p53-paralog DNp73 oncogene is repressed by IFNα/STAT2 through the recruitment of the Ezh2 polycomb group transcriptional repressor

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The DNp73 proteins act as trans-repressors of p53 and p73-dependent transcription and exert both anti-apoptotic activity and pro-proliferative activity. DNp73s are frequently up-regulated in a variety of human cancers, including human hepatocellular carcinomas (HCCs). Increased levels of DNp73 proteins confer to HCC cells resistance to apoptosis and, irrespective to p53 status, a chemoresistant phenotype. Here, we show that interferon (IFN)α down-regulates DNp73 expression in primary human hepatocytes (PHHs) and HCC cell lines. IFNα has been used as pro-apoptotic agent in the treatment of malignancies and there is increasing evidence of IFNα effectiveness in HCC treatment and prevention of recurrence. The precise mechanisms by which class I IFNs exert their anti-proliferative and anti-tumor activity remain unclear. IFNα binding to its receptor activates multiple intracellular signaling cascades regulating the transcription of numerous direct target genes through the recruitment of a complex comprising of STAT1, STAT2 and IFN regulatory factor (IRF)9 to their promoters. We found that, in response to IFNα, the P2p73 promoter undergoes substantial chromatin remodeling. Histone deacetylases (HDACs) replace histone acetyl transferases. STAT2 is recruited onto the endogenous P2p73 promoter together with the polycomb group protein Ezh2, leading to increased H3K27 methylation and transcriptional repression. The reduction of DNp73 levels by IFNα is paralleled by an increased susceptibility to IFNα-triggered apoptosis of Huh7 hepatoma cells. Our results show, for the first time, that IFNα-stimulated gene factor 3 recruitment may serve both in activating and repressing gene expression and identify the down-regulation of DNp73 as an additional mechanism to counteract the chemoresistance of liver cancer cells.

Keywords: transcription; p53 family; epigenetics; chromatin dynamics; apoptosis

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent solid tumors worldwide and represents the third cause of cancer mortality (Fattovich et al., 2004; El-Serag and Rudolph, 2007). Cytotoxic chemotherapy has proven largely ineffective and several mechanisms have been proposed to explain HCC chemoresistance in vivo. p53 status is one important determinant of the response of tumor cells to anti-cancer drugs that trigger apoptosis by inducing DNA damage. In HCC, patients exposed to the aflatoxin B1 mycotoxin a ‘hot spot’ mutation at codon 249, leading to the expression of the R249S DNA binding defective p53 mutant protein, is found in up to 50–70% of cases (Moradpour and Blum, 2005; El-Serag and Rudolph, 2007). In regions where aflatoxin levels in food are low or undetectable, p53 mutation are rarely observed (4–8% of HCCs) and no specific P53 gene mutation pattern can been detected (Moradpour and Blum, 2005; El-Serag and Rudolph, 2007). More recently the p53 family genes TP63 and TP73 have been implicated in both tumorigenesis and in the response to chemo and radiotherapies (Stiewe, 2007; Lunghi et al., 2009). The p53 family genes produce multiple isoforms that differ in their NH2-termini and can be functionally grouped into dominant negative (DN) and trans-activation competent (TA) proteins (Levrero et al., 2000; Melino et al., 2002; Stiewe et al., 2007). p53, TAp73 and TAp63 share the ability to activate transcription of genes involved in cell cycle arrest and apoptosis. The DNp73 proteins lack the N-terminal trans-activation domain and act as trans-repressors of p53 and p63/p73-dependent transcription showing both anti-apoptotic and pro-proliferative potential (Vossio et al., 2002). DNp73 proteins are generated either by alternative exon splicing of the P1p73 promoter transcript (DEx2p73, DEx2/3p73 and DN’p73) or via the alternative use of a second intragenic promoter located in intron 3 (P2p73) (Levrero et al., 2000; Stiewe et al., 2004). DNp73 proteins are up-regulated in a variety of human cancers and accumulate progressively.
in chronic hepatitis, cirrhosis and HCC, conferring to HCC cells a chemoresistant phenotype (Stiewe et al., 2004; Müller et al., 2005; Leverero, 2006).

Interferons (IFN\(_{\alpha}\)) have been used in the treatment of several malignancies including myeloma, melanoma, renal and bladder cancer. IFN\(_{\alpha}\) has also been proposed both in the prevention and treatment of HCC (Chattopadhyay et al., 2007). Although the impact of IFN\(_{\beta}\)-based therapies to prevent HCC development in cirrhotic patients is still highly controversial (Craxì`, 2005; Shiratori et al., 2005; Lok et al., 2009), recent studies have shown clinical efficacy of IFN\(_{\alpha}\) alone and in combination with chemotherapeutic agents in the treatment of advanced HCCs with portal vein tumor thrombosis and/or extrhepatic metastasis (Obi et al., 2006; Uka et al., 2008). Moreover, in randomized clinical trials, IFN\(_{\alpha}\) has been shown to prevent HCC recurrence and to improve survival after curative treatments in early HCCs (Breitenstein et al., 2009; Miyake et al., 2010). The molecular mechanisms underlying IFN\(_{\alpha}\) anti-tumor activity are still unclear. Type I interferons (IFN\(_{\alpha}/\beta\)) are multifunctional cytokines that mediate host defense against microbial challenges, modulate innate and adaptive immune responses and influence both normal and neoplastic proliferation (Pestka et al., 2004) through the activation of multiple intracellular signaling cascades (Uddin and Platanias, 2004) and the transcriptional activation of downstream IFN-sensitive genes (ISGs), including a number of genes involved in the modulation of apoptosis (Chawla-Sarkar et al., 2003). Here, we show that IFN\(_{\alpha}\) down-regulates DNp73 expression in primary human hepatocytes (PHHs) and HCC cell lines through the co-recruitment of STAT2 and the Ezh2 transcription repressor onto the endogenous P2p73 promoter. The reduction of DNp73 levels induced by IFN\(_{\alpha}\) is paralleled by an increased susceptibility to IFN\(_{\alpha}\)-triggered apoptosis of Huh7 hepatoma cells. These results show, for the first time, that IFN\(_{\alpha}\)-stimulated gene factor 3 (ISGF3) recruitment may serve both in activating and repressing gene expression and identify the down-regulation of DNp73 as an additional mechanism to counteract the chemoresistance of liver cancer cells.

Results

**DNp73 expression is repressed in response to IFN\(_{\alpha}\)**

Bioinformatic analysis of the P2p73 promoter (TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html) and MatInspector (http://www.genomatix.de/matinpector.html) with a cut-off for matching to matrices >80%) reveals the presence of two conserved interferon stimulated responsive elements (ISREs), the first in the core promoter, between the transcription start site and the ATG codon (ISRE (I)), and the second located at -1200 bp from the ATG (ISRE (II)) (Figure 1a).

To assess whether these ISRE elements are involved in the regulation of the P2p73 promoter and DNp73 expression in liver cells we used Huh7 HCC cell line, which shows detectable basal levels of DNp73 mRNA and protein. Although Huh7 cells have been described to harbor a defective IFN response to influenza A, vesicular stomatitis virus or Sendai viruses infection (Keskinen et al., 1999), the components of the IFN class I signaling pathway are conserved although relatively high concentrations of exogenous IFN\(_{\alpha}\) are required to activate the ISGs (Melén et al., 2000). The transcriptional activity of class I IFNs is mediated by the binding of the ISGF3 trimeric transcription complex, that is comprised of STAT1, STAT2 and IRF9 (Fu et al., 1990), to the sequence-specific ISREs sites located in the regulatory regions of target genes. STAT2 recruitment in the ISGF3 complex is specifically associated with Type I IFN-dependent signaling (Leung et al., 1995) and can be, therefore, used to track IFN\(_{\alpha}\)-induced ISGF3 binding to target ISRE elements.

We first confirmed that in IFN\(_{\alpha}\)-treated Huh7 cells STAT2 is rapidly phosphorylated (Supplementary Figure S1a) and translocates to the nucleus (Supplementary Figure S1b), where it is recruited onto the promoter region of the IFN-regulated gene ISG15 (Supplementary Figure S1c) to stimulate its transcription (Supplementary Figure S1d). These results are in agreement with previous results obtained in Huh7 cells exposed to exogenous IFN\(_{\alpha}\) and showing the recruitment of STAT1 and STAT2 onto several ISRE-regulated genes (Testoni et al., submitted).

As shown in Figure 1b, the activity of a P2p73 promoter luciferase reporter is strongly repressed in response to IFN\(_{\alpha}\) treatment in Huh7 cells. Using different luciferase reporter constructs carrying P2p73 deletions (Vossio et al., 2002) (Figure 1a), we found that the -306/+76 construct lacking the upstream ISRE (II) site continues to be repressed by IFN\(_{\alpha}\), indicating that the core ISRE (I) site is sufficient to mediate IFN\(_{\alpha}\) repression of the P2p73 promoter (Figure 1b). Similar results were obtained in Huh6 and HepG2 cell lines (Supplementary Figure S2). Next, we showed that in IFN\(_{\alpha}\)-treated Huh7 cells and PHH, DNp73 mRNA levels, measured by an isomorf-specific real-time PCR, decline progressively and are reduced by 80% 8h after stimulation (Figure 1c). Altogether, these results indicate that the activity of the endogenous P2p73 promoter is repressed by IFN\(_{\alpha}\) and that this effect is not influenced by the p53 status.

**IFN\(_{\alpha}\)-induced repression of DNp73 correlates with apoptosis induction**

IFN\(_{\alpha}\) induces apoptosis in hepatoma cells (Fujiooka et al., 2006; Herzer et al., 2009) and induction of TRAIL, the ligand for the DR4 and DR5 death receptors, has been proposed to have a role (Herzer et al., 2009). We found that exposure of Huh7 cells to IFN\(_{\alpha}\) (1000 IU/ml) induces apoptosis, as shown by the increased percentage of sub-G1 population at propidium iodide fluorescence-activated cell sorting (FACS) analysis (Figure 2a) and the accumulation of cleaved poly(ADP-ribose) polymerase (PARP) fragment (Figure 2b). Apoptosis induction was already evident at 12h of...
IFNα treatment in the absence of changes in cell cycle dynamics (Figure 2a, right panel). The accumulation of cleaved PARP in response to IFNα started at 8 h, when the protein levels of DNp73 begin to decrease, and it was evident at 12 h when the peak DNp73 protein down-regulation (70–80%) is observed (Figure 2b). The role of DNp73 in the modulation of IFNα-induced apoptosis is supported by the observation that its over-expression abrogates Huh7 apoptosis response after exposure to IFNα (Figures 2c and d). In view of the established anti-apoptotic activity of DNp73 potential in a variety of human cells (Vossio et al., 2002; Stiewe, 2007) and its frequent up-regulation in human HCCs (Mueller et al., 2005) these results suggest that reduction of DNp73 expression might contribute to IFNα apoptotic activity.

DNp73 is a direct IFNα/STAT2 transcriptional target gene

Next, we investigated the in vivo occupancy of the ISRE sites in the P2p73 promoter by STAT2 in untreated and IFNα-treated cells. Chromatin immunoprecipitation (ChIP) assays show that STAT2 is recruited in vivo in Huh7 cells on ISRE (I) site 30 min after exposure to IFNα (Figure 3a, left panel) and an ISGF3 complex containing the phosphorylated form STAT2 is present on the promoter after 1 h stimulation (Figure 3b, left panel). No STAT2 or P-STAT2 recruitment was observed on the ISRE (II) site (Figures 3a and b, right panel), supporting the notion that the core ISRE (I) element is sufficient to mediate STAT2/IFNα transcriptional activity. These results were also confirmed in human primary hepatocytes, where the phosphorylated form of STAT2 is detected on the core ISRE (I) element after 1 h of IFNα treatment (Figure 3c, left panel). The ISRE (II) site again displays no relevant increase in STAT2 recruitment after IFNα (Figure 3c, right panel). To further confirm the role of ISRE (I) in mediating IFNα repression of P2p73, we tested a −827/+76 luciferase reporter construct carrying a deletion between nucleotide +32 and +44 with respect to transcription start site (tss) (corresponding to the core conserved ISRE (I) nucleotides (Kessler et al., 1988)). As shown in Figure 3d, the −827ΔISRE-luc is only marginally repressed by IFNα. Altogether these results indicate that the recruitment of STAT2 and its phosphorylated form is associated with the transcriptional repression of the P2p73 promoter and that the core ISRE (I) site mediates IFNα transcriptional repression in vivo.
H3K27 methylation mediates IFNα-induced repression of the P2p73 promoter
The notion of IFNα-repressed genes is increasingly recognized (Radaeva et al., 2002) but the mechanistic basis for their transcriptional repression is not clear so far. The well-established model of IFNα signaling through its receptor binding, phosphorylation of STATs and ISGF3 complex formation and recruitment to target promoters is fully demonstrated for activated ISGs (Levy and Darnell, 2002), while the efforts to unveil IFNα-dependent repression of few genes, namely c-MET and hTERT, fail to find a direct transcriptional role of STATs (Xu et al., 2000; Radaeva et al., 2002). In an attempt to correlate the recruitment of STAT2 on the P2p73 promoter and IFNα-induced repression of DNp73 expression we performed additional chromatin immunoprecipitation experiments to assess whether the combinatorial action of other transcription factors and/or the co-recruitment of transcriptional co-repressors might be involved to convert an a priori active chromatin-bound STAT2 complex into a repressive one.

ChIP experiments in Huh7 cells have shown that histone 3 and 4 acetylation (AcH3 and AcH4) are sharply reduced at the ISRE (I) site after exposure to IFNα, concurrent with a decrease in the binding of both the acetyl transferase p300 and serine phosphorylated RNA polymerase II to the same promoter region (Figure 4a, left panel). We also found that histone 3 methylation on lysine 27 is increased at the ISRE (I) site 1 h after IFNα treatment (Figure 4a, right panel).

As H3K27 methylation is the hallmark of the transcriptional repression mediated by Ezh2, a polycomb group (PcG) chromatin-modifying enzyme with histone lysine methyltransferase activity (Caretti et al., 2004; Montgomery et al., 2005; Margueron et al., 2009), we verified its recruitment on the P2p73 promoter in IFNα-treated Huh7 cells (Figure 4a, right panel). Ezh2 is part of the PcG repressive complex 2 whose transcriptional repressive activity is also mediated by the interaction and co-recruitment of HDACs (Van der Vlag and Otte, 1999). As shown in Figure 4a (right panel), Ezh2 recruitment at the ISRE (I) site in the P2p73 promoter
is paralleled by an increase in HDAC1 binding. To functionally link IFNα-induced recruitment of Ezh2 and chromatin changes at the P2p73 promoter with its transcriptional repression we made abrogated Ezh2 expression using specific small interfering RNAs (siRNAs). Depletion by 50% (Figure 4b, lower panel) of Ezh2 in Huh7 cells prevented IFNα-induced repression of the /C0 827/+76 P2p73-luc reporter construct (Figure 4b, upper panel). Altogether these data indicate that in response to IFNα treatment there is a profound re-shuffling of transcription cofactors and chromatin in order to establish a repressive state at the P2p73 promoter.

This is also the first indication of active ISGF3 recruitment onto the promoter region of an IFNα-regulated gene being associated to transcriptional repression. Importantly, our data indicate that recruitment of the phosphorylated ‘active’ form of STAT2 is required for establishing transcriptional repression.

Discussion

IFNα, alone or in combination with chemotherapeutic drugs, has been used with variable efficacy in the treatment of different malignancies including HCC. IFNα has been shown to prevent the growth of preneoplastic lesions and to inhibit the development of HCC in rat models of hepatocarcinogenesis (Nakaji et al., 2004), to exert an inhibitory effect on tumor growth in an HCC cell line xenograft model (Noguchi et al., 2008) and to act negatively on cell proliferation of murine hepatic progenitor cells in vitro and in vivo (Lim et al., 2006). The anti-tumor effects of class I IFNs are thought to be mediated either by indirectly modulating immunomodulatory and anti-angiogenic responses or by directly affecting proliferation or cellular differentiation of tumor cells. Accordingly, in the HCC setting IFNα inhibits cells growth (Fujioka et al., 2006) and induces apoptosis (Herzer et al., 2009) of hepatoma cells in vitro but also displays anti-angiogenesis effects mediated by a reduction of VEGF synthesis and a decrease of intra-tumoral CD31+ endothelial cells (Nakaji et al., 2004). Both direct or indirect anti-tumor effects of IFNs result from the transcriptional regulation of specific subsets of ISGs (Chawla-Sarkar et al., 2003). Oligonucleotide microarray studies have greatly enlarged the spectrum of ISGs that are either activated or repressed in response to IFNα in different normal
and neoplastic cell lineages. In addition to the ISGs implicated in anti-viral, anti-angiogenic, immunomodulatory and cell cycle inhibitory effects, a number of ISGs with apoptotic functions have been identified. These include tumor necrosis factor-α-related apoptosis inducing ligand (TRAIL/Apo2L), Fas/FasL, XIAP-associated factor-1, caspase-4, caspase-8, dsRNA activated protein kinase, 2', 5'-oligoadenylate synthetase, death activating protein kinases, phospholipid scramblase, galectin 9, many IRFs, the promyelocytic leukemia gene and regulators of IFN-induced death (Chawla-Sarkar et al., 2003).

The mechanisms, genes and regulatory networks involved in IFNα-mediated apoptosis in HCC patients and in hepatoma cells are still poorly elucidated. In patients with HBV-related HCC the therapeutic
response to IFNα as adjuvant therapy was higher in the subgroup of male patients with overall poor prognosis displaying low expression of mir26a and mir26b and a distinct transcriptomic signature with the activation of NF-kB and IL6 signaling pathways (Ji et al., 2009). Recently, the DR4/DR5 death receptors ligand TRAIL and the promyelocytic leukemia protein have also been shown to have an important role in mediating, in a p53 independent manner, IFNα-induced apoptosis in HCC cells (Herzer et al., 2009). In our work we correlate IFNα-induced apoptosis with its ability to repress DNP73, a DN isoform of the p53 family member p73. DNP73 exerts a potent anti-apoptotic activity in a variety of human cells (Vossio et al., 2002; Stiewe, 2007) and it is up-regulated in human HCCs (Miller et al., 2005), thus suggesting that its repression might contribute to IFNα anti-apoptotic activity. We show that IFNα down-regulates DNP73 expression in PHHs and HCC cell lines by causing substantial chromatin remodeling of the P2p73 promoter in vivo as assessed by ChIP experiments. HDAC1 is recruited onto the IFN responsive element ISRE (I) in the P2p73 promoter whereas p300 acetyl transferase occupancy and histone acetylation is reduced. These changes are coincident with the binding of a STAT2-containing ISGF3 complex together with the PcG transcription repressor Ezh2, leading to increased H3K27 methylation and transcriptional repression. The role of Ezh2 in mediating IFNα-induced repression of the P2p73 promoter was further confirmed by the ability of specific anti-Ezh2 siRNA to prevent IFNα inhibitory activity. Interestingly, PcG repressive complex 2 and Ezh2 over-expression has been reported in HCCs (Sudo et al., 2005; Steele et al., 2006; Yonemitsu et al., 2009) and associated with more aggressive tumors (Sudo et al., 2005; Steele et al., 2006). Altogether our data represent the first demonstration of recruitment of an ISGF3 complex onto the promoter region of an IFNα-regulated gene being associated to transcriptional repression. Importantly, the recruitment of the phosphorylated ‘active’ form of STAT2 is required for establishing transcriptional repression. In an attempt to speculate about the mechanism by which the P2p73 ISRE (I) might supports a ‘transcriptionally repressive’ vs ‘active’ ISGF3-containing complexes, we analyzed the nucleotide sequence and position of ISRE sites directly bound by STAT2 in ChIP-chip analysis and derived from several ISGs activated or repressed by IFNα in Huh7 cells (Testoni et al., submitted). As shown in Supplementary Figure S3, the graphical representation of the multiple sequences alignment does not show any significant difference in nucleotide composition or conservation between activated and repressed genes. On the other hand, it is worth to note that in the activated targets, all the ISREs are located between 0 and −200 from tss, while in the repressed group, the ISREs are far upstream or downstream. Therefore, it is rather unlikely that the nucleotide composition of ISREs accounts for the ‘choice’ between activating and repressing complexes, whereas we can hypothesize a ‘position effect’ on the role of ISREs sites in triggering a positive or negative transcriptional effect. A genome-wide approach combining in silico analysis, chromatin studies and expression profiling will be needed to confirm this hypothesis.

The identification of genes targeted by IFNα in the liver and the cellular pathways associated with clinical response to IFNα may both help in defining those patients that would benefit more from IFNα treatment in different clinical settings (that is, prevention of recurrence in early HCCs, first/second line treatment of advanced or metastatic tumors). IFNα-induced down-regulation of DNP73 may represent an additional mechanism to counteract the chemoresistance of liver cancer cells.

**Materials and methods**

**Cell cultures and IFNα treatments**

Huh7 (codon 220 mutated p53), HepG2 (wt p53) and Huh6 (wt p53) hepatoma cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum (Gibco-Life Tech, Carlsbad, CA, USA), 1% penicillin/ streptomycine and 1% glutamine (Sigma, St Louis, MO, USA). IFNα was used at a final concentration of 1000 IU/ml and added directly to the culture medium.

**Liver tissue samples and primary culture of human hepatocytes**

PHHs were prepared from adult patients undergoing lobectomy or segmental liver resection for medically required purposes unrelated to this research program. The use of these human hepatic specimens for scientific purposes has been approved by the French National Ethnic Committee. The PHHs used in this study were obtained from two patients: FT304, a 52-year-old female transplant donor and FT310, a 60-year-old female, undergoing liver resection for a cystadenoma. Both patients were negative for hepatitis B virus, hepatitis C virus and human immunodeficiency virus serologic markers. PHHs were prepared and cultured as described elsewhere (Pichard-Garcia et al., 2002). The cells were plated into collagen-coated dishes (BD Biosciences, Billerica, MA, USA) at 1.7 × 10⁶ cells/cm² in a hormonally and chemically defined medium (Pichard-Garcia et al., 2002). Forty-eight hours after plating, cells were cultured in the presence or absence of IFNα at 1000 U/ml for 1 h.

**RNA extraction and qRT–PCR**

Sub-confluent Huh7 cells were treated with 1000 IU/ml of IFNα and total mRNAs were extracted at different time points (RNAeasy mini kit—Qiagen, Germantown, MD, USA). Reverse transcription was performed using the random hexamers method (Superscript II kit—Invitrogen-Life Tech, Carlsbad, CA, USA). RNA quality and quantity were monitored by ethidium-bromide staining and by UV adsorbance. Real-time PCRs were run in a 7900HT machine using Taqman assays (Applied Biosystems, Foster City, CA, USA). #Hs_00165272_ml for DNP73. 18S was used to normalize equal loading of each sample (Applied Biosystems #Hs_99999901_s1).

**Small interfering RNA**

RNA interference was obtained using the α-Ezh2 and Control SmartPool siRNAs (Dharmacon, Lafayette, CO, USA). Cells were harvested 48 h after transfection.

**Western blot analysis**

Sub-confluent Huh7 cells, treated with 1000 IU/ml of IFNα, were harvested at the different time points in 50 mM Tris–HCl pH 8,
120 mM NaCl, 1 mM diethiothreitol, 0.5% NP40, 1 × PIC (Sigma, Inc) for protein extraction. Protein concentration was determined by the bicinchoninic acid protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Protein lysates were transferred onto nitrocellulose membrane and incubated with the following antibodies: P-STAT1 (#9171 Cell Signaling, Danvers, MA, USA), P-STAT2 (#J07-224 Upstate, Lake Placid, NY, USA); DNP73 (#MG-313 Imgenex, San Diego, CA, USA), cleaved PARP (#9541 Cell Signaling) and tubulin (#MS-719 NeoMarkers, Fremont, CA, USA).

DNA transfection and luciferase assay
Luciferase activity was measured in sub-confluent Huh7 cells transfected with the different P2p73 promoter constructs using the Lipofectamine plus reagent (Invitrogen). Briefly, 3 h post-transfection the medium was replaced with 10% serum supplemented DMEM plus 1000 IU/ml of IFNα. After additional 18 h, cells were collected and the cell extracts were analyzed for luciferase activity. The P2p73 promoter deletion mutants and the pcDNA3-DNp73 expression vector were described previously (Vosso et al., 2002; Belloni et al., 2006).

ISRE mutagenesis
−827ΔISRE-luc construct was developed from −827/+76 promoter as described previously. The deletion between nucleotide +32 and +44 after tss was obtained using the Quickchange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA, #200518) according to the manufacturer’s instructions. The construct was confirmed by sequencing.

ChIP and real-time PCR quantification
ChIP analysis was based on the protocol described in Testoni et al. (2006). Briefly, cells (0.5/1 × 10^6) were washed in phosphate-buffered saline and incubated for 10 min with 1% formaldehyde; after quenching the reaction with glycine phosphate-buffered saline and incubated for 10 min with 1%

Conflict of interest
The authors declare no conflict of interest.

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