of a CTCF binding site in *H19* DMR in WT. *Top*, wild type genomic DNA sequence. Bisulfite treated DNA, derived from a WT with LOI and from a normal fetal kidney (FK), was PCR amplified and analyzed by DNA sequencing. Note that the unmethylated cytosine is chemically converted to uracil by bisulfite treatment, leading to a thymine in the chromatogram, whereas methylated cytosine remains unchanged after bisulfite treatment. In WTs with LOI of *IGF2*, all of cytosines in CpG dinucleotides were methylated, whereas in fetal kidney with normal imprinting, the cytosine in CpG dinucleotides was half methylated. The chromatogram is shown at high amplitude for ease of peak discrimination, although they are truncated on the display. The same results were obtained from individual sequencing of cloned PCR products.

tumors overall to matched normal kidney from the same patients ($n = 48$; paired t test).

No Mutations of the *H19* DMR in WT. As frequent mutations were not found in *CTCF*, we examined the DMR upstream of the *H19* gene to which CTCF binds (8, 11). This region in humans is located from 5.2 to 0.3 kb upstream of the start site of transcription. We used direct PCR sequencing to the entire DMR, *i.e.*, from 5.2 to 0.3 kb upstream of the start site, in 15 WTs with LOI of IGF2. No somatic mutations were found anywhere within the region in any tumors (Table 3), although 10 distinct SNPs were identified that were present in both tumor and normal DNA (Table 2). Thus, neither CTCF nor the DMR with which it associates are mutated at appreciable frequencies in WT.

Methylation of the *H19* DMR in WT with LOI Includes the CTCF Binding Region. The *H19* DMR has previously been shown to be methylated on the paternal allele in normal tissues and methylated on both alleles in WT with LOI (6, 7). Hypomethylation of this region has already been shown to be sufficient to insulate *IGF2* from its enhancer, leading to biallelic expression of *IGF2* (8–11). However, the analysis of WT has been performed only at the low level of resolution achievable by the use of restriction endonuclease digestion with CpG-methylation-sensitive enzymes (6, 7). Therefore, we used bisulfite genomic sequencing to analyze in detail the methylation status of the sequence from −5315 bp to −5153 bp upstream of the start site of transcription of *H19*, which contains the canonical CTCF binding sites. Seven of seven WTs with LOI showed hypermethylation in this region (Fig. 1, Table 3). In contrast, all eight normal fetal kidneys examined showed normal half-methylation as expected (Fig. 1 and data not shown).

Furthermore, in half of cases, it was possible to distinguish the alleles directly by the use of a single-nucleotide polymorphism. For example, as shown in Fig. 2, an A/G polymorphism distinguishes maternal and paternal alleles. In these cases, we sequenced individual clones from each sample. In normal fetal tissues, only the paternal allele was methylated, and the methylation extended throughout the 163 bp analyzed, including the core CTCF binding site within this region. As with the direct analysis of the bisulfite-treated product, sequencing of individual clones from tumors with LOI also showed biallelic methylation throughout the entire domain.

Despite the hypermethylation of these sites in all WTs examined with LOI, we were surprised to find that WTs with *normal* imprinting of *IGF2* did not necessarily show a normal pattern of methylation of the *H19* DMR. Thus, of nine tumors examined with normal imprinting of *IGF2*, four showed a normal pattern of monoallelic methylation, as expected, but five tumors showed a biallelic methylation pattern (Table 3).

Table 3 *Genetic and epigenetic analysis of CTCF in WT*

Analysis	Tumors examined	Tumors altered
CTCF mutation in WTs with LOI	15	0
CTCF mutation in WTs with LOH	10	0
H19 DMR mutation in WTs with LOI	15	0
DMR hypermethylation in WTs with LOI	7	7
DMR hypermethylation in WTs with normal imprinting	9	5

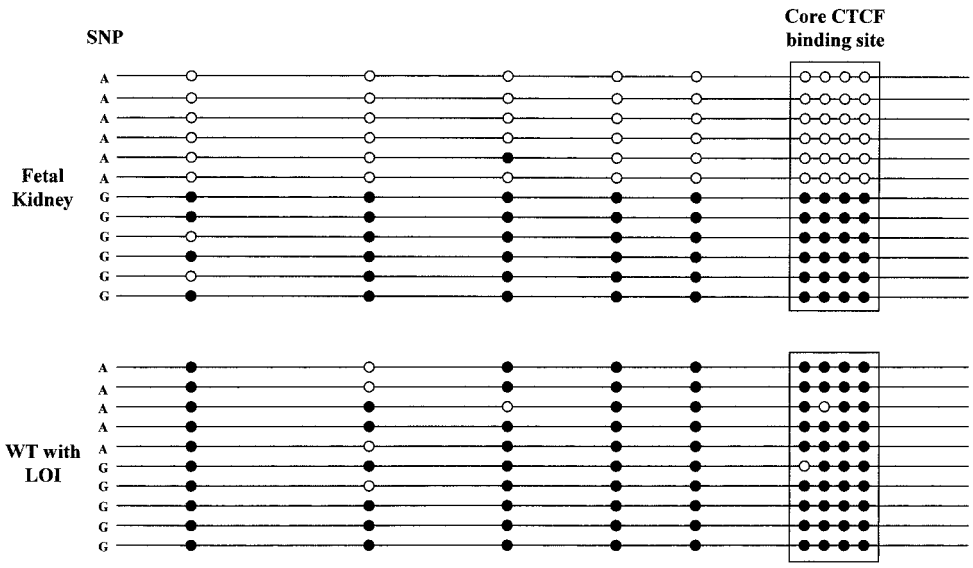

Fig. 2. Allele-specific methylation status in fetal tissue and WT. Bisulfite treatment and PCR of genomic DNA was the same as in Fig. 1, but the PCR products were subcloned prior to sequencing, to link a single-nucleotide polymorphism to the CTCF binding site. Ten to 15 clones were sequenced for each sample. Each *line* represents a separate clone. ●, methylated CpG sites; ○, unmethylated CpG sites. *Boxed area*, the core CTCF-binding site. Monoallelic methylation was observed in fetal tissues, whereas biallelic methylation was seen in WTs with LOI of *IGF2*.

Discussion

There are two major results of this report. First, we show that *CTCF* mutations were not found in any of 15 WTs with LOI. In support of this result, we also examined 10 additional tumors with LOH involving chromosome 16, the chromosome on which *CTCF* is localized. In none of these cases were mutations found, indicating that these mutations may not occur at significant frequency.

However, we and our collaborators have found two rare missense mutations in CTCF zinc fingers 3 and 7 among 59 WTs selected for *16q22* LOH. These mutations were clearly functional, because they resulted in a selective loss of CTCF binding to the *H19* DMR but not to the *β-globin* gene insulator.[5] Nevertheless, the low frequency of these events suggests that genetic disruption of *CTCF* itself is rare in WT, and there must be another tumor suppressor gene on *16q*.

Similarly, there was no significant difference in levels of *CTCF* mRNA in WT with LOI, compared with tumors with normal imprinting, although there was an ~2-fold increased level of expression of *CTCF* in WT overall. We also report here that *cis*-acting CTCF target sequences within the *H19* DMR also did not show mutations in any of 15 tumors analyzed. However, 10 polymorphisms were identified within the *H19* DMR at frequencies of 12–39% (Table 2).

The second major result of this report is that methylation of the *H19* DMR includes CTCF sequences in WTs with LOI. Although this is not a surprising result, inasmuch as altered DNA methylation of this region has been shown at a gross level by us and others (6, 7), we confirmed this observation by bisulfite sequencing. It has been shown previously that methylation can disrupt the action of the CTCF insulator (8–11), and therefore methylation of these sequences is a potential mechanism for LOI in tumors. However, whether this is the initial change in tumors with LOI, or other epigenetic changes are important, remains to be determined. For example, there are two DMRs within the *IGF2* gene that may serve independent roles in the regulation of *IGF2* imprinting in cancer. Recently, altered methylation of a CTCF binding site in the *H19* DMR was described by Nakagawa *et al.* (18) in colorectal cancers with LOI of *IGF2*. However, we had previously shown that LOI of *IGF2* affects both tumor and matched normal mucosa of such patients (14). However, in the study of Nakagawa *et al.* (18), normal methylation was generally observed in the normal mucosa with LOI, again consistent with the idea that CTCF is only one of several factors involved in the disruption of genomic imprinting in cancer. In addition, that study did not examine all potential CTCF binding sites, nor did this; and it will be important to couple such analyses with detailed functional studies of CTCF binding as well as with analysis of DMRs within *IGF2* itself. The results obtained here may also direct future studies to the role of aberrant methylation of *CTCF* target sequences in the deregulation of other potential target sites, such as the *INK4a* and *myc* genes.

Unexpectedly, we found that about half of WTs with normal imprinting of *IGF2* also showed aberrant methylation of the *H19* DMR. This is consistent with the idea that aberrant methylation is necessary but not sufficient, and that CTCF is only one of several factors involved in the disruption of genomic imprinting in cancer. Our finding of the general absence of conventional genetic alterations suggests that, unlike the analysis of conventional tumor suppressor genes in cancer, future studies of imprinting of *IGF2* in cancer must be focused upon epigenetic alterations of the sequences that regulate genomic imprinting.

[5] G. N. Filippova, H. Cui, C. F. Qi, D. I. Loukinov, E. M. Pugacheva, J. E. Ulmer, J. M. Moore, Y. J. Hu, H. Moon, J. Breen, P. E. Grundy, *et al.* Cancer-associated selective disruption of complexes formed by the multifunctional CTCF protein and varying DNA targets involves mutations in the multivalent Zinc finger domain of the protein or binding abrogation by aberrant CpG-methylation of the targets, submitted for publication.

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