The p53 family member p73 modulates the proproliferative role of IGFBP3 in short children born small for gestational age

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ABSTRACT The regulation of insulin-like growth factor–binding protein 3 (IGFBP3) gene expression is complex, because it can be induced by agents that both stimulate and inhibit the proliferation. The principal aim of this study was to investigate whether p73, a member of the p53 gene family, has a role in the regulation of the IGFBP3 expression and whether this regulation occurs in a context of cell survival or death. We demonstrate that IGFBP3 is a direct TAp73α (the p73 isoform that contains the trans-activation domain) target gene and activates the expression of IGFBP3 in actively proliferating cells. As IGFBP3 plays a key role in regulating the growth hormone/insulin-like growth factor type 1 (GH/IGF1) axis, whose alterations in gene expression appear to have a role in the growth failure of children born small for gestational age (SGA), we measured the mRNA expression levels of p73 and IGFBP3 in a group of SGA children. We found that mRNA expression levels of p73 and IGFBP3 are significantly lower in SGA children compared with controls and, in particular, p73 mRNA expression is significantly lower in SGA children with respect to height. Our results shed light on the intricate GH/IGF pathway, suggesting p73 as a good biomarker of the clinical risk for SGA children to remain short in adulthood.

INTRODUCTION Insulin-like growth factor–binding protein 3 (IGFBP3), the main circulating carrier of insulin-like growth factors (IGFs) in postnatal life, inhibits or enhances the effects of IGFs by modulating the amount of free IGF type 1 (IGF-1), by repressing its transfer from the circulation to tissue sites of action, and by regulating the interaction between IGFs and IGF-1 receptor (IGF-1R) (Mohan and Baylink, 2002). The growth hormone (GH)/IGF/IGFBP axis has been extensively studied in neonates with intrauterine growth retardation and in children born small for gestational age (SGA). SGA children represent a group of newborns (5–10%) whose birth weight and/or length are less than −2 SD from the mean for gestational age (Clayton et al., 2007). Most SGA children show catch-up growth during the first 2 yr of life, but ∼15% remain short throughout childhood and adolescence and into adulthood (Klammt et al., 2008; Labarta et al., 2009). The status of the GH/IGF axis at birth or in early postnatal life is not predictive of later growth, and therefore hormone measurements in SGA infants or children are not indicated in routine care (Leger et al., 1996). Aside from its effects on IGF-1/IGF-1R interaction, IGFBP3 also plays a role as growth inhibitor and proapoptotic factor in an IGF-independent...
manner (Firth and Baxter, 2002; Longobardi et al., 2003; Takaoka et al., 2006). Considering that IGFBP3 plays a role in several different contexts, its expression is regulated by many factors. In a previous study, we demonstrated that some polymorphisms in the IGFBP3 promoter region, such as −667 G/A and −396 C/T, influence the basal transcriptional activity of IGFBP3 in SGA children, although IGFBP3 expression remains in the normal range for age and sex (Faienza et al., 2011). GH and insulin regulate the proliferative role of IGFBP3 (Liao et al., 2006; DiGirolamo et al., 2007); proapoptotic and growth inhibitor factors, such as transforming growth factor-β, retinoic acid, tumor necrosis factor-α, vitamin D, antiestrogens, antiandrogens, and tumor suppressor p53, stimulate IGFBP3 expression (Huynh et al., 1996; Nickerson and Huynh, 1999). It is already known that the IGFBP3 gene is a direct target of p53, actively participating in apoptotic pathways triggered by p53. Historically, p53 represents the founding member of a transcription factor family to which also p63 and p73 belong. The three transcriptional factors share common functional characteristics, including the ability to induce cell cycle arrest and apoptosis, but have nonredundant roles (Vousden and Ryan, 2009; Gottlieb and Vousden, 2010). The three genes produce different protein isoforms that are generated through the use of two promoters and alternative splicing events. The TA isoforms (with the transactivation domain) have anti-oncogenic activity, whereas the ΔN isoforms (without the transactivation domain) have a potential anti-apoptotic and proliferative function (Murray-Zmijewski et al., 2006; Deyoung and Ellisen, 2007). It has been reported that ΔNp63α down-regulates IGFBP3 expression (Barbieri et al., 2005), but the role of p73 in the regulation of IGFBP3 expression has not yet been determined.

As the p53 family members are central regulators of cell cycle arrest, apoptosis, and proliferation, and given the functional role of IGFBP3, the principal aim of this study was to investigate whether TAp73 has a role in the regulation of the IGFBP3 expression and whether this regulation occurs in a context of cell survival or death. Consistently, we measured the serum expression levels of TAp73 and IGFBP3 in a group of short children born SGA and in a control group born appropriate for gestational age (AGA) to assess the outcome of TAp73 and IGFBP3 measurements in the clinical risk for SGA babies to remain short in adulthood.

RESULTS

TAp73α activates the transcription of IGFBP3

It is already known that the IGFBP3 gene is a direct target of p53 and contains two p53 responsive elements (RES) located in intron 1 and intron 2: box A and box B, respectively. We identified, upstream and closer to the transcription start site, the already identified p53RE box A and box B (Barbieri et al., 2005) and an additional p53RE in intron 1, which we called box C (Figure 1). To determine whether p53 family members can transactivate IGFBP3 gene expression via this new element, we cotransfected the p53-null H1299 cells with pcDNA3 empty vector or expressing p53, TAp73α, TAp63α, or ΔNp63α and pGL3-Basic-IGFBP3-p53RE-box A, B, or C. The luciferase activities were normalized to Renilla activity. The data represent the average of at least three independent experiments and are shown with SEs.

FIGURE 1: (a) Schematic map of the human IGFBP3 genomic region containing the two known p53REs (box A and box B) and the new p53RE (box C) with related sequences. (b) Luciferase assay. Effect of p53, TAp73α, TAp63α, and ΔNp63α on the transcriptional activity of IGFBP3-p53REs. p53-null H1299 cells were transiently cotransfected with pcDNA3 empty vector or expressing p53, TAp73α, TAp63α, or ΔNp63α and pGL3-Basic-IGFBP3-p53RE-box A, B, or C. The luciferase activities were normalized to Renilla activity. The data represent the average of at least three independent experiments and are shown with SEs.
binding was accompanied by an increase in histone H4 acetylation (Figure 3a). Surprisingly, only TAp73α but not p53 was bound to IGFBP3 box B and box C (Figure 3a). Interestingly, TAp73α binding on boxes A, B, and C was accompanied by histone H4 acetylation, which suggested that TAp73α actively transcribes IGFBP3. At the same time, we examined whether TAp73α can regulate the expression of the endogenous IGFBP3 gene. Total RNA and RNA extracted from T-Rex-293-TAp73α cells, both uninduced and at 24 h after induction of TAp73α expression, were analyzed in RT-qPCR experiments and Western blotting. We clearly demonstrated that TAp73α induced the expression of IGFBP3 mRNA with great efficiency (Figure 3b). In addition, to confirm the contribution of p73 protein in the transcriptional regulation of the IGFBP3 endogenous gene, we selectively suppressed the expression of p73 by transfecting specific p73 short hairpin RNAs (p73-shRNAs) in MCF-7 cells (Figure 4a). The p73-shRNAs we used are designed to target all known p73 variants without targeting related gene family members. Cells were transfected with pRS empty vector (control) or p73-shRNAs for 48 h. As shown in Figure 4a, RT-qPCR and Western blot analyses showed that the cells transfected with p73-shRNA displayed a decrease in the levels of their mRNAs and corresponding proteins. At the same time, in cells that underwent suppression of p73, a marked decrease was observed in IGFBP3 mRNA, further supporting the concept that IGFBP3 is also a p73 target gene (Figure 4b).

**TAp73α activates IGFBP3 expression during cell cycle progression**

As previously reported, TAp73α protein levels undergo a coordinated and complex modulation during physiologic cell cycle progression, reaching a peak in S phase, and their silencing determines a significant suppression of proliferation compared with the control (Figure 5, a and b; Talos et al., 2010). We therefore sought to correlate the transcription of IGFBP3 in arrested and serum-released MCF-7 cells. Cells were grown in low serum for 48 h and were released by serum restimulation for 22 h. The cell cycle profile was monitored by flow-cytometric analyses (Figure 5a) and by immunoblots of cyclins D1, E, and A (Figure 5b), which indicated that MCF-7 cells were arrested in G1 after 48 h of serum starvation and were in S phase 22 h after the refeed. As shown in Figure 5b, TAp73α fell as the cells accumulated in G0/G1 and increased after re-addition of serum, reaching a peak when the cells were in S phase (22 h; Lefkimmiatis et al., 2009). We confirmed that the suppression of p73 in asynchronous and reseeded MCF-7 cells led to a reduction in cell proliferation (the percentage of cells in S+G2 phases decreased). To assess whether the changes we observed in p73 protein levels at G1 and S phases affected the expression of IGFBP3, we performed RT-qPCR (Figure 5c). Interestingly, we found that in G0/G1-arrested cells, when TAp73α protein levels were low, the IGFBP3 expression...
was decreased compared with refed and asynchronous growing cells when TAp73α protein levels are higher. Moreover, the suppression of p73 protein expression by specific p73-shRNAs was paralleled by a further decrease in IGFBP3 mRNA levels both in asynchronous and in starved and refed cells (Figure 5c). All these data strongly suggest that TAp73α, in contrast to p53, regulates the expression of IGFBP3 in actively proliferating cells.

**p73 and IGFBP3 expression are down-regulated in SGA children**

As IGFBP3 plays a key role in regulating the GH/IGF axis, alterations of which in gene expression appear to have a basic role in the growth failure of children born SGA, we assessed the relative contribution of p73 in regulating IGFBP3 expression in SGA children compared with age- and sex-matched AGA controls. Sixteen SGA children and 14 AGA children were analyzed for IGFBP3 mRNA and IGFBP3 protein. Interestingly, as shown in Figure 6a, on average, SGA children expressed both serum p73 mRNAs and IGFBP3 at lower levels than AGA controls (34 and 55% less, respectively; \( p = 0.15 \) and \( p = 0.02 \)). Interestingly, we found that p73 expression is significantly lower in SGA children with respect to height compared with AGA children (62% less; \( p = 0.031 \)), while IGFBP3 expression is equally lower in SGA children with respect to height and weight (67% less, compared with AGA children; \( p = 0.009 \) and 0.07, respectively). Consistently, the circulating IGFBP3 protein levels in children born SGA with respect to height were lower compared with control values (2076 ng/ml vs. 3648 ng/ml; \( p = 0.0002 \); Figure 6b). We found that p53 mRNA expression was lower in SGA children compared with AGA children, but this difference was not statistically significant (unpublished data).

Altogether these experiments suggest that p73 and IGFBP3 are down-regulated in SGA children.

**DISCUSSION**

Short stature, with an incidence of three in 100, is a fairly frequent disorder in children. The child with short stature presents a diagnostic puzzle in which the clinical phenotype, endocrine features, and genetic factors may all contribute to the categorization of the patient into a specific diagnostic group. Mutations in different genes, such as SHOX (short stature homeobox), PTPN11, RAS, and RAF1, have been shown to be associated with the short stature phenotype in patients with differing pathologies.

Persistent short stature is also one of the most frequent complications of being born SGA, as almost 10–15% of such children have a low adult height. Although the pathophysiology of postnatal growth failure in SGA is complex, in contrast to the above-mentioned short statures due to mutations in different genes, mutations or deletions in the genes involved in GH/IGFs/IGFBP3 axis were rarely found. Therefore a detailed knowledge of gene expression profiling may provide crucial elements to clarify the molecular mechanisms that regulate the persistence of short stature in adults born SGA.

This study addresses the question of whether the p53 family member TAp73α may have a role in cell proliferation via mechanisms that involve regulation of IGFBP3 gene expression with the aim of shedding light on the molecular mechanisms governing the GH/IGFs/IGFBP3 axis.

We demonstrate here that IGFBP3 gene expression is promoted by TAp73α as well as by p53 protein. Indeed, we identified a new p53RE in the IGFBP3 gene, which we called box C. This box is selectively bound and transactivated by TAp73α but not by p53. Many factors influence the ability of p53 to function as a sequence-specific transcription factor, including cofactors that may be stress specific, the variation in p53 levels in response to stress, and the specific RE sequence. p53 binding affinities seem to dictate the choice between regulating cell cycle arrest (high-affinity sites) and proapoptotic responses (low-affinity sites). High-affinity p53 binding sites are characterized by a shorter spacer between the two decamers and by the presence of CRRs. This can explain the high-affinity sites described in previously reported studies.

p53RE sites in this region of the IGFBP3 gene were characterized by a further decrease in IGFBP3 mRNA levels both in asynchronous and in starved and refed cells (Figure 5c). All these data strongly suggest that TAp73α, in contrast to p53, regulates the expression of IGFBP3 in actively proliferating cells.
achieved through binding to sequence-specific REs whose consensus sequences are highly similar to that of p53 REs. Among the three REs in the IGFBP3 gene, p53 binds box A with high affinity. This latter RE is composed of two decamers spaced by only one base, and moreover, it is the only one that has the more strongly arranged CATG consensus core sequence, which might explain the p53 binding specificity.

Up to now, the regulation of IGFBP3 gene expression by the p53 family members has been linked to the apoptotic, antiproliferative functions (Buckbinder et al., 1995; Barbieri et al., 2005), although the exact mechanisms underlying cell cycle arrest and apoptosis by IGFBP3 have yet to be fully elucidated. p73 has a significant role in brain development and in both embryonic and adult neurogenesis. p73 has a critical role in maintaining an adequate neurogenic pool by promoting self-renewal and proliferation (Meyer et al., 2004; Talos et al., 2010; Niklison-Chirou et al., 2013). Interestingly, a decrease in IGFBP3 expression has been observed in an Alzheimer’s disease (AD) model cell line and in the hippocampus of AD model transgenic mice (Talos et al., 2010), indicating a protective role for IGFBP3 in primary rat hippocampal neurons. Importantly, IGFBP3 can also be involved in neuronal survival and protection via IGF-1–independent mechanisms. In contrast to reports of the proapoptotic effects of IGFBP3, a number of recent studies demonstrated that IGFBP3 stimulates cell proliferation and protects cells from apoptotic insults in a variety of cell types through IGF-dependent or IGF-independent mechanisms (Martin et al., 2003; Butt et al., 2004; Sung et al., 2014). Of note, we found that the IGFBP3 expression is lower in G0/G1-arrested cells and increases when the cells are refed and that the suppression of p73 by using specific p73sh-RNA decreases IGFBP3 expression (Figure 5c). These data strongly suggest that p73 activates the expression of the IGFBP3 in actively proliferating cells.

Several studies provided evidence that p53 family members have broader roles in cell cycle control than previously expected, and we have previously demonstrated a role for p73 and p63 in supporting cellular proliferation through the transcriptional activation of the genes involved in G1/S and G2/M progression (Lefkimiatis et al., 2009). Moreover, TAp73α is implicated in the control of mitosis and aneuploidy (Fulco et al., 2003; Merlo et al., 2005; Talos et al., 2007), and more recently it has been reported that TAp73α has a crucial role in preventing genomic instability in multiple tissues (Tomasini et al., 2009; Costanzo et al., 2014).

As a growing number of studies indicate that alterations of the genes involved in the signal transduction pathways of GH/IGF/IGFBP3 play a key role in short children who do not respond to GH treatment, we assessed the contribution of p73 in regulating the expression of IGFBP3 in a group of children born SGA. Of note, SGA children are at increased risk for neurodevelopmental disorders, and p73, as reported above, has an important role in brain development. Measuring the mRNA expression levels of IGFBP3 and p73 in children born SGA and in a group of AGA controls, we found that the mRNA expression levels of p73 and IGFBP3 are consistently significantly lower in SGA children compared with AGA controls, and p73 mRNA expression in particular is significantly lower in short children born SGA with respect to height. Of note, we observed in the same children that the circulating IGFBP3 protein levels were lower compared with control values (Figure 6, a and b).

Altogether our experiments demonstrate for the first time a functional link between IGFBP3 and TAp73α and strongly support the hypothesis that the measurement of circulating levels of IGFBP3 in association with analysis of the expression of p73 could allow the identification of patients at risk to remain short in adulthood. This finding is particularly important, since there is no predictive biomarker for this state to date.

**MATERIALS AND METHODS**

**Subjects**

Sixteen children (10 males; mean age 9.08 ± 3.87 yr) out of 20 meeting the inclusion criteria agreed to participate to this study. All children were born at term at the Neonatal Intensive Care Unit (NICU) of the Department of Biomedical Sciences and Human Oncology, Section of Pediatrics, University of Bari, and had a birth length and/or birth weight below −2 SD, height below −2 SD, and no growth failure caused by other disorders (i.e., GH deficiency, metabolic or endocrine diseases). They were followed up for the first 2 yr of life at the Pediatric Endocrinology Unit according to an established follow-up in SGA children and were subsequently invited to participate in the study in the period between January and October 2014. The control group consisted of 14 children born AGA (11 males; mean age 9.53 ± 3.62 yr) with birth length and weight above −2 SD, height below −2 SD, and no growth failure caused by other disorders (i.e., GH deficiency, metabolic or endocrine diseases). They were followed up for the first 2 yr of life at the Pediatric Endocrinology Unit according to an established follow-up in SGA subjects and were subsequently invited to participate in the study in the period between January and October 2014. The control group consisted of 14 children born AGA (11 males; mean age 9.53 ± 3.62 yr) with birth length and weight above −1 SD according to the Italian Neonatal Anthropometric Charts (Bertino et al., 2010), recruited from parents attending the pediatric clinic of the University of Bari for minor trauma (first aid) or for allergic screening. Written informed consent was obtained from all parents, and oral consent was obtained from all children. All the procedures used were in accordance with the guidelines of the Helsinki Declaration on Human Experimentation.

Table 1 shows the clinical and biochemical characteristics of the SGA and AGA groups.
Serum IGFBP3 and IGF-I detection. The serum levels of IGFBP3 from patients were measured by using enzymatically amplified a two-step sandwich-type immunoassay DRG IGFBP-3 (EIA-3300) ELISA kit (DRG Instruments GmbH, Marburg, Germany). Serum levels of IGF-I were measured by the Immulite 2000 IGF-I kit (Siemens Healthcare, Malvern, PA), which is a solid-phase enzyme-labeled chemiluminescent immunometric assay. The IGFBP3 and IGF-I reference values were taken from the Pediatric Reference Ranges supplied by the manuals of the DRG IGFBP-3 (EIA-3300) ELISA kit (DRG Instruments GmbH) and the Immulite 2000 IGF-I kit.

p53REs identification in the IGFBP3 regulatory regions. The computational analysis to identify the p53REs in the IGFBP3 regulatory regions was performed using the PatSearch algorithm implemented in the DNAfan tool (Grillo et al., 2003; Gisel et al., 2004; Sbisà et al., 2007). Using the p53RE syntax pattern previously described (Grillo et al., 2003; Sbisà et al., 2007), we searched for p53REs presence in the IGFBP3 promoter or 5’ UTR or 3’ UTR or in the intronic regions, and we found a new p53RE (named box C: +1649–1689), upstream of the two previously identified p53REs (box A: +3170–3191; box B: +4090–4110; NC_000007.14; Buckbinder et al., 1995).

Cell lines. The human colon carcinoma cell lines HCT116 and HCT116p53−−, the human breast carcinoma cell line MCF-7, the human lung carcinoma cell line H1299, and the embryonic kidney T-Rex-293 were cultured in DMEM plus 10% fetal bovine serum, l-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C, 5% CO₂. The embryonic kidney Flp-In T-Rex-293 cell line from Invitrogen was used to generate stable CAT (chloramphenicol acetyltransferase), p53, and TAp73α expression.

Transfections. Cells (5 × 10⁵) were plated 24 h before transfection. At the time of transfection (60–80% cell confluence), 200 μl of DMEM without serum was incubated with Trans-LT1 Mirus transfection reagent (Tema Ricerca, Bologna, Italy) for 5 min at room temperature. Then the empty pcDNA3 vector (control), pcDNA3-p53wt, pcDNA3-p73α, pcDNA3-TAp63α, pcDNA3-ΔNp63α, the empty pRS (control), or four different p73 shRNAs (Origene, Rockville, MD) were added to the medium containing the transfection reagent and incubated at room temperature for 20 min and subsequently added to the cell cultures for the indicated times.

Protein extraction and Western blot analysis. Cells were plated in 100-mm culture dishes at a density of 1 × 10⁶ cells/ml. After treatment, cells were lysed and extracted as previously described (Tullo et al., 2003). For immunoblotting, the following primary antibodies were used: p53-specific DO-1 (Santa Cruz Biotechnology, Dallas, TX; 1:300), p63 antibody 4A4 (Santa Cruz Biotechnology;
1:400), p73 antibody Ab-2 (ER-15 clone; Oncogene, San Diego, CA; 1:200), antibody anti-cyclin A (Santa Cruz Biotechnology; 1:200), antibody anti-cyclin D1 (Santa Cruz Biotechnology; 1:200), antibody anti-cyclin E (Santa Cruz Biotechnology; 1:200), anti-actin Ab-1 antibodies kit (Calbiochem, San Diego, CA; 1:2000), and IGFBP3 antibody (Santa Cruz Biotechnology; 1:600).

Bound primary antibodies were visualized using Lumi-Light Western Blotting Substrate (Roche, Milan, Italy) on a UVITEC Cambridge Camera.

**RNA extraction from cell lines.** Total cellular RNA was extracted from HCT116, HCT116p53−/−, MCF-7, and 293 T-Rex (CAT, p53wt, p73α) cells using the RNeasy mini kit (Qiagen, Venlo, Netherlands). Purified RNA was then quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA), and RNA quality was determined by running aliquots on the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

**RNA extraction from blood.** Peripheral blood samples were collected in PAXgene Blood RNA Tubes (BD, Hombrechtikon, Switzerland; cat. no. 762162). The sample was gently inverted and stored at −80°C within 2 h of collection. RNA was purified using the manual procedure according to the PAXgene Blood RNA Kit Handbook (BD, cat. no. 762174). Freshly extracted RNA was dosed, and RNA integrity was additionally assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies).

**FIGURE 6:** (a) RT-qPCR of IGFBP3 and p73 mRNA expression in SGA children. Expression data were normalized by HPRT1 expression and measured with respect to one normal sample chosen arbitrarily as a calibrator. The reported data represent the average of at least three independent experiments and are shown with their SEs. *, p value < 0.05. (b) IGFBP3 protein serum levels in SGA children compared for length with AGA children.

|                         | SGA children (n = 16) | AGA children (n = 14) | p Value |
|-------------------------|-----------------------|-----------------------|---------|
| Male/female             | 10/6                  | 11/3                  | —       |
| Gestational age (wk)    | 38.61 ± 1.33          | 39.03 ± 0.98          | ns      |
| Current age (yr)        | 9.08 ± 3.87           | 9.53 ± 3.62           | ns      |
| Birth weight (g)        | 2360.18 ± 410.92      | 3350.12 ± 0.37        | <0.05   |
| Birth weight SD         | −1.98 ± 0.91          | 0.01 ± 0.84           | <0.05   |
| Birth length (cm)       | 45.55 ± 2.13          | 50.15 ± 1.14          | <0.05   |
| Birth length SD         | −1.88 ± 0.89          | −0.03 ± 0.70          | <0.05   |
| Height (cm)             | 123.00 ± 24.1         | 140.02 ± 13.80        | <0.05   |
| Height SD               | −1.85 ± 1.02          | 0.69 ± 0.90           | <0.05   |
| IGF-1 (ng/ml)           | 268.53 ± 183.7        | 201.4 ± 63.78         | ns      |
| IGF-1 SD                | 0.0 ± 1               | 0.45 ± 1.46           | ns      |
| IGFBP3 (ng/ml)          | 2899.28 ± 975.26      | 3864.71 ± 397.32      | <0.05   |
| IGFBP3 SD               | 0.0 ± 1               | 0.0 ± 1               | ns      |

Data are presented as mean and SD. ns = not significant.

**TABLE 1:** Clinical and biochemical characteristics of the study population.
RT-qPCR analysis
Reverse transcription of 200–500 ng of total RNA was performed using Quantitect Reverse Transcription kit (Qiagen), according to the manufacturer's instruction. RT-qPCR experiments were performed in duplicate on the ABI PRISM 7900HT platform (Applied Biosystems, Life Technologies, Carlsbad, CA), using 1 μl cDNA as the template for each reaction with TaqMan Universal PCR Master Mix (Applied Biosystems, Life Technologies). TaqMan assays from Applied Biosystems were used for the amplification of IGFBP3 (Hs00181211_m1), p53 (Hs00153349_m1), p63 (Hs00973430), p73 (Hs01056230_m1), GAPDH (Hs99999905_m1), and HPRT1 (Hs03929098_m1) transcripts. Results were first analyzed in SDS 2.2.1 software and then exported into Microsoft Excel to be further analyzed by using geNorm, which output GAPDH and HPRT1 as best housekeeping genes for cell culture and blood samples, respectively. The reported data represent the average of at least three independent experiments and are shown with their SEs. Two-tailed Student's t tests were performed to assess the statistical significance of gene expression level differences observed between the SGA and AGA children. In this study, a p value < 0.05 was considered to be statistically significant.

Cell cycle analysis. Asynchronous, starved, and refed HCT116 cells, transfected with the empty pRS (control) or four different p73 shRNAs (Origene), were used for this assay. The total cell population, including floating and adherent cells, was harvested at the given time point; washed twice with 1× phosphate-buffered saline; and treated with 150 mg/ml RNase A, 5 mg/ml propidium iodide (PI), and NP-40 0.1% at room temperature for 1 h. The cells were analyzed in a FACScalibur; cell cycle and apoptosis analyses were performed using ModFit analysis software (Becton Dickinson, Franklin Lakes, NJ).

Transfections and luciferase assays. The fragments containing the p53-REs of the human IGFBP3 gene (boxes A, B, and C) were amplified from the human genomic DNA and cloned in the pGL-3 basic plasmid (Promega, Madison, WI). Human H1299 cells (1 × 104) were cultured 24 h before transfection (60–80% confluency). Transient reporter assays were performed as described in Transfections. Each well was cotransfected using TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, WI) according to the manufacturer's instructions, with either empty pcDNA3 vector or containing p53wt, p73α, TAp63α, ΔNp63α, or their mutated forms p53R175H, p73αV156A, TAp63αR279Q, and ΔNp63αR279Q (150 ng); the recombinant reporter vector containing the p53-REs of the human IGFBP3 gene (box A, box B, and box C; 1 μg); and the Renilla pRL-SV40 vector (Promega; 10 ng). Thirty-six hours after transfection, H1299 cells were lysed in passive lysis buffer (Promega), and the luciferase assay was performed using the Dual Luciferase assay system (Promega) according to the manufacturer's instructions. Data were normalized to the Renilla reporter signal. The results reported represent the average of at least three independent experiments and are shown with the SDs.

Chromatin immunoprecipitation assay. T-Rex-293-CAT, T-Rex-293-p53wt, and T-Rex-293-p73 cells were cultured in 15-cm culture dishes for 24 h after the induction of the different proteins. Proteins were cross-linked to DNA in living nuclei, and a chromatin immunoprecipitation assay was performed as previously described (Tullo et al., 2003). Five micrograms of the following antibodies was used to immunoprecipitate the DNA–protein complexes: p53 antibody DO-1 (Santa Cruz Biotechnology), p63 antibody H137 (Santa Cruz Biotechnology), p73 antibodies H-79 and C-20 (Santa Cruz Biotechnology), acetylated H4-histone antibody (Upstate, Lake Placid, NY), or unrelated control anti-Flag antibody (Sigma-Aldrich, St. Louis, MO). DNA fragments were analyzed by PCR using specific primers.

ACKNOWLEDGMENTS
This work was supported by the Ministero dell’Istruzione, Università e Ricerca (projects Virtualab [PON01_01297]).

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