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Promoter histone H3K27 methylation in the control of *IGF2* imprinting in human tumor cell lines

Tao Li[1,3,4,†], Huiling Chen[2,4,†], Wei Li[3], Jiuwei Cui[3], Guanjun Wang[3], Xiang Hu[1], Andrew R. Hoffman[4,‡,*] and Jifan Hu[3,4,*,‡]

[1]Shenzhen Beike Cell Engineering Research Institute, Shenzhen, China [2]Department of Endocrinology, Xiangya Hospital, Central South University, Changsha, Hunan, China [3]Stem Cell and Cancer Center, The First Affiliated Hospital, Jilin University, Changchun, China [4]VA Palo Alto Health Care System and Stanford University Medical School, Palo Alto, CA 94304, USA

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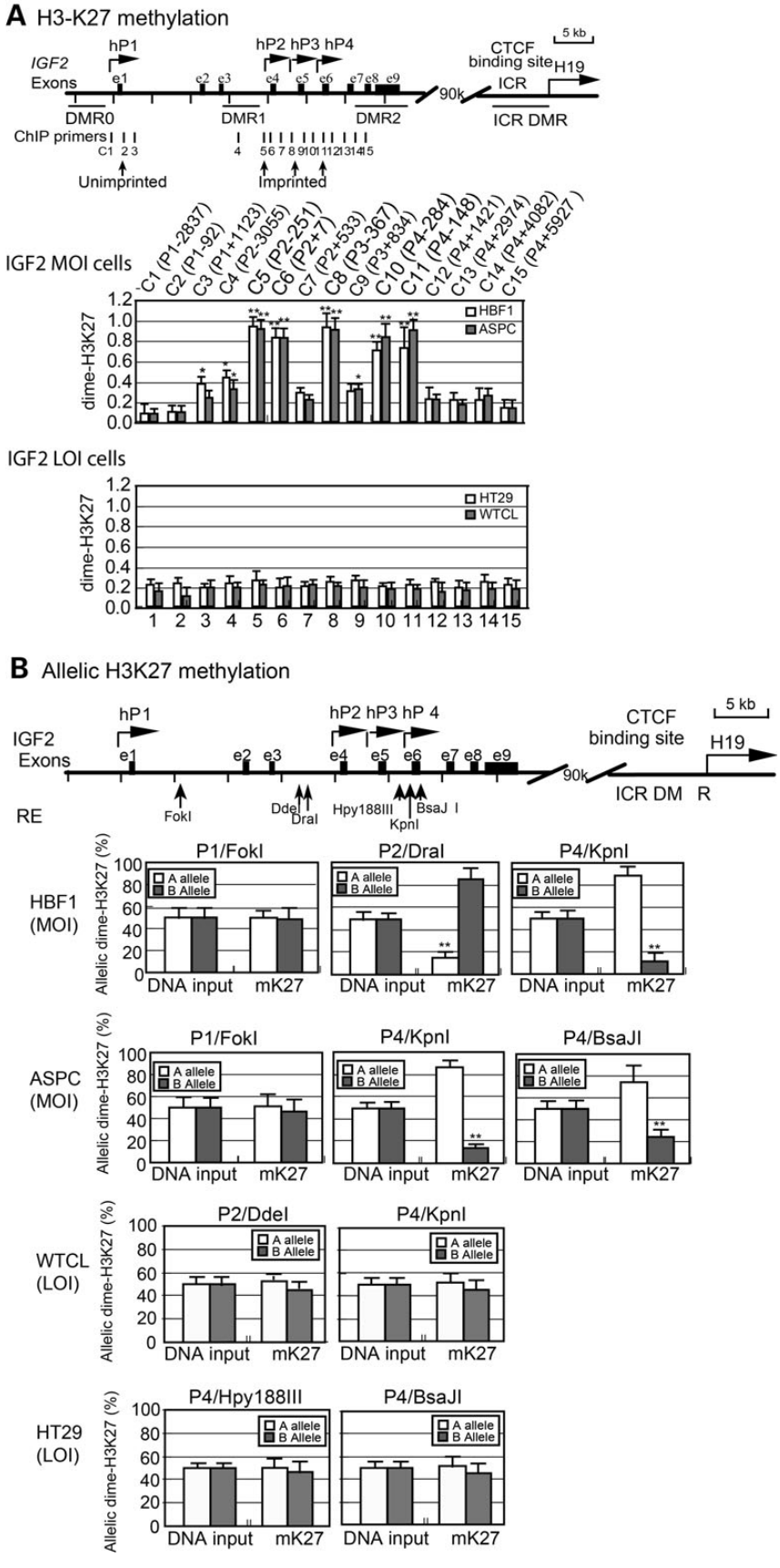
Aberrant imprinting of the insulin-like growth factor II (*IGF2*) gene is a molecular hallmark of many tumors. Reactivation of the normally suppressed maternal allele leads to upregulation of the growth factor that promotes tumor growth. However, the mechanisms underlying the loss of imprinting (LOI) remain poorly defined. We examined the epigenotypes at the gene promoters that control *IGF2* allelic expression. Using chromatin immunoprecipitation, we found that in cells characterized by maintenance of *IGF2* imprinting, three *IGF2* promoters were differentially modified, with the suppressed allele heavily methylated at histone H3K27 while the active allele was unmethylated. In the LOI tumors, however, both alleles were unmethylated, and correspondingly there was no binding of SUZ12, the docking factor of the polycomb repressive complex 2 (PRC2), and of the zinc finger-containing transcription factor (CTCF) that recruits the PRC2. Using chromatin conformation capture, we found that the CTCF-orchestrated intrachromosomal loop between the *IGF2* promoters and the imprinting control region was abrogated in cells with LOI. SUZ12, which docks the PRC2 to *IGF2* promoters for H3K27 methylation, was downregulated in LOI cells. These data reveal a new epigenetic control pathway related to the loss of *IGF2* imprinting in tumors.

INTRODUCTION

Reactivation of the normally suppressed (imprinted) maternal insulin-like growth factor II (*IGF2*) allele, known as loss of imprinting (LOI), is a hallmark of many human tumors, especially childhood tumors (1–8) and cancer stem cells (9). This aberrant biallelic expression may increase the production of IGF-II, promoting the malignant behavior of tumor cells through enhanced cell growth and self-renewal. *IGF2*-overexpressing tumors frequently display loss of *PTEN*, and they are often highly proliferative, exhibiting strong staining for phospho-Akt. These LOI tumors belong to a subclass of neoplasms characterized by poor survival (10). Detection of *IGF2* LOI in circulating white blood cells represents a valuable biomolecular marker for predicting individuals with high risk for colorectal cancer (11).

However, the mechanism underlying loss of *IGF2* imprinting in tumors remains elusive. The genes encoding *IGF2* and *H19* on human chromosome 11p15.5 are reciprocally imprinted, controlled by epigenetic modifications in the differentially methylated region (DMR) of the imprinting control region (ICR) located between these two adjacent genes. In the mouse, the exclusive binding of the insulating factor CTCF to the unmethylated maternal ICR creates a physical boundary, blocking the interaction of downstream enhancers with the remote *IGF2* promoters and silencing the maternal allele (12–21). When this ICR is deleted (22) or mutated (23,24), the suppressed maternal *IGF2* allele is reactivated, leading to LOI. In human tumors, however, a number of epigenetic modifications in the ICR have been described (25–27), and it is unclear whether this enhancer insulation is also causally involved in the abnormal regulation of *IGF2* in tumors.

*To whom correspondence should be addressed at: Department of Medicine, PAIRE, VA Palo Alto Health Care System, Palo Alto, CA 94304, USA. Tel: +1 6504935000; Fax: +1 6508568024; Email: jifan@stanford.edu (J.H.); VA Palo Alto Health Care System, 3801 Miranda Avenue, Palo Alto, CA 94304, USA. Tel: +1 6508583930; Fax: +1 6508568024; Email: arhoffman@stanford.edu (A.R.H.).
†These authors contributed equally to this work.
‡These authors are co-corresponding and senior authors of this report.



Using chromatin configuration capture (3C) methodology, it has been shown that CTCF participates in the formation of a long-range chromosomal loop to the upstream *IGF2* DMRs when it is bound to the maternal ICR (19,28,29). A series of studies from our lab suggest that CTCF may not only function as a physical insulator but also actively participate in the regulation of the imprinted *IGF2* allele (30–33). In this communication, we explored in detail the mechanisms by which the normally suppressed maternal promoters of *IGF2* become reactivated during the process of loss of genomic imprinting.

RESULTS

Loss of promoter H3K27 methylation suppressive mark in tumors

To explore the mechanism underlying aberrant *IGF2* imprinting in tumors, we examined the *IGF2* epigenotypes in the promoter regions that control allelic expression. Unlike the mouse *Igf2* (13,14), loss of human *IGF2* imprinting in tumors may not necessarily be accompanied by changes in DNA methylation in known ICRs (11,25,34,35). We previously examined the DNA methylation status of *IGF2* promoters in tumors and found that *IGF2* promoters were unmethylated on both alleles, thus excluding a role of promoter DNA methylation in controlling allelic suppression (30).

We thus focused on *IGF2* promoter suppression by histone H3K27 methylation to determine whether this epigenetic suppressive mark was altered in tumors in association with LOI. Using chromatin immunoprecipitation (ChIP), we examined H3K27 methylation in the *IGF2* promoters. Promoter 1 (hP1), located immediately downstream of the insulin gene, is not under the control of imprinting mechanisms and is normally biallelically expressed (36). In most tumor cells, hP1 is expressed at very low or undetectable levels, and it does not contribute substantially to *IGF2* expression. We found that H3K27 in the hP1 region was unmethylated in both *IGF2* LOI and maintenance of *IGF2* imprinting (MOI) cells (Fig. 1A, primer set C1). Three downstream promoters (hP2–hP4) are imprinted in normal tissues but often exhibit LOI in tumors. In MOI cells, we found that there was increased H3K27 methylation in these three imprinted promoters (Fig. 1A, middle panel, primer sets C5, C6, C8, C10 and C11), correlating with the suppression of the imprinted allele. In LOI tumor cells, in contrast, H3K27 methylation suppressive mark was not observed in any of the *IGF2* promoters (Fig. 1A, bottom panel), in parallel with the reactivation of the maternal *IGF2* promoters in tumor cells.

We then examined whether the histones at the *IGF2* promoters were differentially methylated at each parental allele. By mapping all available SNPs near the *IGF2* promoters, we found two informative SNPs (*Fok*1 and *Dde*1) downstream of hP1 and three informative SNPs (*Hyp*188III, *Kpn*1 and *Bsa*J1) near hP3–hP4. With the aid of these SNPs, we found that there was no allelic difference in H3K27 methylation at the non-imprinted hP1 (Fig. 1B). At the imprinted hP2 and hP4 promoters, however, we found that H3K27 was monoallelically methylated in normal fibroblasts and in ASPC cells, a cancer cell line characterized by normal *IGF2* imprinting. H3K27 hypermethylation at the promoter was associated with suppression of the maternal allele (30). In LOI tumor cell lines (WTCL and HT29), however, there was no allelic difference in H3K27 methylation, correlating with biallelic expression of *IGF2*.

Lack of SUZ12 binding to the IGF2 promoters

We then looked for factors that are associated with loss of H3K27 methylation at the *IGF2* promoters. H3K27 methylation is catalyzed by methyltransferase EZH2, a critical component of polycomb repression complex 2 (PRC2). Using a mouse imprinting model, we previously demonstrated that PRC2 was recruited to the maternal promoter through the CTCF-mediated docking of a second PRC2 component SUZ12 (30). We therefore examined whether the SUZ12 interaction was also absent in parallel with LOI of *IGF2* in tumors.

Using anti-SUZ12 ChIP, we found enrichment of SUZ12 at the three imprinted *IGF2* promoters in *IGF2* MOI cell lines (HBF1 and ASPC) (Fig. 2A, top panel), indicating an interaction of PRC2 with the imprinted *IGF2* promoters. In the LOI tumor cell lines (HT29 and WTCL), however, this SUZ12 interaction was absent (bottom panel), indicating that PRC2 was not recruited to the three imprinted *IGF2* promoters. As expected, there was no enrichment of SUZ12 at the unimprinted hP1 region (primer sets C1 and C2).

Using available SNPs, we mapped the allelic binding of SUZ12 (Fig. 2B). In fibroblasts (HBF1) and ASPC cells that maintain normal *IGF2* imprinting, SUZ12 interacted monoallelically with *IGF2* promoters and hP4. In the LOI tumors, this differential hP4-SUZ12 allelic enrichment was not observed. Allelic binding of SUZ12 was also absent in the non-imprinted hP1 in both the *IGF2* LOI or MOI cells. Thus, allelic recruitment of the PRC2 complex to the *IGF2* promoters paralleled promoter H3K27 methylation, closely correlating with the imprinting status.

Altered allelic CTCF binding in IGF2 promoters

We previously demonstrated that the PRC2 complex was recruited to the maternal *IGF2* promoters by CTCF, a zinc finger-containing transcription factor (31). CTCF binds to the *IGF2* promoter and the ICR in front of the *H19* gene, forming a

Figure 1. Histone 3 lysine 27 (H3K27) methylation at the *IGF2* promoters. (**A**) H3K27 methylation in *IGF2* promoters as measured by ChIP PCR. Vertical lines: PCR primers (C1–C15) used for ChIP assay. The specific location of each ChIP PCR primer pair was also specified in the parenthesis as the sequence number downstream (+) or upstream (−) of the transcription initiation site. DMR: differentially methylated regions; e1–e9: *IGF2* exons; ICR: imprinted control region; MOI: maintenance of imprinting; LOI: loss of imprinting; hP1: non-imprinted promoter; hP2–hP4: imprinted promoters. Chromatin DNAs were immunoprecipitated with antisera against dimethyl H3K27 (dime-H3K27) and quantitated by ChIP primers. Primer sequences are listed in Supplementary Material, Table S2. The ChIP data are presented as the ratio of the dime-H3K27 ChIP signal over the input signal. Error bars represent the standard error of the average of three independent ChIP assays (each with three PCR reactions). $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the unimprinted C1. (**B**) Allele-specific H3K27 methylation. Allelic ChIP products were separated by polymorphic restriction enzymes (RE). The allelic interaction of CTCF with the *IGF2* promoters (P1 and P4) was identified by using six polymorphic restriction enzymes (*Fok*I, *Dra*I, *Kpn*I, *Dde*I, *Hpy*188III and *Bsa*J1), depending on cells used. The source of the specific parental alleles of 'A' and 'B' is not known. Allelic ChIP enrichment of dime-H3K27 is calculated as the percentage of the A or B allele over the total (A + B) after normalization with the input DNA.

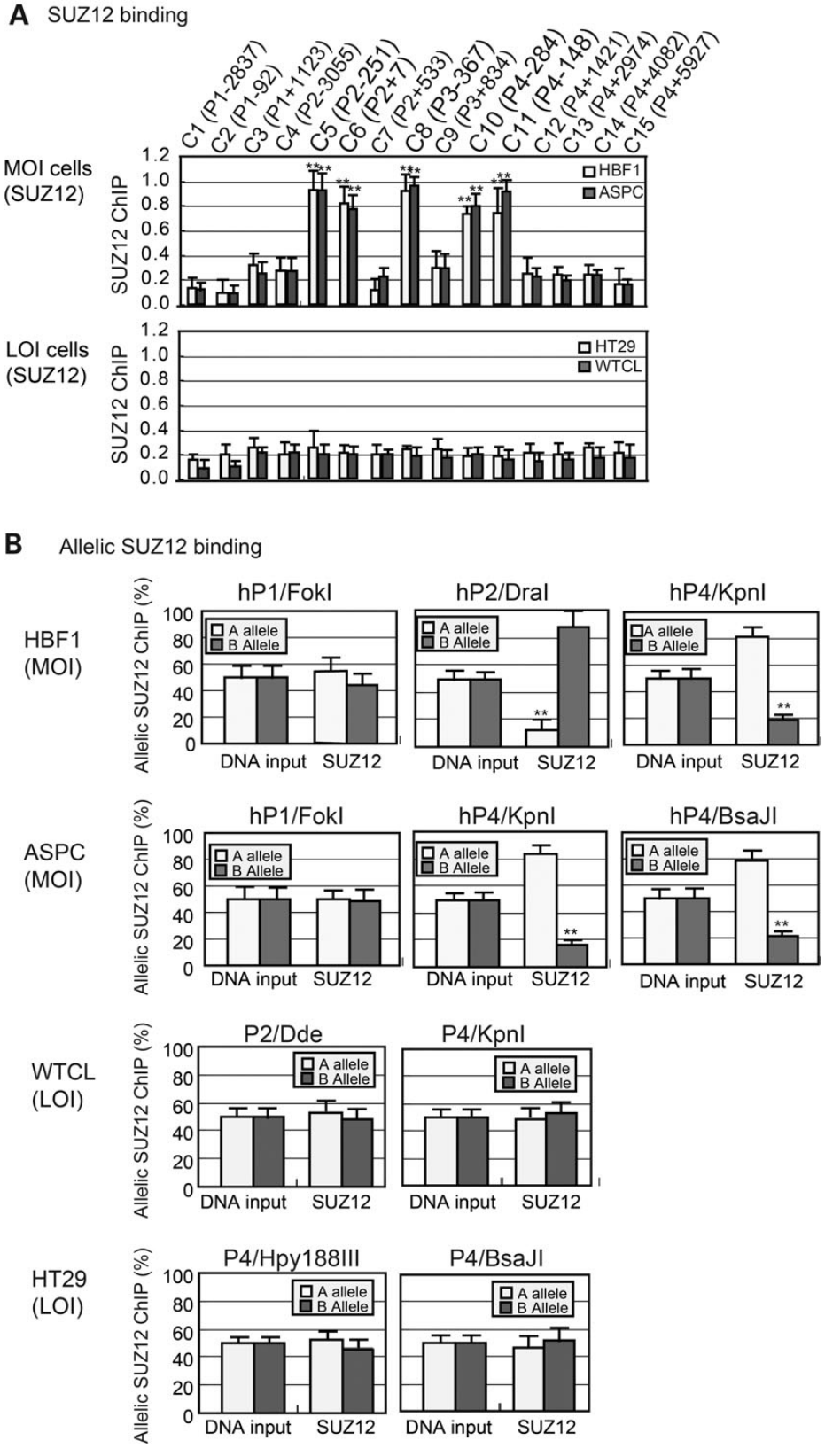


Figure 2. (**A**) SUZ12–*IGF2* promoter interaction. SUZ12 ChIP q-PCR quantitation in *IGF2* MOI and LOI cells. Cells were immunoprecipitated with antisera against SUZ12, followed by PCR amplification with ChIP primers. Input DNA was DNA control before immunoprecipitation. See Figure 1A legend for details. (**B**) Allele-specific SUZ12 binding at the *IGF2* promoters. The polymorphic restriction enzyme sites and PCR primers are the same as in Figure 1B. Allelic binding of SUZ12 is calculated as the percentage of the A or B allele over the total (A + B) after normalization with the input DNA.

long-range chromatin loop structure (28–30,33,37,38) that is essential for the recruitment of PRC2 and the subsequent establishment of the H3K27 methylation code in the maternal promoters. Using ChIP, we found that in sharp contrast to the MOI cell lines (HBF1 and ASPC) (Fig. 3A, top panel), CTCF binding to the imprinted promoters was lost in LOI tumor cell lines (HT29 and WTCL) (bottom panel).

We mapped the allelic binding of CTCF using several polymorphic restriction enzymes (Fig. 3B). In two *IGF2* MOI cell lines (HBF1, ASPC), the unimprinted hP1 did not show significant allelic CTCF binding by using the polymorphic restriction enzyme (*Fok*1). However, using two restriction enzymes (*Dra*1 and *Kpn*1) near the imprinted promoters, we detected monoallelic binding of CTCF, in accord with the monoallelic expression of *IGF2*.

In *IGF2* LOI tumor cells (HT29 and WTCL), however, no differential allelic enrichment of CTCF binding was observed. Using the polymorphic restriction enzymes (*Kpn*1 and *Bsa*J1), we could not detect allelic binding of CTCF to the imprinted promoters. These data suggest the requirement of CTCF promoter binding in the maintenance of genomic imprinting.

Loss of long-range intrachromosomal interaction in tumors

The CTCF–PRC2 promoter complex is formed through a long-range intrachromosomal loop structure between the promoter and the ICR. To further explore the mechanism underlying loss of *IGF2* imprinting in some tumor cell lines, we first used a chromatin conformation capture (3C) method (30,39) to examine the intrachromosomal interaction in cells that have differential *IGF2* imprinting. Cells were fixed with 2% formaldehyde and digested with restriction enzyme *Bam*H1. Chromatin DNAs with spatial proximity were ligated with T4 DNA ligase and amplified with 3C primers that covering the *IGF2* promoters (B1–B3) and the ICR (B4, B5) (Fig. 4A).

The hP1 promoter is not imprinted and contributes very little to the total *IGF2* transcripts in tumors. Using 3C, we did not detect the presence of the long-range intrachromosomal interaction for the unimprinted hP1 (Fig. 4B, B1/B4 and B1/B5). In contrast, the *IGF2* mRNA transcripts from the three most proximal promoters (hP2–hP4) are imprinted (36), and they form intrachromosomal interactions with the CTCF-binding sites in the ICR (30,31). We detected intrachromosomal loops between the ICR and the imprinted promoters hP2 (Fig. 4B, B2/B4 and B2/B5) and hP4 (B3/B4 and B3/B5) in normal skin fibroblasts (HBF1). In tumor cell lines with loss of *IGF2* imprinting, however, these ICR promoter intrachromosomal complexes were not found (Fig. 4C and D). In a previous paper (32), we also showed that the CTCF promoter intrachromosomal loop was essentially preserved in normal tissues (adult liver, fetal liver and kidney) and in all *IGF2* MOI cell lines (GM00498, HCT116, H146, ASPC), but was absent in all *IGF2* LOI tumor cell lines (HT29, H522, WTCL). Taken together, it is clear that the long-range intrachromosomal loop is critical for the MOI (30,31,40).

Induced IGF2 LOI by the protein synthesis inhibitor cycloheximide

To further delineate the molecular mechanisms underlying the LOI of *IGF2*, we inhibited protein synthesis in the MOI cell lines using cycloheximide, predicting that the reduced concentration of putative imprinting factors would alter intrachromosomal interactions, thus recapitulating the LOI as seen in the LOI tumor cell lines (25). We were particularly interested in whether the cycloheximide-treated human fibroblasts and ACPC cells also showed the loss of intrachromosomal interaction and H3K27 methylation.

Both HFB1 and ASPC expressed primarily the A allele, demonstrating a very typical pattern of the MOI (Fig. 5A, lanes 1–2). Treatment with cycloheximide induced various degrees of relaxation of the 'B' allele, depending on cells tested (lanes 3–4).

Using ChIP, we showed that in parallel with *IGF2* LOI, the *IGF2* promoters no longer were associated with SUZ12 and CTCF (Fig. 5B). As a result, H3K27 in the promoter region became unmethylated in these treated cells, indicating the importance of the imprinting factors in maintaining the intrachromosomal loop and the histone imprinting code.

Downregulation of SUZ12 in IGF2 LOI tumors

To further validate the role of chromatin factors in the MOI, we used Western blotting to compare the abundance of CTCF and SUZ12 proteins between the MOI and LOI cells. We did not detect any differences in CTCF abundance between the MOI and LOI cell lines. PRC2 docking factor SUZ12, however, was dramatically downregulated in all LOI tumor cell lines (Fig. 6B). Similarly, the abundance of SUZ12 protein was also low in *IGF2* LOI cells induced by cycloheximide treatment, including HBF1, ASPC, MCF7 and Hep3B (Supplementary Material, Fig. S3). These data suggest that the downregulation of SUZ12 expression may play an important role in the loss of *IGF2* imprinting in these tumors.

DISCUSSION

In this study, we examined molecular mechanisms underlying the loss of *IGF2* imprinting in several tumor cell lines. The three human *IGF2* imprinted promoters contain CpG-rich sequences or CpG islands. However, unlike many other imprinted genes, where the CpG islands are often methylated in inactive promoters, the *IGF2* promoter CpG islands are not differentially modified (25,30). Instead, imprinting of *IGF2* is controlled by a DMR in the ICR (22,24,41).

The ICR region harbors-binding sites for CTCF, an insulator protein that demarcates active and inactive chromatin domains. CTCF binding is methylation sensitive and thus only the unmethylated maternal ICR is available for CTCF binding (13,14). The binding of CTCF to the ICR forms an insulator that prevents the *IGF2* promoters from accessing endoderm-specific enhancers located downstream of the *H19* gene. However, in human tumors, there is often a lack of correlation between DNA methylation and *IGF2* imprinting status (11,25,34,35).

Studies using 3C demonstrate that the binding of CTCF at the ICR is required for intrachromosomal loop interactions on the maternal *IGF2* allele (30,31). CTCF acts as a tethering protein, serving as a molecular glue to secure long-range intrachromosomal (28,30) and interchromosomal (42) interactions. Chromatin looping that brings the ICR and promoters into close contact and recruits PCR2, which induces the H3K27 methylation silencing

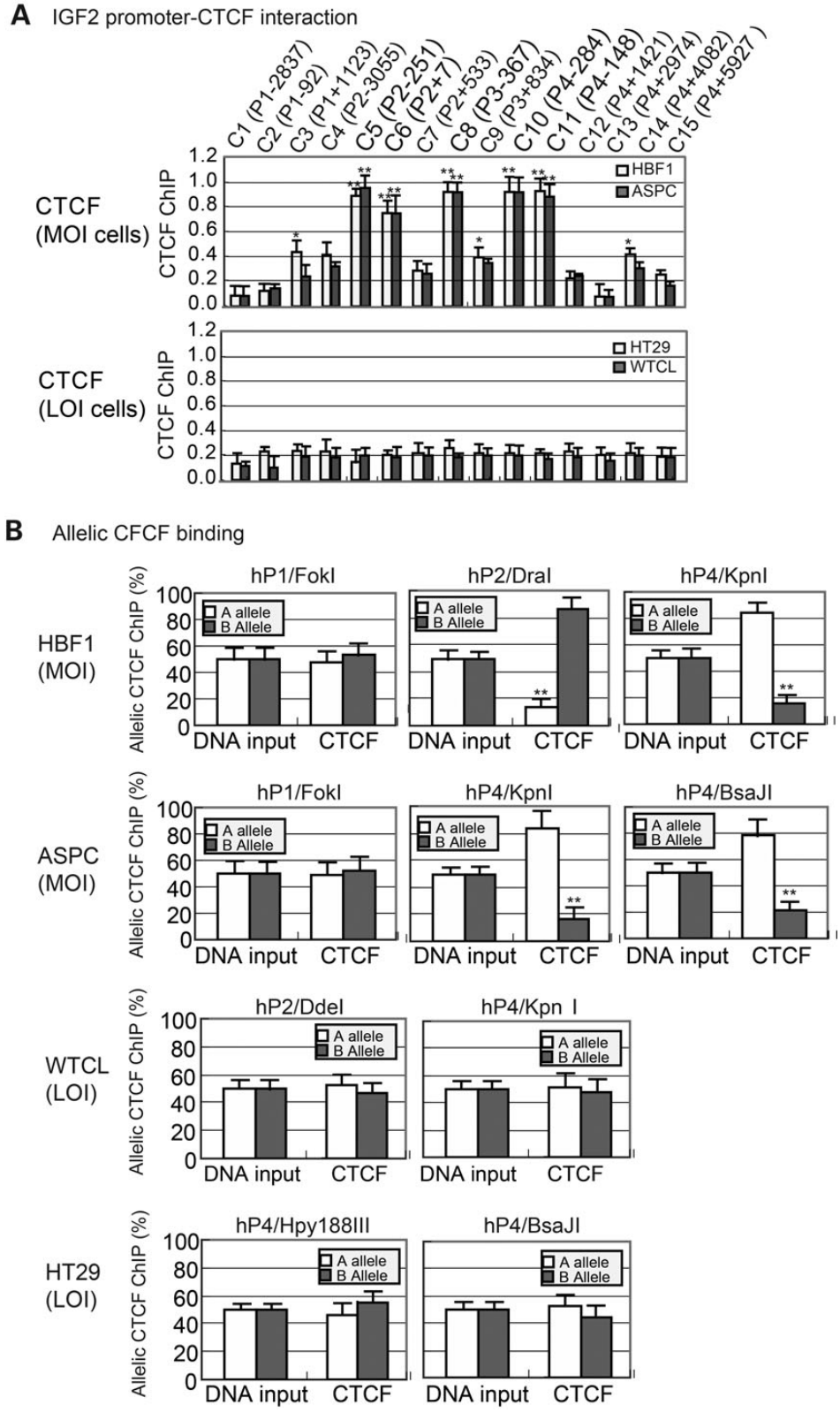


Figure 3. Binding of CTCF to the *IGF2* promoters. (**A**) ChIP q-PCR of CTCF binding in the *IGF2* promoters. Chromatin DNAs in MOI (HBF1 and ASPC) and LOI (HT29 and WTCL) cell lines were immunoprecipitated with antisera against CTCF and quantitated by q-PCR. Input DNA was DNA control before immunoprecipitation. Relative enrichments (fold) of CTCF binding across the promoter regions of human *IGF2*, which were calculated as described previously using input DNA, were plotted along P1 – P4 regions of *IGF2* DNA. See Figure 1A legend for details. (**B**) Quantitative allelic CTCF binding. The polymorphic restriction enzyme sites and PCR primers are the same as in Figure 1B. Allelic binding of CTCF is calculated as the percentage of the A or B allele over the total (A + B) after normalization with the input DNA.

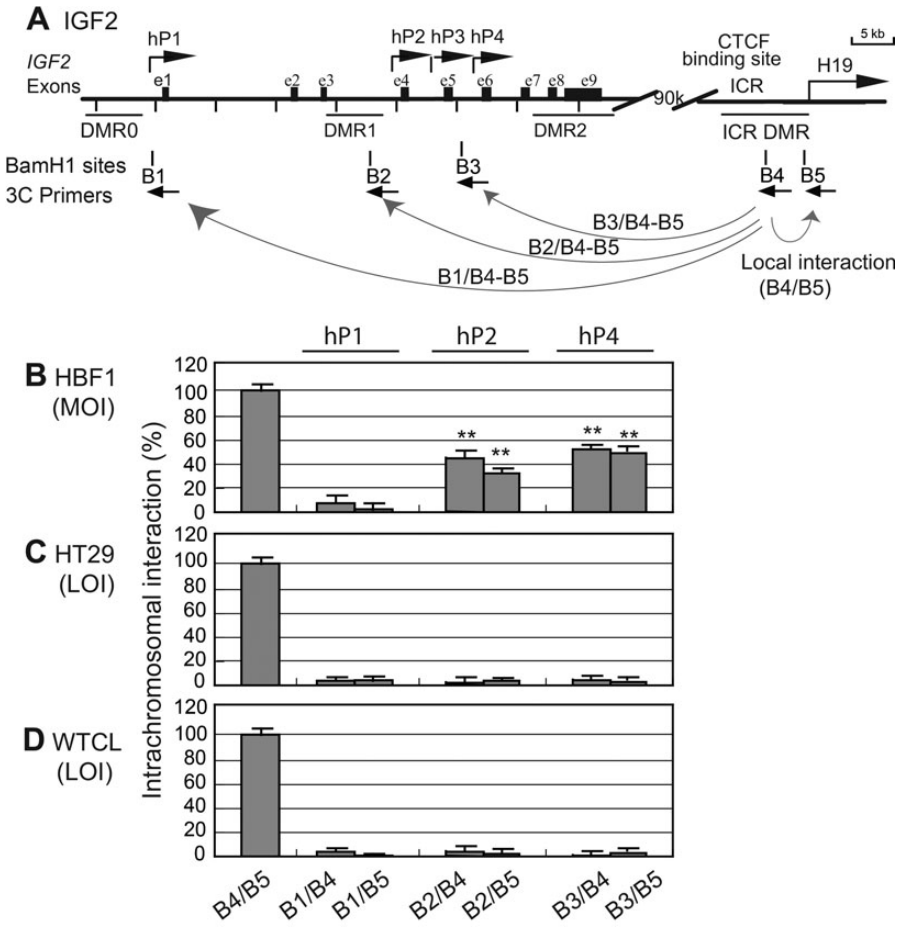


Figure 4. Intrachromosomal interaction between the ICR and *IGF2* promoters. (**A**) Schematic diagram of the *IGF2*/H19 gene locus. DMRs, differentially methylated regions; hP1–hP4, *IGF2* promoters; ICR, imprinting control region; B1–B5, *Bam*H1 sites used for 3C assay. The orientation and location of the 3C primers are shown by arrows under each *Bam*H1 restriction site. (**B**–**D**) Quantitative intrachromosomal interactions. MOI, maintenance of *IGF2* imprinting; LOI, loss of *IGF2* imprinting. PCR primers B4 and B5 (ICR) were used in combination with each target primer in *IGF2* promoters in (A). Each column represents the relative value of the intrachromosomal interaction. B4/B5 is local interaction used as the 3C control.

(30,31). These data suggest a model whereby an intrachromosomal scaffold built with CTCF guides the imprinting signal in the remote ICR to establish the suppressive histone code in the distant *IGF2* promoters.

Thus, the loss of *IGF2* imprinting in human tumors could be caused by a defect in any of the steps during the formation of the CTCF–PRC2–ICR promoter intrachromosomal complex. One defect could be altered CpG DNA methylation in the ICR, where the imprinting signal resides in mouse (13,14). For example, hypermethylation at both parental ICRs could prevent the binding of CTCF and consequently the failure to form the intrachromosomal complex. A detailed analysis of DNA methylation at the *IGF2* locus, however, shows that *IGF2* LOI was not necessarily linked to, and may be independent of, epigenetic marks in the various DMRs, including the ICR (25). In some tumors, *IGF2* LOI persists even when the ICR maintains its normally differentially methylated state. In some tumors, persistent *IGF2* imprinting is accompanied by abnormal epigenetic modifications, for example, hypomethylation or hypermethylation, at CTCF-binding sites.

Data from this study, however, support the concept that aberrant biallelic expression of *IGF2* in human tumors is associated with the loss of the CTCF-orchestrated intrachromosomal complex, which is required for the recruitment of the PRC2 via the co-interaction with SUZ12 (30,31). Without the formation of the intrachromosomal complex, the H3K27 methyltransferase EZH2 cannot be guided to the maternal *IGF2* promoters, where it establishes the suppressive epigenotype. The H3K27 methylation-free promoters then become activated in a similar fashion as in the paternal promoters.

Through the quantitation of chromatin factors in *IGF2* LOI cells either induced by cycloheximide treatment or as found in some malignancies, it appears that the downregulation of SUZ12 is key factor related to the loss of monoallelic expression of *IGF2*. Without SUZ12, the PRC2 cannot be recruited to the maternal *IGF2* promoters, where EZH2 methylates H3K27 and induces the imprinting of the maternal allele (30).

It is also interesting to note that CTCF does not appear to be a key factor involved in loss of imprinting in tumors, as we did not detect significant difference in CTCF expression between the LOI and MOI cell lines (Fig. 6B). However, the binding of CTCF to the *IGF2* promoter cannot be detected in *IGF2* LOI cell lines (Fig. 3). Similarly, the intrachromosomal interaction between the ICR and the *IGF2* promoter is also abolished in

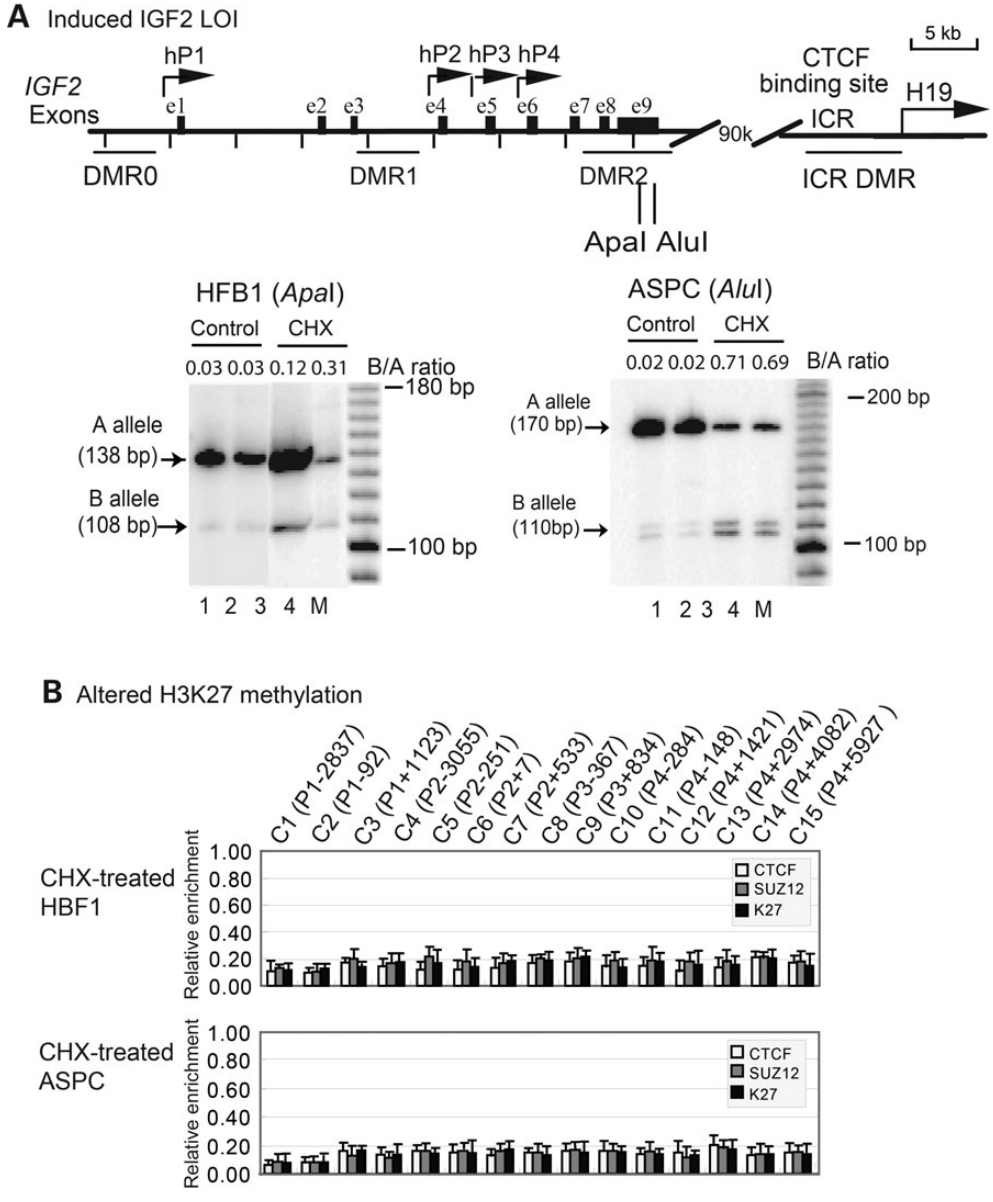


Figure 5. Induced aberrant *IGF2* imprinting by cycloheximide (CHX). (**A**) CHX-induced *IGF2* LOI in human HBF1 fibroblasts and ASPC cells. Cell treated with 2.0 mg/ml CHX for 4 days. The parental alleles of *IGF2* were separated by *Apa*I in HBF1 and *Alu*I in ASPC cells. Lanes 1–2: HBF1 cDNA; lanes 3–4: cell treated with CHX. M, 100 bp marker. B/A ratio, quantitation of the normally imprinted 'B' allele as calculated by the ratio of the 'B' allele over the 'A' allele. Note the monoallelic expression of *IGF2* (MOI) in untreated control cells and various levels of relaxation of the 'B' allele in CHX-treated cells. (**B**) ChIP q-PCR quantitation for altered CTCF and SUZ12 binding and H3K27 methylation. Cells were treated with CHX and immunoprecipitated with antisera against CTCF, Suz12 and dimethyl-H3-K27 (mK27), followed by q-PCR quantitation. Input DNA was DNA control before immunoprecipitation. Note the lack of CTCF and SUZ12 binding and H3K27 methylation of the *IGF2* promoters in CHX-treated cells. See Figure 1A legend for details.

the LOI tumor cell lines. These data thus suggest that CTCF alone may not be able to orchestrate the intrachromosomal complex in this locus. Instead, the coexistence of SUZ12 is needed to coordinate the formation of the intrachromosomal complex in theMOI.

In support of this hypothesis, we have shown in a separate study that virally induced expression of SUZ12 is able to restore normal *IGF2* imprinting in *IGF2* LOI colon cancer cell lines (HT29, HRT18). Transfecting cells with a virally expressed *SUZ12* cDNA, we found that SUZ12 bound to the *IGF2* promoters and coordinated with CTCF to orchestrate a long-range intrachromosomal loop, leading to histone H3-K27 methylation in the *IGF2* promoters and restoring monoallelic expression of *IGF2* in tumor cells (Wang *et al*., unpublished data). It would also be interesting to explore if the downregulation of SUZ12 in tumors has global effects on other target genes and if those genes are also involved in the regulation of IGF2 allelic regulation. As SUZ12 is an essential docking factor in polycomb repressive complex 2 (PRC2), it would be of interest to learn if the downregulated SUZ12 in LOI tumors will affect its chromatin DNA binding and PRC2-mediated gene regulation at other target sites as well.

Recently, the potential role of *IGF2* LOI itself has been explored as a therapeutic approach for tumor-specific gene

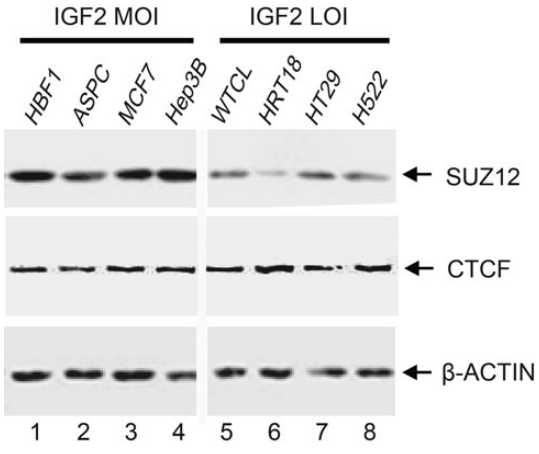


Figure 6. Downregulation of SUZ12 in *IGF2* LOI tumor cells. CTCF and SUZ12 proteins were quantitated by western blotting. Lanes 1–4: *IGF2* MOI cells; lanes 5–8: *IGF2* LOI cells. Note the reduced expression of SUZ12 in *IGF2* LOI tumor cells.

therapy. Oncolytic adenoviruses were constructed by linking the *IGF2*/*H19* enhancer-ICR promoter complex to diphtheria A toxin (DT-A) (43). The viruses induced tumor cell apoptosis specifically in *IGF2* LOI (but not MOI) cell lines and suppressed tumor xenografts in nude mice. It will be interesting to examine if the restoration of *IGF2* imprinting by viral expression of SUZ12 is able to alter tumorigenicity.

It should be noted that cycloheximide treatment is not a perfect model to induce *IGF2* LOI in cells. In addition to inhibiting the synthesis of putative imprinting regulatory factors, like the chromatin factor SUZ12, cycloheximide may globally inhibit the synthesis of many factors involved in maintaining cell growth. Moreover, the degree of *IGF2* LOI depends on the drug concentration and the duration of the treatment. Depending on the cell line tested, the drug may induce a partial loss of *IGF2* imprinting (Fig. 5A, lane 3). More importantly, cycloheximide treatment cannot induce a permanent *IGF2* LOI. Soon after the withdrawal of the drug, the cell assumes a normal *IGF2* imprinting status (25). Thus, other models that specifically inhibit intrachromosomal looping are needed to further test the role of long-range interactions in *IGF2* imprinting.

In summary, our data demonstrate for the first time that aberrant *IGF2* imprinting in tumor cell lines is related to the loss of histone H3K27 methylation suppression mark in the gene promoter. SUZ12 is downregulated in LOI tumor cell lines. In the absence of SUZ12 binding, CTCF is unable to orchestrate a long-range intrachromosomal loop that juxtaposes the ICR close to the gene promoters, enabling the establishment of H3K27 methylation suppression through EZH2, a methyltransferase component of PRC2. In the absence of H3K27 methylation, the maternal allele becomes activated, leading to biallelic expression of IGF2.

MATERIALS AND METHODS

Cell lines and cell culture

Based on the status of *IGF2* imprinting, human cell lines were divided into LOI and 'maintenance of imprinting' (MOI) groups (25,32). Four cell lines with differential *IGF2* imprinting were selected for this study: HT29 (colorectal adenocarcinoma, *IGF2* LOI), WTCL (Wilms' tumor, *IGF2* LOI), HBF1 (human fibroblast, *IGF2* MOI) and ASPC (pancreas adenocarcinoma, *IGF2* MOI) (Supplementary Material, Table S1). Tumor cell lines HT29 and ASPC were purchased from ATCC (Rockville, MD, USA). WTCL was a kind gift from Dr Benjamin Tycko (44), and human fibroblast cells HBF1 were cultured from the skin of a human fetus (45,46).

HT29, ASPC and WTCL cells were maintained in RPMI 1640 and HBF1 in DMEM media (Invitrogen, Carlsbad, CA, USA). Both media were supplemented with 10% fetal bovine serum, and cells were cultured at 37°C, 5% CO_2. Exponentially growing cells were collected by trypsin-EDTA for ChIP and PCR analyses.

Reverse transcription

Reverse transcription (RT) was performed with murine leukemia reverse transcriptase (Invitrogen) using both random hexamers and d(T)17 primers as described previously (45). To eliminate any residual genomic DNA, total RNAs (or total nucleic acids) were treated with DNase I (Takara) for 60 min (2 units/1 μg of RNA) and then extracted with phenol–chloroform before RT. Human multiple tissue panel cDNAs were purchased from CLONTECH (Palo Alto, CA, USA).

Allelic expression of IGF2

Tumor cell lines were first genotyped for heterozygosity of SNPs in *IGF2* mRNA. Tumor cells with informative SNP sites were used for *IGF2* imprinting studies (Supplementary Material, Table S1). *IGF2* transcripts were amplified by RT–PCR (30 cycles of 95°C for 15 s and 60°C for 2 min, followed by a 5-min extension at 72°C) using primers specific for two polymorphic restriction enzymes (*Apa*I, *Alu*I) in the last exon of human *IGF2*. PCR primers used to measure allelic expression of *IGF2* included Apa1: #2505 (CTT GGA CTT TGA GTC AAA TTG GCC T) and #2506 (GAG GAG CCA GTC TGG GTT GTT GCT A); Alu1: #2949 (GTC CCC TCC TCT GCC ATC ACC TGA) and #2950 (GGA TTT TGC CGG AAA TAT TAG CGT). The amplified products were further labeled by primer extension using ^{32}P end-labeled primer. The primer-extended products were digested with *Alu*I and *Apa*I to distinguish two parental alleles and were separated on a 5% polyacrylamide–urea gel. The digested allele was quantitated as the relative value based on PhosphorImager scanning density (47).

ChIP

ChIP assays were performed with a ChIP assay kit (Upstate Biotechnology, NY, USA) by following the protocol provided by the manufacturer with slight modifications as previously described (31). Briefly, 5 million cells were fixed with 1% formaldehyde and then sonicated for 180 s (10 s on and 10 s off) on ice with a Branson sonicator with a 2-mm microtip at 40% output control and 90% duty cycle settings. The sonicated chromatin (0.9 ml) was collected by centrifugation, aliquoted and snap-frozen in liquid nitrogen. To perform ChIP, sonicated chromatin (150 μl) was diluted 10-fold and purified with specific antiserum (2– 5 μl) and protein G-agarose (60 μl). Antibodies to CTCF, SUZ12 and dimethyl-H3-K27 (lysine 27 of histone H3) were

obtained from Upstate Biotechnology. To reduce the ChIP background, we modified the manufacturer's protocol by additional two washing steps following immunoprecipitation. As previously reported (31), anti-IgG was used as the ChIP control in parallel with testing samples.

DNAs were released from the bound chromatins after cross-linking reversal and proteinase K treatment, and were precipitated and diluted in 100 μl of low-TE buffer (1 mM Tris, 0.1 mM EDTA). PCRs (3 μl under liquid wax) contained 1 μl ChIP (or input) DNA, 0.5 mM appropriate primer pairs, and 0.2 U Klen-Taq I (Ab Peptides, MO, USA). Standard PCR conditions were 95°C for 60 s, followed by 35 cycles of 95°C for 15 s, 65°C for 30 s of annealing, and 72°C for 1 min of extension. All primer sets were tested for the absence of primer–dimer products. To avoid heteroduplex formation that may interfere with restriction enzyme digestion, one of each set of primers was end-labeled with [γ-^{32}P]ATP. The γ-^{32}P-labeled primer was added to the PCR mixture (1 μl) at the last cycle of amplification. PCR products were separated on a 5% polyacrylamide–urea gel and quantified by a PhosphorImager scanner (Molecular Dynamics, Sunnyvale, CA, USA). For comparison, the ChIP data were presented as relative values by normalizing to PCR signals of input DNA (i.e. ratio of the ChIP over the input).

ChIP signals were relatively low in *IGF2* LOI cells. Thus, a relatively high PCR cycle was used to amplify the ChIP DNA. After PCR, the DNA products were digested with 1 U of the appropriate polymorphic restriction enzymes in a total volume of 6 μl for 3 h. The digested products were separated on a 5% polyacrylamide–urea gel and quantified by a PhosphorImager scanner (Molecular Dynamics). The data were presented as the relative allele enrichment: the A or B allele/(A + B alleles) × 100%. ChIP was repeated in triplicate independently for each sample.

Allelic ChIP assay

Duplicate PCR reactions (3 μl under liquid wax) contained 1 μl ChIP (or input) DNA, 0.1 mM appropriate primer pairs (Supplementary Material, Tables S1 and S2), 50 mM deoxynucleotide triphosphate, and 0.2 U KlenTaq I (Ab Peptides, St. Louis, MO, USA). Standard PCR conditions were 95°C for 60 s, followed by 30 cycles of 95°C for 10 s and 65°C annealing (and extension) temperature for 90 s and finally 72°C for 10 min. All primer sets were tested for the absence of primer–dimer products. End-labeled PCR primers are listed in Supplementary Material, Table S3. The [γ-^{32}P]-labeled primer was added in 1 μl PCR mixture at the last cycle of amplification. PCR products were digested with appropriate enzymes (New England Biolabs, MA, USA; 1 unit) listed in Supplementary Material, Table S3 in a total volume of 6 ml for 6–12 h under liquid wax. The digested products were separated on a 5% polyacrylamide–urea gel and quantified by a PhosphoImager (Molecular Dynamics). The relative enrichment of CTCF and SUZ12 at each specific site was determined as described previously (34). The allelic levels of modified histones in one parental allele (percentage of both alleles) at each specific site were calibrated with those from input DNA (DNA before ChIP).

Chromatin conformation capture assay

The 3C assay was performed as previously described (30,39). Briefly, MOI (HBF1) and LOI cells (WTCL and HT29) were cross-linked with 2% formaldehyde and lysed with cell lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 0.2% NP-40, protease inhibitors). Nuclei were collected, suspended in 1 × restriction enzyme buffer in the presence of 0.3% sodium dodecyl sulfate (SDS). An aliquot of nuclei (2×10^6) was digested with 800 U *Bam*H1 at 37°C overnight and ligated with 4000 U T4 DNA ligase at 16°C for 4 h. After the treatment with 10 mg/ml proteinase K at 65°C overnight to reverse cross-links and with 0.4 μg/ml RNase A for 30 min at 37°C, DNA was extracted with phenol–chloroform, ethanol precipitated, and used for PCR amplification for the ligated DNA products. PCR primers used in this study were previously described (32).

PCR polymorphism analysis

After genomic mapping, a total of eight available polymorphisms were used to cover the DNA sequence four *IGF2* promoter and exon regions in a fibroblast cell (HFB1) and three tumor cell lines: *Apa*I (exon 4), *Alu*I (exon 4), *Fok*I (promoter hP1), *Dde*I (promoter hP2), *Dra*I (promoter hP4), *Kpn*I (promoter hP4), *Hpy*188III (promoter hP4) and *Bsa*JI (promoter hP4). PCR reaction, restriction enzyme digestion, electrophoresis and quantitation analysis were performed using established methodology as described previously (30,48). Briefly, PCR amplification for polymorphism determination was performed in 96-well microtiter plates, each 3 ml reaction containing 10–20 ng of DNA for genotyping or cDNA for examining allelic expression, 50 nM dNTP, 0.2 mM corresponding primers, 0.1 μCi of [α-^{32}P]dCTP, 0.125 U of Taq DNA polymerase with a hot start PCR. The PCR reaction solution was heated to 98°C for 2 min, amplified for 30–35 cycles at 95°C for 30 s and 65°C for 90 s, and followed by a 5-min extension at 72°C. PCR products were then digested with the polymorphic restriction enzymes (1 unit) in a 10 μl volume at 37°C for 4 h. Each digested product was detected or quantitated by PhosphorImager 445SI scanner (Molecular Dynamics) after electrophoresis in a 5% polyacrylamide urea gel.

Cell treatment with cycloheximide

Human fibroblasts cells HBF1 and pancreatic cell line ASPC were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 100 U/ml of penicillin and 100 mg/ml of streptomycin, and grown at 371°C with 5% CO_2. ASPC, a pancreatic cancer cell line keeping normal *IGF2* imprinting in cell culture, was maintained in RPMI 1640 medium as recommended by ATCC. Cells were seeded in six-well plates at the density of $2–10^5$ cells/well. Twenty-four hours following plating, cells were replaced with fresh medium and were treated with the concentrations 2.0 mg/ml of cycloheximide as previously described (25). Tumor cells were collected after Day 4 and analyzed for *IGF2* imprinting and ChIP assay.

Western blotting

Expression of CTCF and SUZ12 proteins were determined by western blotting as previously described (30). Cells were lysed with boiling 1% SDS, 10 mM Tris–HCl, pH 7.4, sonicated for 30 s and centrifuged at 15 000 *g* for 5 min. Supernatant lysates with equal amounts of protein were used for immunoblotting of CTCF protein. The proteins were examined by SDS–PAGE

and western immunoblotting with the anti-CTCF and anti-Suz12 antibodies (1:1000; Upstate Biotechnology) and the ECL detection system (Amersham) by following the instructions of the manufacturer.

Statistical analysis

All experiments were performed in triplicate, and the data are expressed as mean ± SD. The comparative C_T method was applied in the quantitative real-time RT–PCR assay according to the $\Delta\Delta C_T$ method (25,30). The data were analyzed with one-way analysis of variance, and results were considered statistically significant at $P \leq 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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