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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 IFN-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.
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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 NH_2-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, 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; Műller *et al*., 2005; Levrero, 2006).

Interferons (IFNs) have been used in the treatment of several malignancies including myeloma, melanoma, renal and bladder cancer. IFNα has also been proposed both in the prevention and treatment of HCC (Chattopadhyay *et al*., 2007). Although the impact of IFN-based therapies to prevent HCC development in cirrhotic patients is still highly controversial (Craxì and Cammà, 2005; Shiratori *et al*., 2005; Lok *et al*., 2009), recent studies have shown clinical efficacy of IFNα alone and in combination with chemotherapeutic agents in the treatment of advanced HCCs with portal vein tumor thrombosis and/or extrahepatic metastasis (Obi *et al*., 2006; Uka *et al*., 2008). Moreover, in randomized clinical trials, IFNα 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 IFNα anti-tumor activity are still unclear. Type I interferons (IFNα/β) 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 IFNα 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 IFNα is paralleled by an increased susceptibility to IFNα-triggered apoptosis of Huh7 hepatoma cells. These results show, for the first time, that IFN-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 IFNα

Bioinformatic analysis of the P2p73 promoter (TFsearch (http://www.cbrc.jp/research/db/TFSEARCH.html) and MatInspector (http://www.genomatix.de/matinspector.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 IFNα 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 IFNα-induced ISGF3 binding to target ISRE elements.

We first confirmed that in IFNα-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 IFNα and showing the recruitment *in vivo* of both 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 IFNα 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 IFNα, indicating that the *core* ISRE (I) site is sufficient to mediate IFNα 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 IFNα-treated Huh7 cells and PHH, DNp73 mRNA levels, measured by an isoform-specific real-time PCR, decline progressively and are reduced by 80% 8 h after stimulation (Figure 1c). Altogether, these results indicate that the activity of the endogenous P2p73 promoter is repressed by IFNα and that this effect is not influenced by the p53 status.

IFNα-induced repression of DNp73 correlates with apoptosis induction

IFNα induces apoptosis in hepatoma cells (Fujioka *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 IFNα (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 12 h of

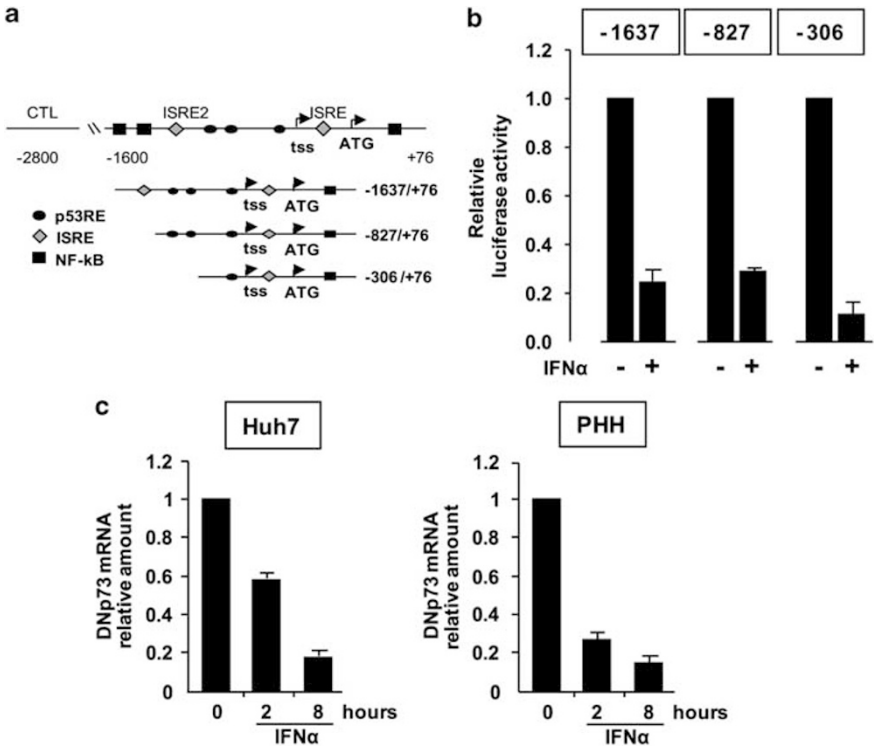

Figure 1 DNp73 expression is repressed in response to IFN-α. (**a**) Schematic representation of the human P2p73 promoter (upper panel) and its deletion mutants (lower panel); putative p53, NF-kB and ISRE sites are indicated. ISRE elements: ISRE (I): −201/−186; ISRE (II): −1236/−1222. Nucleotide positions are relative to the ATG present in exon 3′. (**b**) Huh7 cells were transfected with 300 ng of the different P2p73 promoter constructs, exposed to IFNα (1000 UI/ml) for 18 h and cell extracts from untreated and IFNα-treated cells were tested for luciferase activity. The −306/+76 construct continues to be repressed by IFNα indicating that the *core* ISRE (I) site is sufficient for IFNα-induced P2p73 repression. Results are expressed as relative activity to the untreated control after normalization for transfection efficiency using the dual luciferase assay system. Histograms show the mean values of three experiments each performed in triplicate; bars indicate s.d. (**c**) RNAs from Huh7 cells and PHHs left untreated or treated with IFNα 1000 UI/ml for the indicated times were PCR analyzed with primers specific for DNp73 transcripts. Results are normalized with the expression level of the 18S RNA and expressed as mRNA amounts relative to the untreated control. Histograms show the mean of three experiments; bars indicate s.d.

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 (Műller *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*.

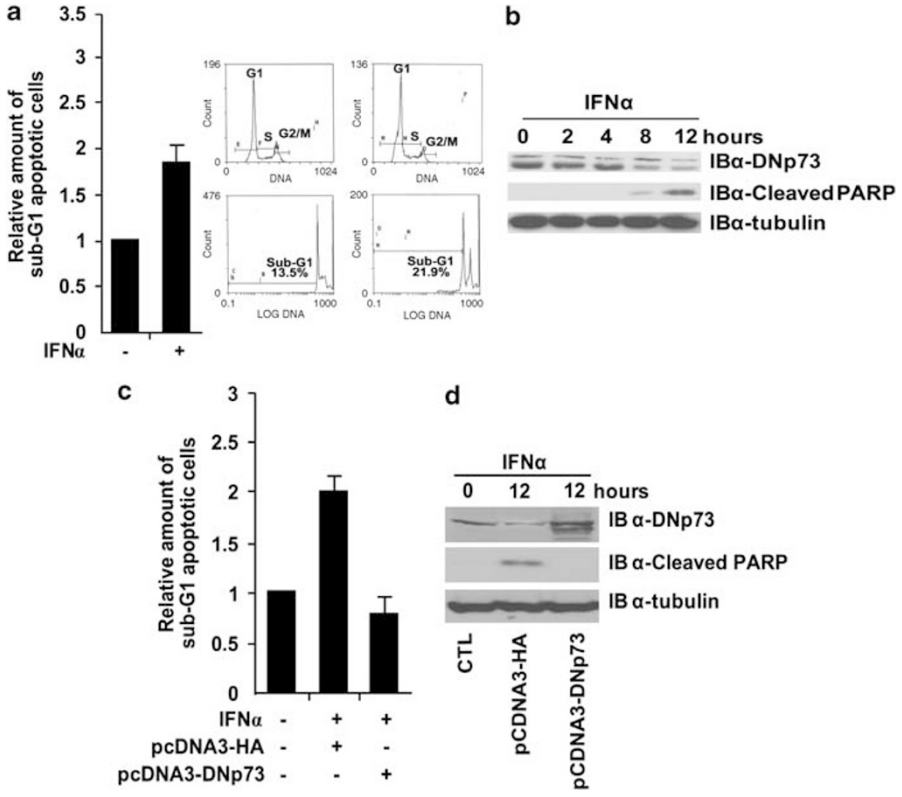

Figure 2 DNp73 repression and apoptosis induction in IFNα-treated cells. (**a**) Untreated and IFNα-treated (1000 UI/ml for 12 h) Huh7 cells were stained with propidium iodide and FACS analyzed for DNA content. Histograms (left panel) show the mean sub-G1 values of three experiments; bars indicate s.d. The right panel shows sub-G1 population and cell cycle distribution from one representative experiment. (**b**) Apoptosis temporally correlates with the reduction of DNp73 protein levels in response to IFNα. Whole cell extracts from untreated and IFNα-treated (1000 UI/ml for the indicated times) Huh7 cells were immunoblotted with anti-cleaved PARP and anti-DNp73 antibodies. Tubulin levels detected by immunoblotting were used to normalize equal loadings from lysate samples. (**c**) DNp73 overexpression abrogates IFNα-induced apoptosis. Huh7 cells were transfected with 300 ng of pCDNA3-DNp73 or pCDNA3-HA expression vectors (24). After IFNα treatment (1000 UI/ml for 12 h) cells were stained with propidium iodide and FACS analyzed for DNA content. Histograms (left panel) show the mean sub-G1 values of three experiments; bars indicate s.d. (**d**) Whole cell extracts from IFNα-treated Huh7 cells not transfected, transfected with the control pCDNA3-HA and with pCDNA3-DNp73 expression vectors were immunoblotted with anti-DNp73 and anti-cleaved PARP antibodies. Tubulin levels detected by immunoblotting were used to normalize equal loadings from lysate samples.

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 methyl transferase 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

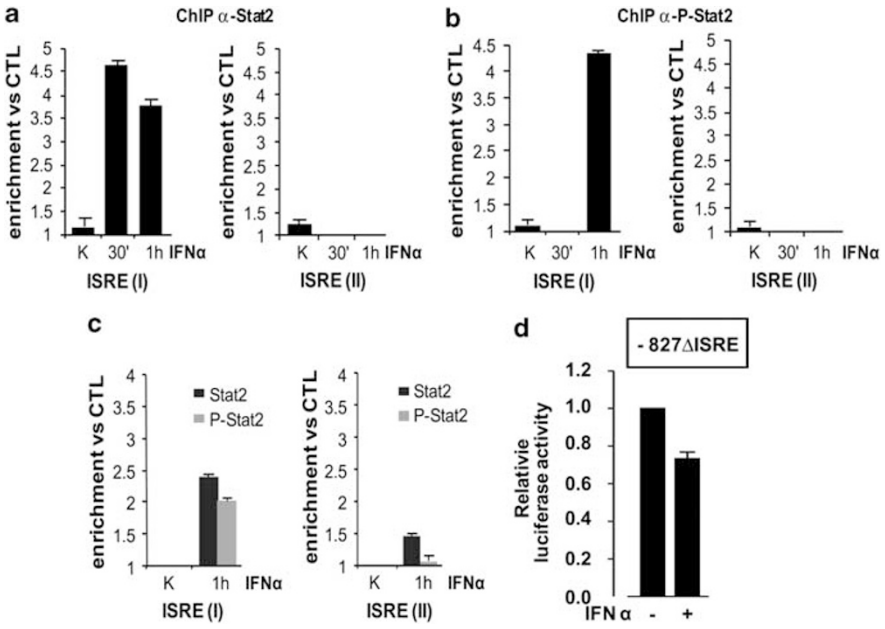

Figure 3 DNp73 is a direct STAT2 transcriptional target gene. (**a**) STAT2 is recruited onto the ISRE (I) element in the P2p73 promoter in response to IFNα. Chromatin from untreated and IFNα-treated (1000 UI/ml for the indicated times) Huh7 cells was immunoprecipitated with either the relevant control IgG or an anti-STAT2 antibody and PCR analyzed with primers amplifying the regions containing ISRE (I) (left panel) and ISRE (II) (right panel) sites in the P2p73 promoter. Results are expressed as specific enrichment (% of input values on ISRE sites divided for % of input values on the control (CTL) region) (see Materials and methods section for more details). (**b**) ISRE (I) bound complexes after IFNα treatment are enriched in phospho-STAT2. Chromatin was prepared as in (**a**), immunoprecipitated with a specific anti-phosphorylated-STAT2 antibody and PCR analyzed with primers amplifying the regions containing ISRE (I) (left panel) and ISRE (II) (right panel) sites in the P2p73 promoter. ChIP-specific enrichment is calculated as in (**a**). (**c**) STAT2 and phospho-STAT2 are recruited *in vivo* onto the ISRE (I) site in the P2p73 promoter in PHHs exposed to IFNα. Chromatin was prepared from untreated and IFNα-treated (1000 UI/ml for the indicated times) PHH, immunoprecipitated with either anti-STAT2 or anti-phospho-STAT2 antibodies and PCR analyzed with P2p73 ISRE (I) (left panel) and ISRE (II) (right panel)-specific primers. Results are expressed as in (**a**). (**d**) ISRE (I) deletion abrogates IFNα-driven P2p73 repression. Huh7 cells were transfected with 300 ng of the −827ΔISRE-luc, exposed to IFNα (1000 UI/ml) for 18 h and cell extracts from untreated and IFNα-treated cells were tested for luciferase activity. Results are expressed as relative activity to the untreated control after normalization for transfection efficiency using the dual luciferase assay system. Histograms show the mean values of three experiments each performed in triplicate; bars indicate s.d.

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 −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

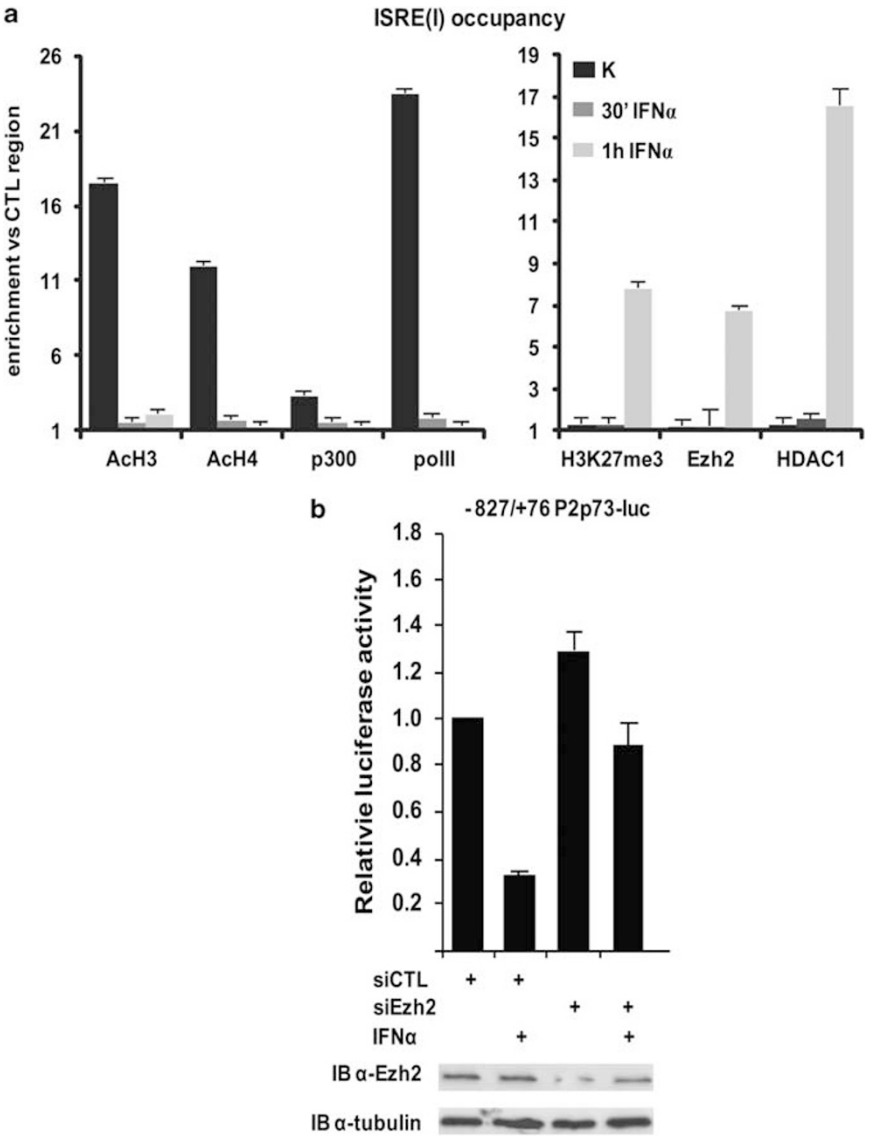

Figure 4 Ezh2 PcG protein contributes to repress P2p73 promoter in response to IFNα. (**a**) Left panel: IFNα-induced changes in histones marks and chromatin-modifying enzymes occupancy at the ISRE (I) site. Chromatin from untreated and IFNα-treated Huh7 cells was immunoprecipitated with the indicated antibodies and PCR analyzed with P2p73 ISRE (I)-specific primers in the P2p73 promoter. Results are expressed as specific enrichment, as described in legend of Figure 3a. Right panel: HDAC1 and Ezh2 are recruited onto the ISRE (I) site in the P2p73 promoter. Chromatin from untreated and IFNα-treated (1000 UI/ml for the indicated times) Huh7 cells were immunoprecipitated with anti-HDAC1, anti-H3K27me3 or anti-Ezh2 antibodies and PCR analyzed with primers amplifying the regions containing ISRE (I). (**b**) siRNA-mediated depletion of Ezh2 prevents IFNα-induced repression of the P2p73 promoter. Untreated and IFNα-treated (1000 UI/ml) Huh7 cells were co-transfected with 300 ng of the $-827/+76$ P2p73-luc reporter construct and 50 nM control (CTL) or anti-Ezh2-specific siRNA pools. Cell cultures were harvested 48 h after transfection and cell extracts were tested for luciferase activity as described in Materials and methods. Results are expressed as relative activity to the IFNα-untreated cells transfected with the control siRNA pool after normalization for transfection efficiency using the dual luciferase assay system. Histograms show the mean of three experiments each performed in triplicate; bars indicate s.d. Lower panel: whole cell extracts from untreated and IFNα-treated Huh7 cells co-transfected with the CTL and anti-Ezh2 siRNA pools were immunoblotted with anti-Ezh2 antibody. Tubulin levels detected by immunoblotting were used to normalize equal loadings from lysate samples.

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 (Műller *et al*., 2005), thus suggesting that its repression might contribute to IFNα 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 Ethic 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 1.7×10^5 cells/cm^2 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_010652727_m1 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 diothiothreitol, 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 (#07–224 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 (Vossio *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 ($0.5/1 \times 10^8$) were washed in phosphate-buffered saline and incubated for 10 min with 1% formaldehyde; after quenching the reaction with glycine 0.125 M, cells were sonicated and chromatin fragments of an average length of 1 kb recovered by centrifugation. Immunoprecipitations were performed with ProtG–Sepharose (KPL) and 3–5 μg of the indicated antibodies. After immunoprecipitation, washings and reverse cross-linking, samples were extracted twice with phenol/chloroform, once with chloroform and ethanol precipitated in the presence of 30 μg of glycogen. Quantitative real-time PCR was performed using SYBR-Green ROX mix, and an ABI Prism 7900 Sequence Detection System according to the manufacturer's instructions (Applied Biosystems, Inc). The primers used were: ISRE (I)for: CGGGTTTTGTTGTTGGATTCA; ISRE (I)rev: GCATTTT CGCTTTTCCCATCT; ISRE (II)for: AAGCAGGCAGGTG GTTTGG; ISRE (II)rev: CGGAGACCCTTTCCCCTTT; CTLfor: CCCCCACTCAGCCTTTCC; CTL_rev: TGCAGC CCCAAAGTGCTT. Dissociation curves after amplification showed that all primer pairs generated single products. The amount of PCR product amplified was calculated as percent of a standard curve of the input. A 3000-bp upstream region from the P2p73 ATG was used as negative internal control and percent of input values on ISRE sites were divided for percent of input values on CTL region to obtain the specific enrichment.

Antibodies used in ChIP

The antibodies used in ChIP were phospho-STAT2 (#07-224 Upstate), STAT2 (sc-476), p300 (sc-585), PolII (sc-9001 Santa Cruz Biotechnology, Santa Cruz, CA, USA), Ezh2 (#3147 Cell Signaling), HDAC1 (#H3284 Sigma), AcH3 (#07-352), AcH4 (#06-866 Upstate) and H3K27me3 (#ab6002 Abcam, Cambridge, CA, USA).

FACS analysis of DNA content

After IFNα treatment, cells were harvested, fixed in EtOH 70%, washed and re-suspended in phosphate-buffered saline 1 × . Propidium iodide (50 μg/ml) and RNase A (1 mg/ml) were added and incubated for 1 h at 37 °C before FACS reading (Nicoletti *et al*., 1991).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)