Epigenetic silencing of the XAF1 gene is mediated by the loss of CTCF binding

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XAF1 is a tumour suppressor gene that compromises cell viability by modulating different cellular events such as mitosis, cell cycle progression and apoptosis. In cancer, the XAF1 gene is commonly silenced by CpG-dinucleotide hypermethylation of its promoter. DNA demethylating agents induce transcriptional reactivation of XAF1, sensitizing cancer cells to therapy. The molecular mechanisms that mediate promoter CpG methylation have not been previously studied. Here, we demonstrate that CTCF interacts with the XAF1 promoter in vivo in a methylation-sensitive manner. By transgene assays, we demonstrate that CTCF mediates the open-chromatin configuration of the XAF1 promoter, inhibiting both CpG-dinucleotide methylation and repressive histone posttranslational modifications. In addition, the absence of CTCF in the XAF1 promoter inhibits transcriptional activation induced by well-known apoptosis activators. We report for the first time that epigenetic silencing of the XAF1 gene is a consequence of the loss of CTCF binding.

The tumour-suppressor gene X-linked inhibitor of the apoptosis (XIAP)-associated factor 1 (XAF) favours apoptosis by inhibiting XIAP1–5, which is one of the most important members of the inhibitors of apoptosis protein (IAP) family. In addition, XAF1 also presents XIAP-independent proapoptotic actions that contribute to its tumour suppressor gene activity6–8. XAF1 expression is absent or decreased in gastric9, ovarian10, pancreatic11, esophageal12, colon13, melanoma14 and urogenital tumours15–17. Although loss of heterozygosity has been showed to be associated to XAF1 expression absence18, promoter CpG dinucleotide hypermethylation appears to be the principal cause of altered XAF1 expression9,12,17. Exposure to demethylating agents such as 5-azacytidine readily induces the reestablishment of XAF1 expression, thereby increasing the sensitivity to drug-induced apoptosis12,19,20. In xenograft models, ectopic XAF1 expression impedes tumour formation and prolongs the survival of tumour-bearing mice21,22. However, the molecular mediators of the hypermethylated state and decreased expression are currently unknown.

CTCF is a multitask protein involved in gene regulation. This protein functions as a transcriptional regulator, enhancer blocker and chromatin barrier23. These actions are secondary to its main function as a genome-wide organizer of chromatin architecture24,25. The biological actions of CTCF are explained by its ability to function as a DNA-binding protein scaffold. CTCF interacts with its DNA-binding sites in a methylation-sensitive fashion, thereby impeding the methylation of imprinting control regions26,27. In cancer, it has been described that CTCF is able to modulate the histone posttranslational modification (HPM) status and CpG methylation from several tumour suppressor genes28.
Here, we demonstrated that CTCF directly regulates XAF1 expression by binding to a methylation-sensitive CTCF-binding site in its promoter. The absence of CTCF promotes epigenetic silencing of the XAF1 promoter by both accelerated CpG-dinucleotide methylation and the transition from active to repressive HPMs. Importantly, in cancer cell lines, the lack of CTCF regulation on the XAF1 promoter via methylation on its cognate binding site partially blocks its transcriptional responsiveness to two well-known transcriptional activators, TNF-α or IFN-α. These findings uncover for the first time an epigenetic mechanism involved in establishing the repressive configuration of the XAF1 promoter and, consequently, transcriptional unresponsiveness.

Results
Specific CpG-dinucleotide methylation impedes full XAF1 responsiveness to either TNF-α or IFN-α in MCF-7 cells. As expected based on previous reports showing that XAF1 promoter is hypermethylated in cancer,9,12,17 here, pre-exposure to demethylating agents increased the transcriptional activation of XAF1 in basal conditions (Supplementary Fig.Ia). To test XAF1 dynamic expression, we used two well-known XAF1 transcriptional activators, TNF-α and IFN-α.29–31 Demethylating conditions were required to display full transcriptional activation of XAF1 at both the mRNA and protein levels.

Figure 1. XAF1 expression is induced by either TNF-α or IFN-α in demethylating conditions. (a) MCF-7 cells were pre-treated with 5-Aza-2′-deoxycytidine (5μM) and Trichostatin-A (0.2μM) for 3 days before stimulation with TNF-α (20ng/mL) for 24h. Quantitative PCR (qPCR) analysis of XAF1 and CTCF mRNA expression was performed. HPRT mRNA was used as loading control. Results are presented in terms of fold change. The means from three independent experiments were plotted with ±SEM, *P < 0.05. (b) MCF-7 cells were treated as shown in (a). Using a specific antibody, XAF1 was immunoprecipitated from equal quantities of total extracted proteins for each condition. XAF1, CTCF and GAPDH protein levels were measured by Western blot. (c) MCF-7 cells were pre-treated as in (a) before stimulation with IFN-α at the indicated concentrations. mRNA expression of both XAF1 and CTCF was analysed by qPCR after normalizing with HPRT mRNA. The mean from three independent replicates were plotted with ±SEM, *P < 0.05. (d) MCF-7 cells were pre-treated and stimulated as shown in (c). Western blot analysis was performed as shown in (b), 5-Aza-2′-deoxycytidine (5-A-DC), Trichostatin-A (TSA).
after TNF-α (Fig. 1a,b) or IFN-α (Fig. 1c,d) exposure. To extend these observations to another unrelated cancer cell line, we used ACHN cells, which have previously been shown to be responsive to IFN-α in demethylating conditions. As observed with MCF-7 cells, we observed a dramatic increase in XAF1 responsiveness in demethylating conditions (Fig. 2a). As a positive control, we used the Colo205 cell line that presents an unmethylated XAF1 promoter. Even without previous exposure to epigenetic modifiers, we observed a clear XAF1 transcriptional activation by TNF-α exposure (Supplementary Fig. 1b).

We then reasoned that differential dinucleotide CpG methylation between control cells and cells treated with demethylating agents could help us to identify which DNA segments are important for the full responsiveness of XAF1. To this end, we performed bisulphite genomic sequencing using a specific set of primers: CTCF, MYC, IGF2, and XAF1.
primers to amplify the \( XAF1 \) promoter. Exposure to 5-aza-2′-deoxycytidine (5-A-DC) and trichostatin-A (TSA) induced consistent demethylation of three CpG dinucleotides in MCF-7 cells (Fig. 2b; A, B and C). These results indicate that full transcriptional activation of the \( XAF1 \) gene is associated with a specific CpG-dinucleotide methylation state of its promoter.

CTCF interacts with the \( XAF1 \) promoter when cells are stimulated with TNF-\( \alpha \) or IFN-\( \alpha \). CTCF is known to regulate the expression of diverse tumour suppressor genes by directly binding to promoter sequences\(^{28} \). We searched for transcription binding sites in a window of \(-22 \) to \(-500\) bp relative to the transcription start site and the previously described binding sites for IRF-1, ISRE, p53 and the uncharacterized CTCF binding site. Histone posttranslational modification (HPM).

**Figure 3. Features of the \( XAF1 \) promoter.** (a) The \( XAF1 \) promoter visualized in the UCSC genome browser. The picture illustrates the CpG-methylation status from different types of cell lines. Additionally, the profiles of several histone posttranslational modifications such as H3K4Me1, H3K4Me3 and H3K27Ac are presented from different cell lines. Several transcription factor binding sites obtained from ChIP-Seq data are also shown. At a higher resolution, the CTCF binding site in the \( XAF1 \) promoter in glioblastoma and fibroblast cells is shown. (b) Schematic representation of the \( XAF1 \) promoter showing the CpG-dinucleotide positions from \(-22 \) to \(-500\) bp relative to the transcription start site and the previously described binding sites for IRF-1, ISRE, p53 and the uncharacterized CTCF binding site. Histone posttranslational modification (HPM).
As in MCF-7 cells, we observed a dramatic increase in the interaction of CTCF with the XAF1 promoter when the cells were stimulated with either TNF-α or IFN-α after exposure to demethylating agents (Supplementary Fig. 1c). These results support a methylation-sensitive association of CTCF with the XAF1 promoter.

**XAF1 expression is regulated by CTCF.** To further define the role of CTCF on XAF1 mRNA expression, we used specific siRNAs to downregulate CTCF expression in a series of loss-of-function experiments. We verified the efficacy of these siRNAs at both mRNA and protein levels (Supplementary Fig. 1d). Because previous reports have shown that demethylating agents increase XAF1 induction by IFN in ACHN cells\(^\text{22}\), we used this cell line to analyze the effect of these siRNAs on XAF1 transcriptional responsiveness to TNF-α or IFN-α. As described above, demethylating conditions are necessary to uncover the CTCF-binding site (Fig. 2b,c). We clearly observed lower levels of XAF1 mRNA in cells transfected with the siRNAs against CTCF than those transfected with control siRNAs (Fig. 4a). Additionally, we confirmed the regulatory effect of CTCF on the XAF1 promoter using the secreted alkaline phosphatase (SEAP) reporter gene assays. In these assays, the enzymatic activity drove by the XAF1 promoter region comprising −3000 bp to +350 bp relative to the transcription start site.
CTCF protects the XAF1 gene from epigenetic silencing. The insulating action of CTCF protects several genes from epigenetic silencing\(^{26,34}\). In particular, it has been described that the absence of CTCF in tumour suppressor gene promoters induces their epigenetic silencing, which supports the role of CTCF in cancer\(^{35–37}\). To test the possible epigenetic-mediated regulation of CTCF on the XAF1 gene, we compared the XAF1 promoter activity in a genomic integrated context by measuring a GFP reporter gene. For this, we compared the wild type XAF1 promoter with the CTCF-deletion (Δ-CTCF-XAF1) construct. Supporting the insulating role of CTCF on the XAF1 gene, cells with the Δ-CTCF-XAF1 promoter showed lower GFP levels than those with the wild-type XAF1 promoter after 60 days of continuous culture (Fig. 5a). To further support this finding, single-cell clones for each transfection were isolated and propagated for an additional 35 days. As expected by the previous result, silencing of GFP expression levels was mainly observed in single-cell clones with the integrated Δ-CTCF-XAF1-promoter (Fig. 5a), pointing toward an epigenetic-protective effect of the CTCF binding site. A possible alternative explanation for the difference in GFP expression levels between transfections could be attributed to a distinct number of integration events. To exclude this possibility, the transgene copy number was measured by real-time PCR as previously reported\(^{38}\). The difference between GFP expression levels driven by the Δ-CTCF-XAF1-promoter and wild-type-XAF1-promoter was independent of the transgene copy number (Supplementary Fig. 2b). To gain insight into the epigenetic mechanism involved in GFP silencing of the Δ-CTCF-XAF1-promoter, we hypothesized that loss of the CTCF-binding site could promote 1) accelerated CpG methylation or 2) acquisition of a repressive chromatin configuration based on HPMs (or both). To test the first hypothesis, sequencing of the sodium bisulphite-modified genomic DNA from single-cell clones for each transfection was performed. To discriminate endogenous XAF1 promoter amplification, a nested-PCR strategy was performed in which the first set of primers annealed to plasmid sequences surrounding the exogenous XAF1 promoter (Fig. 5b and Supplementary Fig. 2c). We observed that the Δ-CTCF-XAF1-promoter is more susceptible to dinucleotide-CpG methylation than the wild-type-XAF1-promoter in a genomic-integrated context (Fig. 5b). To test our second hypothesis, ChIP assays were performed using specific antibodies directed to H3K4-2me or H3K9-3me posttranslational modifications in single-cell clones from each stable transfection. To interrogate the relative enrichment of repressive or active HPMs in the transgene, we designed a pair of primers that anneal to the plasmid sequence immediately after the exogenous XAF1 promoter (Fig. 5c and Supplementary Fig. 2c). Lower levels of the H3K4-2me posttranslational modification, a marker for transcription activity, were observed in cell single clones from the Δ-CTCF XAF1-promoter (Fig. 5c). As expected, the Δ-CTCF XAF1 promoter was enriched with the repressive H3K9-3me modification (Fig. 5c). Overall, these findings support the notion that CTCF regulates DNA methylation in the XAF1 promoter; thus, loss of CTCF in its cognate-binding site induces DNA-methylation and polarization from active to repressive HPM, which in turn induces transcriptional repression.

XAF1 expression is modulated by CTCF in apoptotic conditions. It has been well described that XAF1 expression reactivation has a crucial role in apoptosis induced by TNF-α/cycloheximide (CHX) or IFN-α/TNF-related apoptosis-inducing ligand (TRAIL)\(^{6,22}\). To test if CTCF could regulate XAF1 expression in apoptotic conditions, MCF-7 cells were exposed to either TNF-α/CHX or IFN-α/TRAIL. Cytotoxicity induced by the co-treatment of either TNF-α/CHX or IFN-α/TRAIL was analysed by cell viability assays (Supplementary Fig. 2d). As expected, we observed the transcriptional activation of XAF1 after exposure to both regimens (Fig. 6a,b). To assess the biological relevance of CTCF-mediated XAF1 transcription, single-cell clones with the wild-type or Δ-CTCF-XAF1 promoter were exposed to inducers of apoptosis. After that, FACS was used to measure GFP-reporter gene activity. Whereas the wild-type promoter activity correlated with the XAF1 transcriptional activation, the Δ-CTCF-XAF1 promoter did not present any transcriptional activity (Fig. 6c). Several reports have shown that XAF1 is an IFN-stimulated gene in cancer cells\(^{30,39,40}\). Because its promoter is commonly hypermethylated in these...
cells, transcriptional activation of the XAF1 gene could be dependent on IFN-α-mediated demethylation and could thus rely on CTCF. Supporting this hypothesis, we found that single-cell clones with the Δ-CTCF-XAF1 promoter were unable to respond to IFN-α, indicating that CTCF could be relevant in the IFN-α-mediated induction of XAF1 (Fig. 6c).

Discussion

In cancer, it has been described that the XAF1 gene is transcriptionally silenced by CpG-dinucleotide hypermethylation in its promoter. Exposure to demethylating agents induces XAF1 transcriptional activation, thereby compromising cell viability by promoting apoptosis, mitotic catastrophe or cell cycle inhibition. Thus, CpG methylation in the XAF1 promoter represents the main epigenetic mechanism involved in XAF1 silencing and, consequently, in resistance against apoptosis. However, the deregulation of epigenetic mechanism is implicated in a variety of diseases, including cancer. CTCF is a multi-task protein involved in chromatin regulation, with profound consequences in gene expression. In a panel of breast cancer cell lines, heightened CTCF expression was associated with apoptosis.
resistance. The protective action of CTCF is explainable, in part, by negative regulation of the Bax gene, which increases the apoptotic threshold. It has also been clearly demonstrated that CTCF regulates the chromatin configuration of many tumor suppressor genes, affecting their transcription rates. Here, we describe that CTCF interacts with the XAF1 promoter, thereby regulating its chromatin configuration and, consequently, its transcriptional responsiveness to activators. We were able to demonstrate two biological scenarios. First, CTCF maintains an open-chromatin configuration in the XAF1 promoter, as assessed by the presence of both active HPMs (Fig. 5c) and de-methylated CpG dinucleotides (Fig. 5b), allowing high transcriptional responsiveness to activators (Figs 1 and 6c). Second, the loss of CTCF regulation in the XAF1 promoter, explained by the fact that CTCF interaction with its cognate binding site in the XAF1 promoter is methylation sensitive (Fig. 2b,c), induces polarization from active to repressive HPMs (Fig. 5c) and accelerates CpG-dinucleotide methylation (Fig. 5b). This closed chromatin state represses transcriptional activation (Fig. 6c) and possibly contributes to apoptotic resistance. Thus, CTCF is a determinant that confers a permissive chromatin configuration to the XAF1 gene, which is critical for apoptotic program culmination.

In X-chromosomal inactivation, certain genes escape from the epigenetic silencing mechanism. CTCF mediates this escape by inhibiting the propagation both of methylation and of repressive HPMs from surrounding silent regions. In this scenario, CTCF mediates the shift between an open and closed

Figure 6. The CTCF binding site in the XAF1 promoter mediates XAF1 responsiveness to activators in apoptotic conditions. (a) MCF-7 cells were treated with 5-Aza-2′-deoxycytidine (5 μM) and Trichostatin-A (0.2 μM) for 3 days before stimulation with TNF-α in the presence of cycloheximide (TNF-α + CHX) (left panel). qPCR analyses of XAF1 and CTCF mRNA expression were performed. HPRT mRNA was used as loading control. Data are presented as fold change. Results are presented as means ± SEM from three independent experiments, *P < 0.05. (b) MCF-7 cells were pre-treated as shown in (A) before the addition of IFN-α in the presence of TRAIL (IFN-α + TRAIL) (right panel). The expression of XAF1 and CTCF and HPRT was determined by qPCR. HPRT was used as loading control. (c) Stable single-cell clones containing either pegFPN1-XAF1 or pegFPN1-D-CTCF-XAF1 constructs were stimulated with either TNF-α + CHX or IFN-α + TRAIL. After, GFP protein levels were measured using FACS. Data are represented as the mean SD of four single-cell clones from each transfection, *P < 0.05.
chromatin configuration by functioning as a scaffold protein to attract different enzymatic complexes involved in HPMs\(^{35,48,49}\). Concordantly, epigenetic silencing of CTCF-regulated genes is observed when CTCF is unable to interact with its cognate DNA-binding site by a methylation-sensitive interaction that favours the presence of repressive, HPMs\(^{27,35,50}\). Importantly, reports showing the biological weight of different HPMs in the XAF1 promoter are missing. In the present report, assessing long-term transgenic behaviour allowed us to uncover the actions of CTCF on the chromatin configuration of the XAF1 promoter (Fig. 5). We observed for the first time that the absence of local CTCF in the XAF1 promoter induces the transition from active to repressive HPMs. We envision that the loss of CTCF affinity to its cognate binding-site in the XAF1 promoter could be the first driving event for the transcriptional repression of the XAF1 gene. Additional experiments are needed to support this, but it has been reported that CTCF posttranslational modifications impair its ability to interact with DNA targets\(^{51–54}\). Thus, lack of CTCF in its cognate site allows its methylation, impeding re-association of CTCF to it even with new posttranslational modifications arise. Consequently, this induces accelerated CpG-dinucleotide methylation and polarization from active to repressive HPM and a consequent XAF1 transcriptional silencing.

It has been described that the nuclear matrix plays an important role in the regulation of gene transcription. Chromatin is anchored by short stretches of DNA sequences called matrix attachment regions (MARs). MARs range in size from 100 to 2000bp and are rich in AT dinucleotide pairs and repetitive sequences. Both chromatin loop formation and the transcriptional activation of genes surrounded by MARs are dependent on nuclear matrix anchorage\(^{55}\). This is explainable by the fact that transcriptional factors are present in the nuclear matrix\(^{56}\). CTCF associates with the nuclear matrix\(^{57,58}\) and mediates the anchoring of DNA sequences to it, as observed in the 5\(^{′}\)-HS4 chicken \(\beta\)-globin insulator\(^{58}\). In addition, the association of CTCF with the nuclear matrix depends on nucleophosmin/B23\(^{59}\). However, it has been observed that IFN-\(\gamma\) induces transcriptional activation of major histocompatibility complex genes, which coincides with the reorganization of chromatin loops\(^{60}\). Interestingly, DNA anchorage to the nuclear matrix after IFN-\(\gamma\) exposure was associated with CTCF binding sites\(^{61}\). Therefore, CTCF reconfigures genomic regions by forming loops that affect the transcriptional gene landscape. In the present paper, we demonstrate that in cancer cells, CTCF is unable to associate with its cognate DNA-binding site in the XAF1 promoter if it is methylated (Fig. 7a), thus effectively rendering it unresponsive to well-known...
activators (Fig. 7a). However, after demethylating the cognate site, CTCF is now able to associate with the XAF1 promoter to enhance transcriptional activation (Fig. 2). One intriguing possibility is that CTCF could be able to attract DNA to the nuclear matrix, mediating faster chromatin loop formation in the nuclear matrix after exposure to exogenous stimuli. Although not tested, we envision that CTCF could be able to attract the XAF1 promoter to the nuclear matrix by its association with nucleophosmin/B23, thereby inducing both chromatin loop formation and transcriptional activation of the XAF1 gene (Fig. 7b). In cancer, this putative mechanism would not occur due to the absence of CTCF in its DNA-binding site via a methylation-sensitive mechanism (Fig. 7a).

Finally, we demonstrate for the first time that CTCF is critical to maintaining key CpG-dinucleotides demethylated in the XAF1 promoter (Fig. 5b). This could be explainable by previous reports showing that CTCF associates with and activates PARP-1, which negatively regulates DNMT1, thus maintaining the CpG dinucleotides within the CTCF-binding sites free from methylation. Additionally, a pool of PARP is located in the nuclear matrix and is implicated in chromatin loop formation. Although not tested, an interesting hypothesis would be the possibility that PARP-1 is a mediator of the effects of CTCF (Fig. 7b). Further experiments are required to test this.

In conclusion, we demonstrate a novel functional CTCF binding site in the XAF1 promoter. The association of CTCF with its binding site induces an open chromatin configuration by enriching active HPMs and maintaining CpG-dinucleotides free from methylation. In cancer, methylation negatively affects the interaction between CTCF and the XAF1 promoter, disabling the protective epigenetic actions of CTCF against the closed-chromatin configuration. Our finding are consistent with CTCF acting as a key regulatory element in the well-accepted observation that CpG-dinucleotide methylation on the XAF1 promoter inhibits its transcriptional activation. The absence of CTCF regulation of the XAF1 gene may constitute a selective advantage during clonal evolution by means of increasing the apoptotic threshold.

Methods

Cell culture and reagents. MCF-7 (HTB22) cells were acquired from the American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). ACHN (CRL-1611) cells were acquired from the ATCC, and maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS. The cells were grown in a humidified incubator that was maintained at 37 °C with 5% CO₂. Demethylating conditions were established by treating the cell lines for 3 days with 0.2 μM Trichostatin-A (TSA) and 5 μM 5-aza-2’-deoxycytidine (5-A-DC) (SIGMA). Daily, the medium was replaced with fresh medium containing 5-A-DC and TSA. The transfection of constructs was performed using Lipofectamine 2000 (Invitrogen). TNF-α and IFN-α were purchased from R&D and PROSPEC, respectively.

Constructs. Genomic DNA isolated from peripheral human blood was used as a template. Primers used in this work are listed in Supplementary Table S1. Specific primers were designed to amplify by PCR the region from −1200 to +350 bp relative to the transcription start site (TSS) from the XAF1 gene (XAF1.2). The PCR product was purified and cloned into pTZ57r/t (Thermo). Then, XAF1.2 was subcloned into the Pearson-2N1 (Clontech) expression vector to produce Pearson-2N1-CTCF-promoter. Deletion of the CTCF-binding site from the Pearson-2N1-XAF1-promoter plasmid was performed using the QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). Following the manufacturer's protocol, we generated Pearson-2N1-Δ-CTCF-XAF1. Specific primers were designed to amplify by PCR the genomic region from −3000 to +350 bp relative to the transcription start site from the XAF1 gene (XAF1 promoter). The PCR product was cloned using GeneJet PCR cloning kit (Fermentas) and was then subcloned into pSEAP2-Basic (Clontech), a secreted alkaline phosphatase (SEAP) gene reporter expression vector, to produce the wild-type-XAF1-promoter-SEAP construct. Using a QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies), we generated the Δ-CTCF-XAF1-promoter-SEAP construct, which lacked the CTCF binding site. To generate an inducible system for CTCF overexpression, CTCF was amplified from cDNA using Pfu polymerase (Stratagene) and cloned into pQCXIP (Clontech). It was then subcloned into pTRE-Tight-Bi-AcGFP1 (Clontech) to produce pTRE-Tight-Bi-AcGFP1-CTCF. All plasmids were confirmed by capillary sequencing.

Transient and stable transfection of MCF-7 cells. MCF-7 cells were seeded in 12-well plates one day before transfection. The cells were co-transfected with 0.625 μg of either wild-type-XAF1-promoter-SEAP or Δ-CTCF-XAF1-promoter-SEAP plasmids and 0.625 pg of pMetLuc (Clontech), which is a plasmid encoding secreted Metridia luciferase used for transfection normalization. After 24h, the transfection medium was changed out for fresh medium. After 48h, the medium was collected to measure both SEAP and Luciferase activities using the Great EscAPE SEAP chemiluminescence kit (Clontech) and Ready-To-Glow-Secreted Luciferase Reporter Assay (Clontech), respectively. For inducible CTCF overexpression, MCF-7 cells were transfected with 2 μg pTet-On plasmid (Clontech), which encodes the rTet repressor protein. The cells were selected in G418 (1000 μg/mL) for 4 weeks. The pool of the resulting colonies was then expanded under G418 selection and cotransfected with 2 μg pTRE-Tight-Bi-AcGFP1-CTCF with 1 μg pQCXIP empty plasmid. Stable cell clones were selected with puromycin after two weeks of selection.
MCF-7 cells were seeded in 6-well plates. After 1 day, the cells were transfected with 2 \( \mu \)g of either peGFP-N1-XAF1-promoter or peGFP-N1-\( \Delta \)-CTCF-XAF1-promoter plasmids. After 48 h, the cells were selected with G418 (1000 \( \mu \)g/mL) for 4 weeks. Then, G418-resistant cells were analysed by fluorescence activate cell sorting (FACS). The resistant cells were further cultured for 30 days in the absence of G418 and were analysed by FACS. Then, single cell clones were isolated. The single cell clones were continuously cultured further in the absence of G418 for 35 days, and reporter gene expression was evaluated by FACS.

**Transient transfection of small interfering RNAs (siRNAs) against CTCF.** ACHN and MCF-7 cells were seeded in 6-well plates. After one day, the cells were treated in demethylating conditions, as indicated above. The cells were then transfected with 0.1 \( \mu \)M human CTCF small interfering RNAs (siRNAs; Qiagen) using Lipofectamine® 2000 (Invitrogen). After 24 h, the transfection medium was replaced with fresh medium containing demethylating agents. RNA isolation was performed 48 h post-transfection using TRIzol reagent (Invitrogen). RNA was converted to cDNA using random primers and SuperScript® VILO (Invitrogen).

**Bisulphite DNA sequencing analysis.** DNA was extracted from either MCF-7 cells or MCF-7 stable cell lines using the FlexiGene DNA Kit (Qiagen). DNA (1.5 \( \mu \)g) was bisulphite converted using the Zymo EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's protocol. The bisulphite-converted DNA was subjected to PCR amplification using specific primers to the XAF1 promoter (XAF-EnBis/XAF-EnBia). A nested-PCR amplification strategy was used to amplify DNA converted from stable cell lines using specific primers against the plasmid sequence (1.2GFPPBis/1.2GFPPBia) in the first PCR reaction to avoid amplification of the XAF1 endogenous promoter. The product from this PCR was used in a second round of PCR amplification using specific primers against the XAF1 promoter, as described above. PCR products were gel purified and cloned using the GeneJET PCR cloning kit (Fermentas), and positive clones were sent for sequencing.

**Chromatin immunoprecipitation.** Cells (3 \( \times \) 10\(^6\)) were fixed with 1% formaldehyde and neutralized by adding 0.125 M glycine. The cells were then lysed in cell lysis buffer (10 mM EDTA, 50 mM TRIS-HCl pH 8, 1% SDS, protease inhibitor cocktail). The cell lysate was sonicated to obtain soluble chromatin with a mean length of 400 bp. Chromatin immunoprecipitation (ChIP) was performed using a specific antibody raised against CTCF (C02-2899; Cell Signaling Technology) or CTCF (07-729; Millipore). Specific antibodies against H3K9me3 (ab8898 Abcam) and H3K4me2 (7766 Abcam ab) were used to perform ChIP assays on soluble chromatin from single cell clones. The DNA recovered after ChIP was subjected to PCR amplification using specific primers to the XAF1 promoter, as described above. PCR products were gel purified and cloned using the GeneJET PCR cloning kit (Fermentas), and positive clones were sent for sequencing.

**Immunoblotting.** Protein fractions were subjected to either 15% or 18% SDS-PAGE and transferred to Immobilon P membranes (Millipore). Next, the membranes were incubated with the indicated antibodies overnight, and the blots were visualized using the Immobilon Western kit (Millipore) with a peroxidase-labelled secondary antibody, according to the manufacturer's protocols.

**Protein immunoprecipitation assay.** The cells were washed with PBS, scraped and centrifuged at 2,000 rpm for 3 min. The cells were lysed using 1 mL of TNTE-5 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% Triton, 1 mM EDTA and 1X protease inhibitor cocktail) and incubated at 4°C for 15 min. The lysates were centrifuged at 14000 \( g \) for 10 min. The supernatants were incubated with 10 \( \mu \)L of recombinant protein G agarose beads (Life Technologies) for 1 h. After incubation, the lysates were centrifuged at 14000 \( g \) for 30 sec. The supernatants were incubated overnight at 4°C with 3 \( \mu \)L of primary antibody with constant agitation. Next, 20 \( \mu \)L of recombinant protein G agarose beads was added to each lysate, and the lysates were then incubated with constant agitation for 1 h on ice. The lysates were next centrifuged at 14000 \( g \) for 10 sec. The resulting pellets were washed twice with TNTE-1 buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Triton, 1 mM EDTA and 1X protease inhibitor cocktail), followed by boiling in Laemmli sample solution (100 mM Tris pH 6.8, 20% Glycerol, 2% SDS, 0.05% bromphenol blue and 100 mM DTT) for further analysis.

**Cell viability assay.** Cell viability was measured colorimetrically using the MTS-PMS assay (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay; Promega) according to the manufacturer's protocol. Briefly, the cells were seeded in 96-well plates, and 24 h after treatment, the reagents from the kit were added to the culture medium. After 2 h of incubation, the absorbance was measured at a wavelength of 490 nm using a microplate reader.

**RT-qPCR.** Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 2 \( \mu \)g of total RNA was used for cDNA synthesis with random hexamers. Quantitative PCR was carried out using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) using
Statistical analysis. GraphPad Prism version 5.0 for Mac Os X (La Jolla, CA) was used to perform statistical analyses. One-way analysis of variance was performed, and the Bonferroni post-test was used at 95% confidence intervals to determine significant differences.

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Author Contributions

G.U.M.-R. performed all plasmid constructs and ChIP assays, conceived experiments, analyzed and performed western-blots and statistical analyses. V.M. interpreted data and reviewed the manuscript. G.V.-A. performed gain-of-function and loss-of-function assays, RT-PCRs, transgene assays, bisulfite genomic sequencing and analyzed data. K.V.-S. performed gene reporter assays. L.J.-H. and L.M.-G. partly supported by CONACYT grant 132931 to Jorge Melendez-Zajgla. The authors thank Jose Luis Melendez-Zajgla. Georgina Victoria-Acosta was a recipient of a PhD fellowship provided by Consejo Nacional de Ciencia y Tecnología (CONACYT) [grant number 267949], and this study is part of her doctoral thesis from the Biomedical Sciences Doctorate Program, Universidad Nacional Autonoma de Mexico. This work was partly supported by CONACYT grant 132931 to Jorge Melendez-Zaigla. The authors thank Jose Luis Cruz-Colin for his help in cell culture techniques.

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Author Contributions

G.V.-A. performed gain-of-function and loss-of-function assays, RT-PCRs, transgene assays, bisulfite genomic sequencing and analyzed data. K.V.-S. performed gene reporter assays. L.J.-H. and L.M.-G. performed western-blots and statistical analyses. V.M. interpreted data and reviewed the manuscript. G.U.M.-R. performed all plasmid constructs and ChIP assays, conceived experiments, analyzed and
interpreted data, and wrote the manuscript. J.M.-Z. conceived experiments, analyzed and interpreted
data, reviewed the manuscript and wrote the final version.

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Corrigendum: Epigenetic silencing of the XAF1 gene is mediated by the loss of CTCF binding

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In the Supplementary Information file originally published with this Article, Supplementary Figures 1c and 1d were incorrectly labelled as Figure 1a and 1b. In addition, Supplementary Figures 2b, 2c and 2d were incorrectly labelled as Figure 1c, 1d and 1e. Lastly, Supplementary Figures 1a, 1b and 2a were omitted. These errors have been corrected in the Supplementary Information that now accompanies the Article.

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