the FEBS Journal

Sp1 binds to the external promoter of the *p73* gene and induces the expression of TAp73γ in lung cancer

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Keywords
lung cancer; P1 promoter; p73 isoforms; Sp1; TAp73γ; ΔNp73

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(Received 16 February 2010, revised 1 May 2010, accepted 12 May 2010)

doi:10.1111/j.1742-4658.2010.07710.x

The *p73* gene possesses an extrinsic P1 promoter and an intrinsic P2 promoter, resulting in TAp73 and ΔNp73 isoforms, respectively. The ultimate effect of *p73* in oncogenesis is thought to depend on the apoptotic TA to antiapoptotic ΔN isoforms' ratio. This study was aimed at identifying novel transcription factors that affect TA isoform synthesis. With the use of bioinformatics tools, *in vitro* binding assays, and chromatin immunoprecipitation analysis, a region extending −233 to −204 bp upstream of the transcription start site of the human *p73* P1 promoter, containing conserved Sp1-binding sites, was characterized. Treatment of cells with Sp1 RNAi and Sp1 inhibitor functionally suppress TAp73 expression, indicating positive regulation of P1 by the Sp1 protein. Notably Sp1 inhibition or knockdown also reduces ΔNp73 protein levels. Therefore, Sp1 directly regulates TAp73 transcription and affects ΔNp73 levels in lung cancer. TAp73γ was shown to be the only TA isoform overexpressed in several lung cancer cell lines and in 26 non-small cell lung cancers, consistent with Sp1 overexpression, thereby questioning the apoptotic role of this specific *p73* isoform in lung cancer.

Introduction

Lung cancer is one of the most common and fatal types of cancer in developed countries. Despite scientific advances, the overall number of associated deaths has only slightly decreased during the last 20 years [1]. The well-known tumour suppressor gene *p53* has been found to be mutated in 70–90% of lung cancer cases and in less than 50% of all cancer cases [1]. However, the involvement of *p73*, its structural and functional homologue, in this type of cancer is not clearly understood [2].

The *p73* gene is a member of the *p53* family that encodes an N-terminal transactivation domain (TA), a highly conserved DNA-binding domain (DBD), and a C-terminal oligomerization domain [3]. Despite its high degree of sequence similarity with *p53*, especially in the DBD, and its ability to activate various *p53*

Abbreviations
ChIP, chromatin immunoprecipitation; DBD, DNA-binding domain; EMSA, electrophoretic mobility shift assay; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; TA, transactivation domain; TSS, transcription start site; VEGF, vascular endothelial growth factor.

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targets [4] as well as to induce apoptosis in cancer cells [5], *p73* has unique characteristics that differentiate it from a classical Knudson-type gene. Unlike *p53*, *p73* rarely mutates in cancer [6], and $p73^{-/-}$ mice do not develop spontaneous tumours, but show severe abnormalities in neuronal development [7]. The gene produces numerous isoforms as a result of: (a) alternative splicing in the 3′-end (leading to the formation of α, β, γ, δ, ε, ζ and η isoforms) [8–12]; (b) the use of an extrinsic promoter (P1) and an alternative, intrinsic promoter (P2) in the 5′-end (leading to the formation of TA and ΔN classes of isoforms, respectively) [13]; and (c) alternative splicing in the 5′-end (resulting in truncated transcripts p73Δex2, p73Δex2/3, and ΔN′-p73, which partially or entirely lack the TA, collectively called ΔTA) [14]. The numerous isoforms derive from several combinations between differential N-terminal domain and C-terminal domain [15].

Despite the rarity of *p73* mutations, overexpression of p73 isoforms is common in several types of cancer [14,16], including lung cancer [2]. Elevated levels of expression of p73 isoforms have also been correlated with lung cancer, as ΔNp73 overexpression predicts a poorer prognosis in patients with squamous cell carcinoma and adenocarcinoma [17]. In addition, TAp73 is overexpressed in lung cancer tumour tissues [18,19].

The 'two genes in one' idea has been suggested for *p73*, whereby the same gene is thought to generate products with opposing roles, mainly the apoptotic TA isoform(s) and the antiapoptotic ΔN isoforms. In general, TAp73 isoforms regulate the transcription of ΔNp73 isoforms, which, in turn, act as dominant negative regulators of both TAp73 and p53, thus giving a dominant negative feedback loop [13]. Consequently, the ultimate effect of p73 isoforms in cancer progression is attributed to the TA/ΔN ratio, rather than the overexpression of a specific p73 isoform or a specific class of p73 isoforms *per se* [20,21].

In line with this concept, the selective promoter activation could result in the activation of either oncogenic or tumour suppressor isoform(s) of this gene, thereby shifting the TA/ΔN equilibrium towards an oncogenic or a tumour suppressor direction. For example, the *p73* P1 promoter contains functional E2F1-binding sites [22], through which the E2F1 transcription factor induces TAp73 overexpression and consequent apoptosis [23,24]. It has been reported that the *p73* P1 promoter is not completely inactivated by site-directed mutagenesis of its functional E2F1 sites [23], implying that additional transcription factor(s) play a significant role in its regulation. This study focused on the identification of novel transcriptional factors that control the use of the *p73* P1 promoter and, subsequently, the relative expression of p73 isoforms in lung cancer by using lung cancer cell lines and tumour samples. Sp1 was found to activate the transcription of TAp73γ in lung cancer via highly conserved Sp1-binding sites on the *p73* P1 promoter. In addition, TAp73γ and Sp1 are co-overexpressed both *in vitro* and *in situ* in lung cancer. Sp1 also affected the ΔNp73 levels in lung cancer.

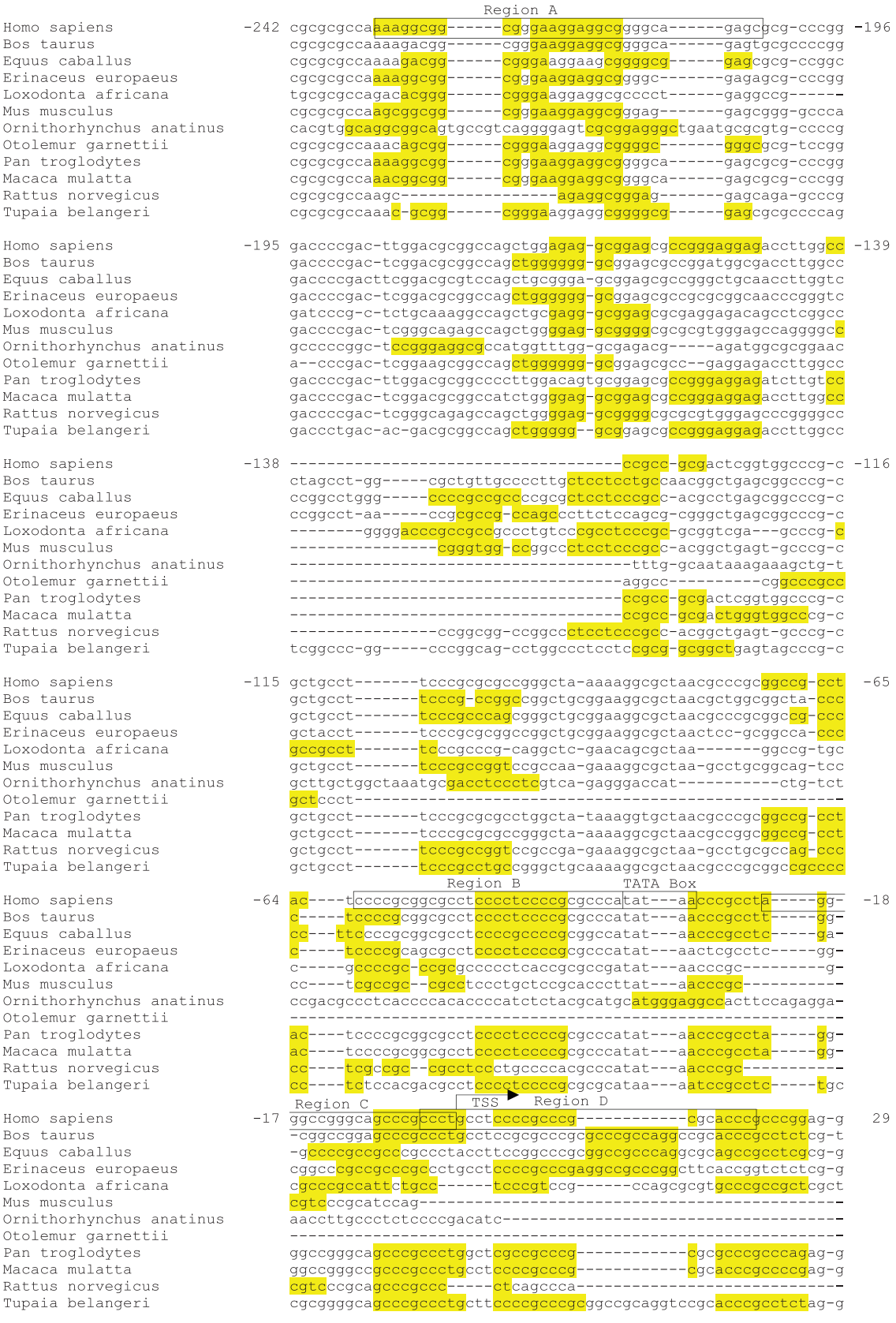
Results

The *p73* P1 promoter has multiple putative Sp1-binding sites

In order to identify transcription factors that control the use of the *p73* P1 promoter, we searched for conserved binding sites located in regions of its sequence that show high homology among various species, including *Bos taurus*, *Equus caballus*, *Erinaceus europaeus*, *Loxodonta africana*, *Macaca mulatta*, *Mus musculus*, *Ornithorhynchus anatinus*, *Otolemur garnettii*, *Pan troglodytes*, *Rattus norvegicus* and *Tupaia belangeri*. The transcription start site (TSS) of the human transcripts ENST00000346387, ENST00000354437, ENST00000357733, ENST00000378290, and ENST00000378295, which is located at chr1:3558989 (Ensembl v54, May 2009), was selected. The analysis focused on the first 250 bp upstream of the TSS, which shows most conservation among mammals. Four conserved human *p73* P1 promoter regions (A–D), containing potential Sp1-binding sites, were identified (Fig. 1). Region A is located –233 to –204 bp upstream of the human *p73* P1 TSS, and contains two putative Sp1-binding elements. Regions B, C, and D, which are located −61 to −33, −20 to −1, and −4 to +20 bp upstream of the TSS, respectively, all contain one putative Sp1-binding element. Our *in silico* prediction of candidate Sp1 motifs in regions A, C and D is in accordance with a previous study, in which MATINSPECTOR V2.2 at the TRANSFAC website was used [25]. Furthermore, CONTRA analysis also suggested another candidate Sp1 motif in region B. Our study demonstrated a canonical, conserved TATA box at position −32, based on the mapping of the TSS by Ensembl, which is identical to the TATA box previously described for the human *p73* P1 promoter [22].

Regions A, B and C on the *p73* P1 promoter can bind Sp1 *in vitro*

We evaluated the affinity of the *in silico*-identified region A, B, C and D oligonucleotides for *in vitro*


Region A
Region B
TATA Box
Region C
TSS
Region D
Homo sapiens -242 cgcgcgccaaaaggcgg------cgggaaggaggcgcgggca------gagcgcg-cccgg -196
Bos taurus cgcgcgccaaaagacgg------cgggaaggaggcgcgggca------gagtgcgccccgg
Equus caballus cgcgcgccaaaagacgg------cgggaaggaagcgcgggcg------gagcgcg-ccggc
Erinaceus europaeus cgcgcgccaaaaggcgg------cgggaaggaggcgcgggc-------gagagcg-cccgg
Loxodonta africana tgcgcgccagacacggg------cgggaaggaggcgcccct------gaggccg------
Mus musculus cgcgcgccaagcggcgg------cgggaaggaggcgcgggag-------gagcggg-gccca
Ornithorhynchus anatinus cacgtggcaggcggcagtgccgtcaggggagtcgcggagggctgaatgcgcgtg-ccccg
Otolemur garnettii cgcgcgccaaacagcgg------cgggaaggaggcgggggc-------gggcgcg-tccgg
Pan troglodytes cgcgcgccaaaaggcgg------cgggaaggaggcgcgggca------gagcgcg-cccgg
Macaca mulatta cgcgcgccaaacggcgg------cgggaaggaggcgcgggca------gagcgcg-cccgg
Rattus norvegicus cgcgcgccaagc------------------agaggcgggag-------gagcaga-gcccg
Tupaia belangeri cgcgcgccaaac-gcgg------cgggaaggaggcgggggcg------gagcgcgccccag
Homo sapiens -195 gaccccgac-ttggacgcggccagctggagag-gcggagcgccgggaggagaccttggcc -139
Bos taurus gaccccgac-tcggacgcggccagctgggggg-gcggagcgccggatggcgaccttggcc
Equus caballus gaccccgacttcggacgcgtccagctgcggga-gcggagcgccgggctgcaaccttggtc
Erinaceus europaeus gaccccgac-tcggacgcggccagctgggggg-gcggagcgccgcgcggcaacccgggtc
Loxodonta africana gatcccg-c-tctgcaaaggccagctgcgagg-gcggagcgcgaggagacagcctcggcc
Mus musculus gaccccgac-tcgggcagagccagctgggagg-gcgggggcgcgcgtgggagccaggggcc
Ornithorhynchus anatinus gcccccggc-tccgggaggcgccatggtttgg-gcgagacg-----agatggcgcggaac
Otolemur garnettii a--cccgac-tcggaagcggccagctgggggg-gcggagcgcc--gaggagaccttggcc
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Macaca mulatta gaccccgac-tcggacgcggccatctgggggag-gcggagcgccgggaggagaccttggcc
Rattus norvegicus gaccccgac-tcgggcagagccagctgggggag-gcgggggcgcgcgtgggagcccgggggcc
Tupaia belangeri gaccctgac-ac-gacgcggccagctgggggg--gcggagcgccgggaggagaccttggcc
Homo sapiens -138 ----------ccgcc-gcgactcggtggcccg-c -116
Bos taurus ctagcct-gg-----cgctgttgccccttgctcctcctgccaacggctgagcggcccg-c
Equus caballus ccggcctggg-----ccccgccgccccgcgctcctcccgcc-acgcctgagcggcccg-c
Erinaceus europaeus ccggcct-aa-----ccgcgccg-ccagccctttctccagcg-cgggctgagcggcccg-c
Loxodonta africana --------ggggacccgccgccgccctgtcccgcctcccgc-gcggtcga---gcccg-c
Mus musculus ----------cgggtgg-ccggccctcctcccgcc-acggctgagt-gcccg-c
Ornithorhynchus anatinus ----------tttg-gcaataaagaaagctg-t
Otolemur garnettii ----------aggcc----------cggcccgcc
Pan troglodytes ----------ccgcc-gcgactcggtggcccg-c
Macaca mulatta ----------ccgcc-gcgactgggtggccg-c
Rattus norvegicus ----------ccggcgg-ccggccctcctcccgcc-acggctgagt-gcccg-c
Tupaia belangeri tcggccc-gg-----cccggcag-cctggccctcctccgcg-gcggctgagtagcccg-c
Homo sapiens -115 gctgcct-------tcccgcgcgccgggcta-aaaaggcgctaacgcccgcggccg-cct -65
Bos taurus gctgcct-------tcccg-ccggccggctgcggaaggcgctaacgctggcggcta-ccc
Equus caballus gctgcct-------tcccgcccagcgggctgcggaaggcgctaacgcccgcggccg-ccc
Erinaceus europaeus gctacct-------tcccgcgcggccggctgcggaaggcgctaactcc-gcggcca-ccc
Loxodonta africana gccgcct-------tccgcccg-caggctc-gaacagcgctaa-------ggccg-tgc
Mus musculus gctgcct-------tcccgccggtccgccaa-gaaaggcgctaa-gcctgcggcag-tcc
Ornithorhynchus anatinus gcttgctggctaaatgcgacctccctcgtca-gagggaccat----------ctg-tct
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Homo sapiens -64 ac----tccccgcggcgcctccctcccgcgcccatat---aacccgccta-----gg- -18
Bos taurus c-----tccccgcggcgcctccctcccgcgcccatat---aacccgcctt-----gg-
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Otolemur garnettii ----------
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Tupaia belangeri cc----tctccacgacgcctccctcccgcgcgcataa---aatccgcctc-----tgc
Homo sapiens -17 ggccgggcagcccgccctgcctcccgcccg----------cgcacccgcccggag-g 29
Bos taurus -cggccggagcccgccctgcctccgcgcccgcgcccgccaggccgcacccgcctctcg-t
Equus caballus -gccccgccgccgccctaccttccggcccgcggccgcccaggcgcagccgcctcgcg-g
Erinaceus europaeus cggccgccgcccgcctgcctccccgcccgaggccgcccggcttcaccggtctctcg-g
Loxodonta africana cgcccgccattctgcc------tcccgtccg------ccagcgcgtgcccgccgctcgct
Mus musculus cgtccccgcatccag----------
Ornithorhynchus anatinus aaccttgccctctccccgacatc----------
Otolemur garnettii ----------
Pan troglodytes ggccgggcagcccgccctgcctcgccgcccg----------cgcgcccgcccagag-g
Macaca mulatta ggccgggccgcccgccctgcctccccgcccg----------cgcacccgccccgag-g
Rattus norvegicus cgtccccgcagcccgccc-----ctcagccca----------
Tupaia belangeri cgcggggcagcccgccctgcttccccgcccgcggccgcaggtccgcacccgcctctag-g

synthesized Sp1 protein using electrophoretic mobility shift assay (EMSA) experiments. *In vitro*, Sp1 can bind to region A, B and C oligonucleotides (Fig. 2A, lanes 6 and 11, and Fig. 2B, lane 6, respectively). Self-competition experiments, as well as competition experiments using an excess of unlabelled control oligonucleotide (containing a control Sp1 binding site) for region A radiolabelled oligonucleotide, abolished the formation of the Sp1–radiolabelled region A oligonucleotide complex (Fig. 2A, lanes 7 and 8, respectively). The addition of the mSp1 oligonucleotide (containing a mutated Sp1 binding site) did not affect protein–DNA binding (Fig. 2A, lane 9), whereas the addition of antibody against Sp1 strongly supershifted the Sp1–DNA complex (Fig. 2A, lane 10). Similar experiments for regions B (Fig. 2A) and C (Fig. 2B) confirmed specific *in vitro* Sp1–DNA binding. Notably, the binding activity of the region A oligonucleotide was markedly higher than those of all other oligonucleotides that were tested, possibly indicating that both putative Sp1 binding elements in region A are active. Therefore, region A appears to be a better binding site for Sp1. In contrast, region D failed to bind *in vitro* synthesized Sp1 protein (Fig. 2B, lanes 11–15), and it was excluded from further analysis.

Binding of endogenous Sp1 from lung cancer cell lines to the *p73* P1 promoter

In order to validate the ability of endogenous Sp1 to bind to the *p73* P1 promoter within the cellular environment, we performed additional EMSA experiments using nuclear extracts from 11 representative lung cancer cell lines. We used only region A radiolabelled oligonucleotide, as it was found to bind *in vitro* to Sp1 more effectively. We observed that the binding of endogenous Sp1 to region A in the fibroblast cell line IMR90 was almost equal to that in the normal HNBE cells (Fig. 2C, lanes 1 and 2). A marked increase in the level of region A oligonucleotide–Sp1 complexes was noted in the anaplastic carcinoma cell line (Fig. 2C, lane 3), and the levels of the complexes appeared to remain equivalently high in the small cell lung cancer cell line (Fig. 2C, lane 4), the squamous cell carcinoma cell lines (Fig. 2C, lanes 5–7), the adenocarcinoma cell lines (Fig. 2C, lanes 8–10), and the large cell lung carcinoma cell line (Fig. 2C, lane 11). The region A oligonucleotide–Sp1 complex was supershifted in the representative cell line A549 (Fig. 2C, lane 12), demonstrating the specificity of region A for Sp1 of the nuclear cell lysates. The Sp1–DNA binding pattern for the region A oligonucleotide is consistent with that of the control oligonucleotide (Fig. 2D).

Binding of Sp1 to the *p73* P1 promoter within the cellular environment is further supported by chromatin immunoprecipitation (ChIP) assays. Sp1 antibody immunoprecipitated the *p73* P1 promoter in A549 cells in a dose-dependent manner (Fig. 2E). In contrast, no PCR signal was observed when the irrelevant β-actin antibody was used for ChIP. The sheared and cross-linked DNA that was produced prior to the immunoprecipitation step (input) was used as a positive control PCR template.

TAp73 synthesis is regulated by Sp1 through region A in lung cancer cell lines

Next, we tested the ability of Sp1 to regulate TAp73 expression *in vivo* by treating the standard TAp73-expressing cell line A549 with either Sp1 small interfering RNA (siRNA) or an Sp1 protein inhibitor. The resulting changes in TAp73 expression were monitored by western blot analysis. The known Sp1 target vascular endothelial growth factor (VEGF) [26] was used as a positive control. A549 cells were transiently transfected with Sp1 siRNA, and the nonsilencing control siRNA was the negative control for Sp1 siRNA interference. As shown in Fig. 3A, treatment with Sp1 siRNA resulted in the downregulation of TAp73 and VEGF levels as compared with the corresponding levels in the siRNA-untreated cells, revealing positive regulation of the *p73* P1 promoter by Sp1. In contrast, TAp73 and VEGF levels were not affected by treatment with negative control Sp1 siRNA. Similarly, TAp73 levels gradually decreased after a 48 h treatment of A549 cells with increasing concentrations of the Sp1 inhibitor mithramycin A (Fig. 3B), which not only interferes with the transcription of genes containing GC-rich regions in their promoters, but also, at high concentrations, reduces recruitment of Sp1 to its own promoter [27].

We then performed transient transfection of A549 cells with region A double-stranded phosphorothioate oligonucleotides, which are able to antagonize region A for Sp1 binding, in order to examine whether

Fig. 1. The P1 *p73* promoter has multiple putative Sp1-binding sites, conserved among 12 mammalian species. Alignment using CONTRA analysis revealed four conserved, putative Sp1 element-containing regions, spanning from −233 to −204 bp (region A), −61 to −33 bp (region B), −20 to −1 bp (region C) and −4 to +20 bp (region D) relative to the TSS of the human *p73* P1 promoter. The four regions are box-highlighted, and the human Sp1-binding sites are yellow-shaded. The TATA box is also box-highlighted.

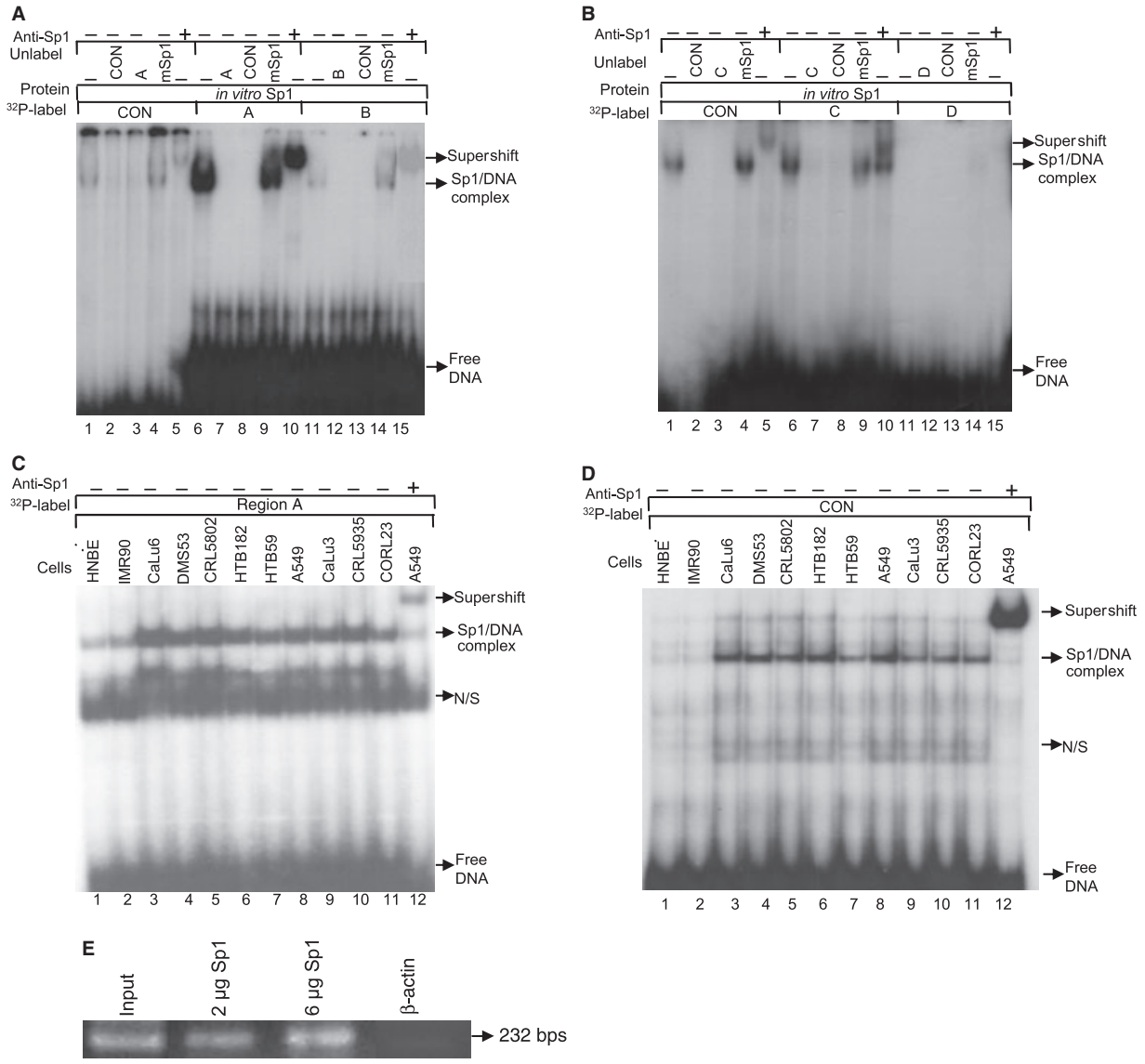

Fig. 2. Sp1 binds to the *p73* P1 promoter both *in vitro* and *in vivo*. (A) The ^{32}P-labelled region A target was incubated with the *in vitro* Sp1 protein either alone (lane 6) or in the presence of cold region A oligonucleotide (self-competition reaction) (lane 7), cold control oligonucleotide (CON) (competition reaction with positive control) (lane 8), or cold mutant Sp1 oligonucleotide (mSp1) (competition reaction with negative control) (lane 9). In lane 10, the protein–DNA complexes are supershifted with polyclonal antibody against Sp1 (supershift reaction). Lanes 11–15 correspond to a similar set of reactions for the ^{32}P-labelled region B target. Lanes 1–5 correspond to the positive control reactions for the Sp1-containing oligonucleotide (CON). (B) Lanes 6–10 correspond to a similar set of reactions for the ^{32}P-labelled region C target, and lanes 11–15 correspond to a similar set of reactions for the ^{32}P-labelled region D target. Lanes 1–5 correspond to the positive control reactions for the Sp1-containing oligonucleotide (CON). EMSAs using *in vitro* Sp1 and region A, B, C or D oligonucleotides revealed that regions A, B and C can bind to Sp1. (C) Lanes 1–11 contain radiolabelled region A oligonucleotide incubated with nuclear extracts from 11 lung cancer cell lines and electrophoresed on polyacrylamide gel. The specificity of the region A oligonucleotide–Sp1 protein complex is confirmed by a supershift reaction with polyclonal antibody against Sp1 in the representative A549 cell line (lane 12). (D) Lanes 1–11 show the corresponding positive control EMSA experiments demonstrating specific binding of endogenous Sp1 of the same cell lines to radiolabelled control Sp1 oligonucleotide (CON). The CON–Sp1 protein complex was supershifted in the representative cell line, A549 (lane 12). Unlabel., unlabelled oligonucleotides; ^{32}P-label, ^{32}P-labelled oligonucleotides; N/S, nonspecific DNA–protein complexes. (E) ChIP assay with DNA from A549 cells. Immunoprecipitation was performed with 2 µg and 6 µg of antibody against Sp1. PCR primer pairs were specific for the −265 to +61 bp region of the *p73* P1 promoter. Chromatin incubated with antibody against β-actin was used as a negative immunoprecipitation control, whereas input was used as a positive PCR control.

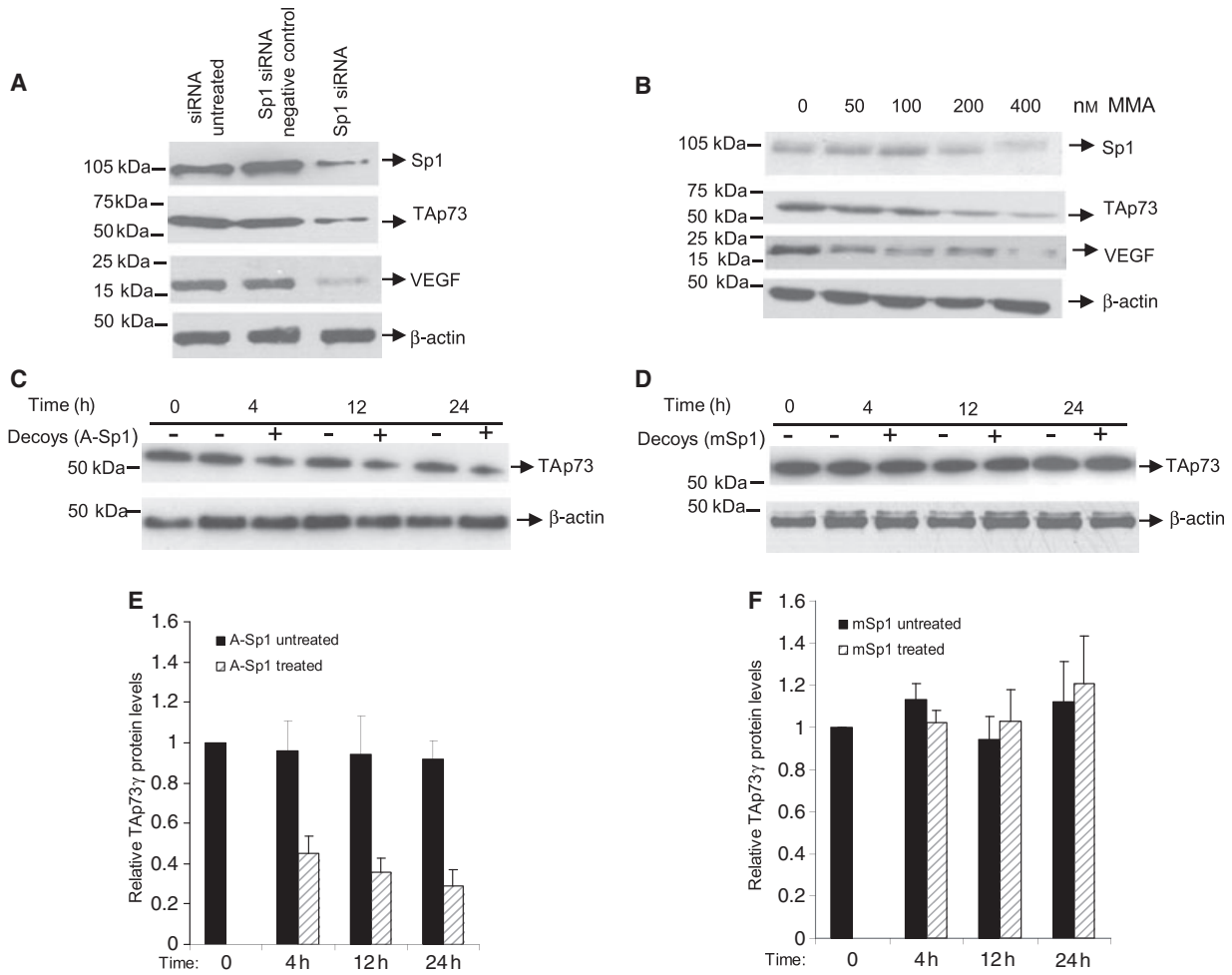

Fig. 3. Sp1 mediates TAp73 overexpression through P1 activation. (A) Transient transfection with Sp1 siRNA results in the reduction of Sp1 and TAp73 levels in A549 cells. VEGF levels were used as a positive control for Sp1 siRNA interference. β-actin levels were used as a loading control. Nonspecific Sp1 siRNA was used as a negative control. (B) A 48-h treatment of A549 cells with increasing concentrations of the Sp1 inhibitor mithramycin A results in reductions in Sp1 and TAp73 levels. VEGF levels were used as a positive control (C) A549 cells were transiently transfected with region A decoy, total protein extracts were prepared from these cells after 4, 12 and 24 h of decoy treatment, and the TAp73 levels were estimated by western blot analysis. (D) Similar transient transfection experiments with mutant Sp1 (mSp1) decoys were performed as a negative control of interference. The experiment was performed in triplicate. (E) TAp73 levels were quantified by IMAGEQUANT and compared with the corresponding levels of the untreated cells. As shown in the graph, TAp73 levels decreased with time upon region A decoy treatment. (F) Quantification of the TAp73 levels and comparison with the corresponding levels of decoy-untreated cells (black bars) demonstrated no change in TAp73 levels of mSp1-treated cells over time (grey bars). The protein amounts in all experiments were normalized to β-actin.

region A of the *p73* P1 promoter is specifically responsible for Sp1-mediated TAp73 expression in lung cancer cells. Mutant (mSp1) double-stranded phosphorothioate oligonucleotides were used as the corresponding negative control. Region A phosphorothioate oligonucleotides were able to reduce TAp73 expression over a 24 h treatment period (Fig. 3C,E), whereas mSp1 phosphorothioate oligonucleotides failed to affect TAp73 expression (Fig. 3D,F). In contrast, region B and C phosphorothioate oligonucleotides had a negligible effect on TAp73 expression, even after 48 h of treatment (data not shown).

TAp73γ and Sp1 are co-overexpressed in lung cancer cell lines and non-small cell lung cancers (NSCLCs)

Western blot analysis for TAp73 isoforms using total protein extracts from 15 lung cancer cell lines revealed that the abundantly expressed TAp73 isoform in all

tested cell lines was TAp73γ, whereas TAp73α and TAp73β were not detected. The level of TAp73γ was low in the normal HNBE cells, slightly increased in the fetal lung fibroblast cell lines (CCL171 and IMR90), and substantially increased in the lung epithelial anaplastic carcinoma cell line (CALU6), the small cell carcinoma cell line (DMS53), the squamous lung cancer cell lines (CRL5802, HTB182, HTB58, HTB59, and SKMES1), the adenocarcinoma cell lines (A549, CALU3, CRL5935, and SKLU1), and the large cell lung cancer cell line (CORL23). The corresponding Sp1 expression pattern was consistent with that of TAp73γ (Fig. 4A), as well as with the Sp1–DNA binding pattern revealed by the EMSA experiments. Quantification of TAp73γ and Sp1 levels is shown in Fig. 4B.

To verify our findings *in situ*, we analysed the expression of TAp73 isoforms in a group of 26 lung cancer patients. TAp73γ was exclusively overexpressed in 68.42% (13/19) of squamous cell lung cancer samples and in 57.14% (4/7) of adenocarcinoma samples as compared with their corresponding adjacent normal tissues. TAp73α and TAp73β were undetectable in the tumour tissues of all patients. Sp1 levels were also examined, and Sp1 was found to be overexpressed in 57.89% (11/19) of squamous cell lung cancer samples and in 42.86% (3/7) of adenocarcinoma samples. Sp1 and TAp73γ were co-overexpressed in 42.86% (3/7) of adenocarcinoma samples, in 52.63% (10/19) of squamous cell lung cancer samples, and in 50% (13/26) of total lung cancer samples. Figure 4C shows TAp73 and Sp1 levels in representative squamous cell carcinoma and adenocarcinoma samples (Fig. S1). The mean TAp73γ levels showed an approximately 12-fold increase in tumour tissues with respect to the corresponding normal levels. Similarly, an approximately eight-fold increase in the mean Sp1 levels was observed in the examined tumour samples (Fig. 4D).

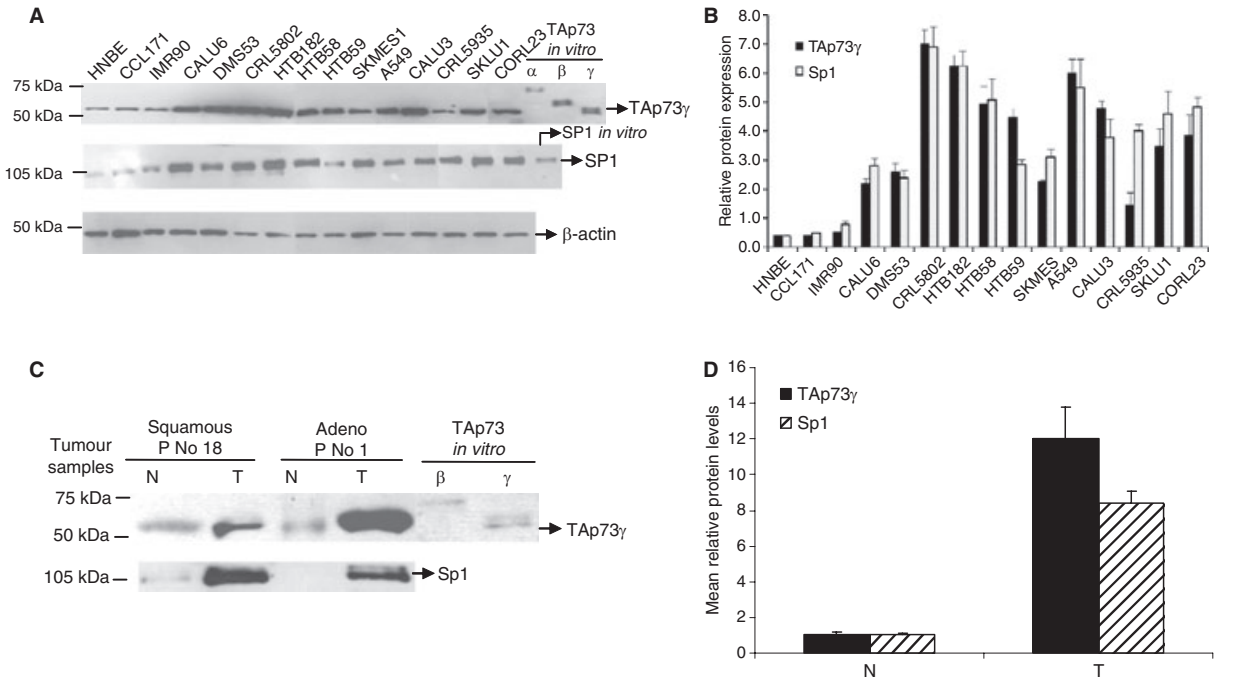

Fig. 4. TAp73γ and Sp1 are co-overexpressed in lung cancer cell lines and tumour samples. (A) Western blot analysis of total extracts from 15 lung cancer cell lines revealed coelevation of Sp1 and TAp73γ protein levels in these cells. *In vitro*-translated TAp73α, TAp73β and TAp73γ were used as controls for the identification of TAp73 isoforms, *in vitro* Sp1 was used as a control for the expression of Sp1, and β-actin was used as a loading control. (B) Sp1 and TAp73γ levels were quantified by IMAGEQUANT and expressed relative to the normal HNBE cell line. (C) Western blot analysis demonstrated a significant increase in both TAp73γ and Sp1 levels in the representative squamous cell carcinoma (patient No. 1) and adenocarcinoma (patient No. 18) samples as compared with the corresponding normal tissues. *In vitro*-synthesized TAp73β and TAp73γ were used as controls, for the identification of the exact TAp73 isoform expressed in these samples. (D) The mean levels of TAp73γ and Sp1 in 26 NSCLCs samples were compared with the corresponding mean levels in the normal samples. Relative mean TAp73γ levels showed an almost 12-fold increase (grey bars), and relative mean Sp1 levels showed a greater than eight-fold increase (black bars). The experiment was performed in triplicate.

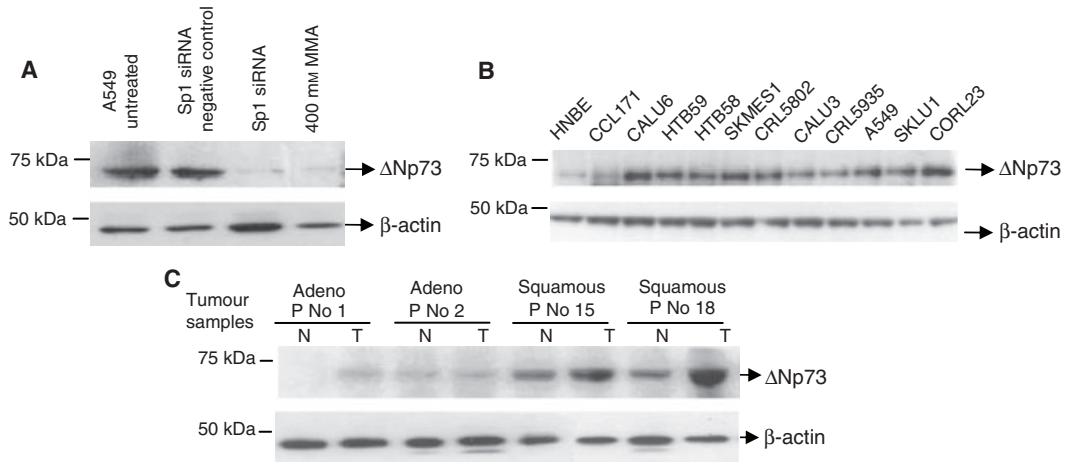

Fig. 5. ΔNp73 levels are affected by Sp1, and ΔNp73 is overexpressed in lung cancer cells. (A) Transient transfection with Sp1 siRNA resulted in the downregulation of both ΔNp73 proteins in A549 cells. Nonspecific Sp1 siRNA was used as a negative control, and β-actin levels were used as a loading control. The Sp1 inhibitor mithramycin A at 400 nM also caused a marked decrease in ΔNp73 levels. (B) Western blot analysis of total extracts from 12 lung cancer cell lines revealed elevated ΔNp73 levels in these cells. (C) Western blot analysis demonstrated an increase in ΔNp73 in representative squamous cell carcinoma and adenocarcinoma samples relative to the adjacent normal tissues.

ΔNp73 levels are affected by Sp1 and enhanced in lung cancer cells

As the outcome of the action of TAp73 is dependent on the presence of the dominant negative ΔNp73 [13], an important issue to be considered is whether Sp1 also affects ΔNp73 levels in the context of lung cancer. It is also important to investigate whether ΔNp73 is co-overexpressed, along with TAp73γ, in lung cancer. In this respect, we first assessed the effect of Sp1 siRNA treatment of A549 cells on ΔNp73 levels. As shown in Fig. 5A, ΔNp73 levels were markedly reduced in the Sp1 siRNA-treated A549 cells as compared with the untreated cells, in contrast to the ΔNp73 levels of nonsilencing control-treated A549 cells, which remained unchanged. Similarly, ΔNp73 levels showed a marked decrease upon treatment of the A549 cell line with 400 nM mithramycin A (Fig. 5A). A CONTRA analysis was performed in order to examine whether a direct interaction of Sp1 with the *p73* P2 promoter is possible. Interestingly, our analysis showed a conserved region of 124 bp upstream of the ΔN-TP73 TSS. A highly conserved Sp1 candidate site was found at position –17 to –26. This sequence was flanked by two candidate TATA boxes at positions −3 to −9 and –26 to +32. Another Sp1 site was identified at the 5′-end of the conserved promoter region (−115 to −124). The TSS was located at chr1:3597096 (Ensemble v54, May 2009) of the ΔN-TP73 promoter (transcripts ENST00000378280 and ENST00000378285) (data not shown).

Next, ΔNp73 levels were monitored in 12 lung cancer cell lines, as well as in four representative paired samples of the 26-membered panel of lung cancer patients. Figure 5B shows that ΔNp73 protein expression was low in the normal HNBE and fetal lung fibroblast CCL171 cell lines, whereas it was significantly increased in the lung epithelial anaplastic carcinoma cell line (CALU6), in the squamous lung cancer cell lines (HTB59, HTB58, and SKMES1), in the adenocarcinoma cell lines (CALU3, CRL5935, A549, and SKLU1), and in the large cell lung cancer cell line (CORL23). In agreement with the data concerning cell lines, as well as previous data on clinical samples [17,19], ΔNp73 was also overexpressed in the representative tumour samples as compared with their corresponding normal tissues (Fig. 5C). Thus, ΔNp73 levels are not only enhanced in lung cancer cells, along with those of TAp73γ, but are also affected by Sp1.

Discussion

In the search for transcription factors that affect the use of the *p73* P1 promoter, we identified a region −233 to −204 bp upstream of the TSS of the human *p73* P1 promoter containing conserved, functional Sp1-binding sites. Reduction of the endogenous Sp1 levels or inhibition of Sp1 binding to this region downregulates TAp73 expression in lung cancer cells. Importantly, Sp1 also affected the expression of ΔNp73 in lung cancer cells.

Sp1 has traditionally been considered to be a ubiquitous transcription factor, responsible for the basal/

constitutive activation of a wide range of viral and mammalian genes. However, novel data strongly correlate deregulated Sp1 expression with tumour development, growth and metastasis, as it is significantly overexpressed in pancreatic, breast, thyroid and colon tumours, and it transactivates genes with a substantial role in cancer progression, cell cycle regulation, and antiapoptotic procedures [28]. Our study makes Sp1 the second transcription factor identified, so far, after E2F1 as directly controlling the *p73* P1 promoter. In addition, it indicates an association between Sp1 overexpression and TAp73 overexpression in lung cancer.

Sp families of transcription factors can form complexes with TAp73 isoforms [29]. Recently, it was shown that TAp73 isoforms interfere with Sp1 transcriptional activity, thus acting as repressors of Sp1-mediated activation of genes, such as those encoding enhancer II of the core protein of hepatitis B virus [30], human telomerase reverse transcriptase [31,32], the potent angiogenic factor VEGF [33] and the cell cycle G_2/M checkpoint controller cyclin B [34]. It is proposed that this repression may be achieved via formation of Sp1–TAp73 complexes, resulting in the abrogation of Sp1 binding to corresponding elements on target gene promoters [30,32]. This tumour suppression mechanism parallels that of p53 [35,36]. The above-mentioned negative effect of p73 on Sp1-mediated transcription is specific only to the TAp73 isoforms, and not the ΔNp73 [31] or ΔTAp73 isoforms [30,32], and its efficiency fluctuates depending on the type of TAp73 isoform, with TAp73β being the most effective suppressor and TAp73γ being the least effective [32]. It remains to be elucidated whether TAp73 interference in the Sp1-mediated transactivation of oncogenes also applies to lung cancer, suggesting that the interactions between Sp1 and TAp73 isoforms extend beyond the level of transcriptional control of the *p73* P1 promoter.

In this study, we also demonstrated that the full-length p73 isoform overexpressed in cancer cells both *in vitro* and *in situ* is TAp73γ. TA isoforms were found to be elevated in lung cancer samples in the past, but the exact TAp73 isoform(s) overexpressed were not determined [2,19]. To the best of our knowledge, this is the first time that this particular isoform has been found to be specifically and exclusively overexpressed in cancer cells. Typically, TAp73 isoforms activate genes that mediate either cell cycle arrest or apoptosis, such as *p21*, *bax*, *mdm2*, *gadd45*, *cyclin G*, *IGFBP3*, and *14-3-3*, and trigger cell death [5]. *In vivo* evidence supports the proposed role of TA isoforms as tumour suppressors, as *TAp73*$^{-/-}$ mice are tumour-prone and develop tumours upon treatment with carcinogens,

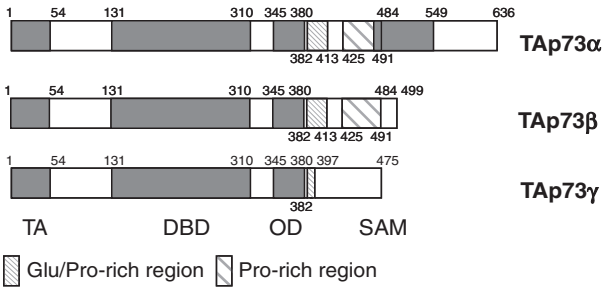

Fig. 6. Comparison between the primary structure of TAp73α, TAp73β, and TAp73γ. Alternative splicing results in the loss of the Pro-rich domain and in the truncation of the Glu/Pro-rich domain, which contains a newly identified N-terminal transactivation domain. OD, oligodimerization domain; SAM, sterile α-motif (based on [40]).

with lung adenocarcinoma being the most frequent cancer diagnosed in these knockout animals [37]. Therefore, our finding raises questions about the presumed role of TAp73γ in cancer, suggesting that its function may diverge from the traditionally proposed apoptotic function of TAp73 isoforms. Indeed, TAp73γ has been almost ineffective in activating the *p21Waf1/Cip1* promoter and inhibiting colony formation of Saos cells, in contrast to the more efficient TAp73α and TAp73β [9]. Similarly, it only poorly transactivates a p53-binding consensus sequence-containing promoter in *p53*-null cell lines [11].

The failure of TAp73γ to exert the same drastic transactivation activities as the more extensively studied TAp73α and TAp73β might be associated with differences in its C-terminal domain (Fig. 6). In this respect, a newly highlighted difference in TAp73γ is that its C-terminal domain is basic and forms weak sequence-specific DNA–protein complexes, whereas the corresponding domains of TAp73α and TAp73β are neutral and form strong DNA–protein complexes, reflecting differential promoter binding and target gene transactivation [38]. Another difference in the C-terminal domain of TAp73γ is that, owing to the excision of exon 11 during alternative splicing, it lacks most of the Glu/Pro-rich domain and the Pro-rich domain, which are located in a region extending from 382 to 491 amino acids and are thought to enhance the transactivation activities of TAp73α and TAp73β [39,40]. In addition, lack of exon 11 in TAp73γ results in the truncation of a second transactivation domain, located within amino acids 381–399, which was recently shown to regulate genes involved in cell cycle progression [41]. The above data imply a transactivational deficit for TAp73γ as compared with other TAp73 isoforms, which could influence its apoptotic function.

In agreement with previous clinical studies [19], we demonstrated that ΔNp73 levels are also elevated in

lung cancer cell lines and in exemplary tumour samples. Furthermore, and for the first time, we showed that ΔNp73 levels are reduced *in vitro* upon inhibition or knockdown of the Sp1 transcription factor. The effect of Sp1 on ΔNp73 expression may be direct, as highly conserved, putative Sp1-binding sites on the *p73* P2 promoter were identified by bioinformatic analysis. This possibly means that Sp1 controls both TAp73 and ΔNp73 expression via regulation of their respective promoters. Alternatively, it is possible that this effect may be indirect, as the overexpression of P2-derived ΔN isoforms could be attributed to the overexpression of TAp73, which is known to activate the P2 promoter [13]. In this case, downregulation of ΔNp73 expression upon Sp1 inhibition or reduction could be caused by subsequent downregulation of TAp73 expression. Furthermore, the possibility that the *p73* P1 promoter is able to produce a fraction of ΔNp73 molecules in lung cancer cannot be excluded, as the P1-derived ΔN′ transcripts, which have been reported to be expressed in lung cancer tumours [19], are also translated to ΔNp73 [14]. In other words, as ΔNp73 proteins are the translational products of both P1-derived ΔN′ and P2-derived ΔN transcripts, the decreased ΔNp73 levels may be attributed, at least in part, to the reduced activity of the *p73* P1 promoter. Finally, it is also possible that the influence of Sp1 on ΔNp73 levels might be the combinational and/or synergistic result of all the above-mentioned processes. Therefore, all of these issues should be addressed in the future.

Taken together, our findings make it clear that there is a link between the expression of Sp1 and p73 isoforms in lung cancer. Not only does Sp1 have the potential to affect the TA and ΔN protein isoform levels, but its deregulated expression is also implicated in lung cancer. On the other hand, TAp73 overexpression in lung cancer could be linked to oncogene-induced DNA damage, as induction of p73 is DNA damage response-dependent [42,43]. The mechanisms that underlie the interplay between Sp1 and full-length or N-terminal-truncated p73 isoform(s) should be further investigated.

Experimental procedures

Bioinformatics

The CONTRA [44] web tool was used for *tp73* P1 promoter analysis, as follows. The direction of transcription of *tp73* was identified, and the most upstream TSS of all *tp73* Ensembl [45] transcripts was selected. One thousand base pairs of the UCSC multiz 28-way 5000 upstream alignment, homologous to the human *tp73* P1 promoter genomic sequences, were used for the initial analysis. The sequences were compared against the V$SP1_Q2_01 TRANSFAC position weight matrix of Sp1 target motifs with a core cut-off of 0.90 and a similarity matrix cut-off of 0.75. The sequence alignment and its accompanying information regarding potential Sp1 sites were downloaded and viewed by JALVIEW [46]. Through BIOEDIT [47], the alignments were imported to Microsoft Word 2003 (http://www.microsoft.com/) for further manipulation.

Cell lines and culture conditions

The following human lung carcinoma cell lines used in this study were obtained from the American Type Culture Collection (Rockville, MD, USA): HNBE, CCL171, IMR90, CALU6, DMS53, CRL5802, HTB182, HTB58, HTB59, SKMES1, A549, CALU3, CRL5935, SKLU1 and CORL23. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). To evaluate the effects of mithramycin A (Sigma-Aldrich, St Louis, MO, USA), 60–70% confluent cells were incubated with 50–400 nM mithramycin A in 60-mm cell culture dishes for 48 h.

Patient characteristics and tumour specimens

Tumour specimens and their corresponding normal tissues were derived from 26 lung cancer patients, 18 males and eight females. Of the 26 patients, 19 were diagnosed with squamous cell carcinoma and seven with adenocarcinoma. The patients' mean age was 68.6 years. All of the above-mentioned patients underwent surgical tumour excision at the Cardiothoracic Centre of Broadgreen, Liverpool, UK. The study protocol was approved by the Liverpool Ethics Committee and all of the patients provided written, informed consent.

Preparation of total cell lysates and nuclear extracts

For the preparation of total cell lysates, cells were lysed in lysis buffer (20 mmol·L^{-1} Tris, pH 7.6, 0.5% Triton X-100, 250 mmol·L^{-1} NaCl, 3 mmol·L^{-1} EDTA, 3 mmol·L^{-1} EGTA, 10 g·mL^{-1} Pefabloc, 2 mmol·L^{-1} sodium orthovanadate, 10 g·mL^{-1} aprotinin, 10 g·mL^{-1} leupeptin, and 1 mmol·L^{-1} dithiothreitol). Lysates were incubated on ice for 30 min and then centrifuged at $8000 \times g$ at 4 °C for 10 min. The supernatant was aliquoted and stored at −70 °C.

For the preparation of nuclear extracts, cells were pelleted and homogenized in ice-cold hypotonic buffer (25 mM Tris, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride) with a Teflon–

glass homogenizer. The nuclear fraction was pelleted, washed with isotonic buffer (25 mM Tris, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 0.2 mM sucrose) and lysed with extraction buffer (25 mM Tris, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride). Nuclear debris was removed by centrifugation at 55 000 ***g*** for 1 h at 4 °C. Estimations of the protein concentrations for both total cell lysates and nuclear extracts were performed using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Protein extraction from tumour samples

Frozen tissue samples mixed with ice-cold RIPA buffer [1 × $NaCl/P_i$, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (v/v) SDS] (Sigma-Aldrich) containing protease inhibitors (Roche Applied Science, Hague Road, IN, USA) at a tissue/buffer volume ratio of 1 : 1. The mixture was incubated on ice for 1 h and homogenized with frequent vortexing. The homogenate was centrifuged at 13 000 ***g*** for 15 min at 4 °C, and the resulting supernatant was collected in a clean Eppendorf tube.

In vitro proteins

In vitro Sp1 was purchased from Promega (Madison, WI, USA). TAp73α, TAp73β and TAp73γ were synthesized from the corresponding expression plasmids [9], using the TnT *in vitro* translation system (Promega).

EMSAs

Annealed oligonucleotides representing regions A, B, C and D of the human *p73* P1 promoter were used (Invitrogen). A consensus Sp1-binding site and a mutant Sp1-binding site were used as positive and negative control, respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Oligonucleotide sequences are summarized in Table 1. Annealed oligonucleotides were end-labelled with [^{32}P]ATP[γP], using T4 polynucleotide kinase (New England Biolabs, Ipwich, MA, USA), following the manufacturer's instructions. Radiolabelled products were purified on Microspin G-25 columns (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. The reaction mixture was prepared by mixing 2000 c.p.m. of γ-^{32}P-labelled oligonucleotide with 20 μg of nuclear cell protein in binding buffer (50 mM Hepes, pH 8.0, 500 mM NaCl, 0.5 M phenylmethanesulfonyl fluoride, 0.5 $mg{\cdot}mL^{-1}$ BSA, 20% glycerol, 1 mM EDTA) plus 1 mM dithiothreitol and 150 $\mu g{\cdot}mL^{-1}$ poly(dI-dC) (Sigma-Aldrich), and left at room temperature for 30 min. The reaction mixtures were subsequently electrophoresed on a 6% polyacrylamide gel at 150 V for 90 min, and the gel was dried and visualized by autoradiography. For the supershift assay, the reaction mixture was incubated with antibody against human Sp1 (PEP2) (Santa Cruz Biotechnology) for 30 min at 4 °C.

ChIP assay

Cells were crosslinked at a final concentration of 1% formaldehyde for 10 min at 37 °C. Crosslinked cells were washed twice in ice-cold $NaCl/P_i$ and collected by centrifugation for 5 min at 300 × ***g*** The pellet was resuspended in 600 μL of buffer (50 mM Tris/HCl, pH 8.0, 85 mM KCl, 0.5% NP40) and incubated for 10 min on ice. The solution was centrifuged for 5 min at 1700 × ***g***, and the pellet was resuspended in 600 μL of lysis buffer (50 mM Hepes/KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors. Lysate was sonicated to shear DNA to an average fragment size of 500–1000 bp, and the debris was pelleted by centrifugation for 5 min at 11 000 × ***g*** and 4 °C. The soluble chromatin material was further treated with salmon sperm DNA/protein A agarose 50% slurry (Upstate Biotechnology, Lake Placid, NY, USA). After overnight incubation with antibody against Sp1 (sc-59x) (Santa Cruz Biotechnology), the immune complexes were

Table 1. Oligonucleotides used in EMSA experiments. F, forward; R, reverse.

Name	Position (relative to the TSS)	Direction	Oligonucleotides (5′- to 3′)
Region A	−233 to −204 bp	F	aaaggcggcgggaaggaggcggggcagagc
		R	gctctgccccgcctccttcccgccgccttt
Region B	−61 to −33 bp	F	cccgcggcgcctcccctccccgcgccca
		R	tgggcgcggggaggggaggcgccgcggg
Region C	−20 to −1 bp	F	aggggccgggcagcccgccct
		R	agggcgggctgcccggcccct
Region D	−4 to +20 bp	F	ccctgcctccccgcccgcgcaccc
		R	gggtgcgcgggcggggaggcaggg
Cold control oligonucleotide	None	F	attcgatcggggcggggcgag
		R	ctcgccccgccccgatcgaat
mSp1	None	F	attcgatcggttcggggcgag
		R	ctcgccccgaaccgatcgaat

treated with 60 μL of salmon sperm DNA/protein A agarose 50% slurry (Upstate Biotechnology) for 2 h at 4 °C. The beads were then washed sequentially for 5 min at room temperature in 1 mL of lysis buffer without SDS, in 1 mL of lysis buffer plus 500 mM NaCl, in 1 mL of buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA, pH 8.0, 250 mM LiCl, 1% NP40, 1% sodium deoxycholate), and finally twice in Tris/EDTA. Immune complexes were eluted with 200 μL of elution buffer (1% SDS, 50 mM Tris/HCl, pH 7.5, 10 mM EDTA) and incubated for 5 min at 65 °C. The pooled elutes were incubated with RNase for 1 h and with proteinase K for 4 h at 65 °C to reverse crosslinks. DNA was extracted by phenol/chloroform treatment and precipitated with 10 μg of glycogen and ethanol overnight at – 20 °C. The pelleted DNA was resuspended in 10 μL of nuclease free water and amplified by PCR. The PCR primers used were as follows: forward, 5′-TCG CCG GGC TCT GCA GGA G-3′; and reverse, 5′-GTT TCG CTG CGT CCC CTT CGC-3′.

siRNA transient transfection

Sp1 Validated Stealth RNAi DuoPak and its medium-GC content siRNA control (Invitrogen) were used for knockdown of *p73* and *VEGF*. A549 cells were harvested in six-well plates and transfected with Lipofectamine RNAiMAX according to the manufacturer's instructions. Lipofectamine-containing medium was replaced after 6 h. Cells were collected following a 48-h incubation at 37 °C, and total proteins were isolated for western blot analysis.

Double-stranded oligonucleotide functional analysis

We used phosphorothioate oligonucleotides for regions A, B and C (Invitrogen) to transiently transfect the A549 cell line, as previously described [48]. Cells were harvested in six-well plates and transfected with 150 nM Sp1-decoy oligonucleotides, using Fugene transfection reagent (Roche Applied Science), according to the manufacturer's instructions. After 4 h, the medium was replaced with fresh medium, without Fugene and oligonucleotides. The cells were collected 4, 12 and 24 h after transfection, and total proteins extracted from these cells were subjected to western blot analysis.

Western blot analysis

Protein extracts (10 μg) were electrophoresed on an 8% SDS/polyacrylamide gel under reducing conditions, transferred to nitrocellulose membranes, and blocked for 2 h at room temperature. The blots were subsequently incubated overnight at 4 °C with the following primary antibodies: rabbit immunoglobulin against human Sp1 (PEP2) (Santa Cruz Biotechnology), mouse immunoglobulin against human β-actin (Abcam, Cambridge, UK), mouse immunoglobulin against human full length p73 (which has been shown to recognize TAp73α, TAp73β, and TAp73γ [49]), rabbit immunoglobulin against human VEGF (sc-507) (Santa Cruz Biotechnology), and mouse immunoglobulin against human ΔNp73 (Abcam), in 1 : 600, 1 : 1000, 1 : 4000, 1 : 500 and 1 : 500 dilutions, respectively. The blots were incubated with the appropriate secondary horseradish peroxidase-conjugated antibodies (Santa Cruz, Santa Cruz, CA, USA) in corresponding dilutions of 1 : 8000, 1 : 5000, 1 : 10 000, 1 : 3000 and 1 : 10 000 for 2 h at room temperature. Detection of protein levels was carried out using an enhanced chemiluminescence system (Pierce, Rockford, IL, USA). The protein amounts were normalized to β-actin and quantified using IMAGEQUANT software (GE Healthcare, Little Chalfont, UK).

Acknowledgements

We thank G. Melino for kindly providing us with the TAp73 constructs. S. Logotheti and V. Zoumpourlis were supported by 05NON-EU-3. A. Daskalos, T. Liloglou and J. K. Field were supported by the Roy Castle Lung Cancer Foundation, UK. B. Vojtesek was supported by grants IGA MZ CR NS/9812-4 and MZOMOU2005. V Gorgoulis is financially supported by European commition grants FP-7 GENICA and FP-7 INLACARE.

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Supporting information

The following supplementary material is available:
Fig. S1. (A) TAp73 and Sp1 protein expression in 26 lung cancer patients. N, normal tissue; T, tumour tissue; PNo, patient number. (B) TAp73γ/Sp1 ratio in lung cancer patient samples.

This supplementary material can be found in the online version of this article.

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