

## Report

# The Fatty Acid Synthase Gene is a Conserved p53 Family Target from Worm to Human

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## KEY WORDS

TAp73 $\alpha$ ,  $\Delta$ Np63 $\alpha$ , CEP-1, FAS, direct target gene, cellular proliferation

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## ABSTRACT

The discovery that the p53 family consists of three members (p53, p63 and p73) in vertebrates and of a single homolog in invertebrates has raised the challenge of understanding the functions of the ancestor and how they have evolved and differentiated within the duplicated genes in vertebrates. Here, we report that the fatty acid synthase (FAS) gene, encoding for a key enzyme involved in the biogenesis of membrane lipids in rapidly proliferating cells, is a conserved target of the p53 family throughout the evolution. We show that CEP-1, the *C. elegans* p53 homolog, is able to bind the two p53 family responsive elements (REs) identified in the worm *fasn-1* gene. Moreover, we demonstrate that *fasn-1* expression is modulated by CEP-1 in vivo, by comparing wild-type and CEP-1 knockout worms. In human, luciferase and chromatin immunoprecipitation assays demonstrate that TAp73 $\alpha$  and  $\Delta$ Np63 $\alpha$ , but not p53, TAp73 $\beta$  and TAp63 $\alpha$  bind the two p53 REs of the human *FASN* gene. We show that the ectopic expression of TAp73 $\alpha$  and  $\Delta$ Np63 $\alpha$  leads to an increase of *FASN* mRNA levels, while their silencing produces a decrease of *FASN* expression. Furthermore, we present data showing a correlation between  $\Delta$ Np63 $\alpha$  and *FASN* expression in cellular proliferation.

Of relevant importance is that *fasn-1* is the first CEP-1 direct target gene identified so far in *C. elegans* and our results suggest a new CEP-1 role in cellular proliferation and development, besides the one already described in apoptosis of germ cells. These data confirm the hypothesis that the ancestral functions of the single invertebrate gene may have been spread out among the three vertebrate members, each of them have acquired specific role in cell cycle regulation.

## INTRODUCTION

The p53 gene family consists of three genes in vertebrates, p53, p63 and p73, while a single gene is present in invertebrates. The p53 gene, the first member of the family to be identified, encodes for a transcription factor, involved in maintaining genomic integrity by regulating genes controlling cell growth and apoptosis in response to cellular stress signals.<sup>1,2</sup> Later on, p63 and p73 were identified as p53 homologous genes, since they encode proteins showing significant aminoacid identity with p53 in the three main functional domains (transactivation, central DNA binding and tetramerization domains) and that are able to transactivate some of the p53-responsive genes and induce cell cycle arrest and apoptosis.<sup>3,4</sup>

For long time p53 gene was believed to have a single promoter and to produce a single protein. Recently it has been demonstrated that, like p73 and p63, p53 has a complex transcriptional pattern, encoding different mRNA variants through both the existence of two promoters and the use of alternative splicing mechanisms.<sup>5</sup> Through the use of the alternative promoter, in intron 2 for p73 and p63, and in intron 4 for p53, the three genes give rise to two types of proteins: the proteins containing the transactivation (TA) domain and the isoforms lacking the TA domain, called  $\Delta$ Np73,  $\Delta$ Np63 and  $\Delta$ 133p53, respectively.<sup>5-7</sup> The  $\Delta$ Np63 and  $\Delta$ Np73 isoforms, contain a second activation domain responsible for the transactivation activity of these proteins.<sup>8,9</sup> Moreover, p53 and p73 produce other isoforms lacking the TA domain (the  $\Delta$ N40 isoform for p53 and Ex2p73, Ex2/3p73 and  $\Delta$ N'p73 for p73) through N-terminal alternative splicing mechanisms.<sup>5,10</sup> p53, p73 and p63 undergo alternative splicing mechanisms also at the 3' end of their primary transcripts, producing different C-terminal isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ , for p53,  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$  and  $\zeta$  for p73 and  $\alpha$ ,  $\beta$ , and  $\gamma$  for p63).<sup>4,5,11-13</sup> Several structural and functional differences between p53, p73 and p63 emerged rapidly. Differently from p53, p63 and p73 have an extended C-terminal region, coding for the sterile alpha domain (SAM), a protein-protein interaction domain found in proteins involved in development regulation.<sup>14</sup> In addition,

important functional differences derive from the analysis of p53, p73 and p63 null mice. In fact, while p53-null mice develop normally but are cancer prone, p73-null mice do not develop a tumor phenotype, but exhibit profound neurological, pheromonal, inflammatory and homeostasis defects whereas p63-null mice show striking defects in epithelia development.<sup>7,15</sup> Thus, current data suggest that, while p53 main role lies in the inhibition of tumour progression, p73 and p63 appear to be much more strongly involved in development and differentiation.<sup>16,17</sup>

A single p53 family homologous gene was described in invertebrates: *Mya arenaria*,<sup>18</sup> *Loligo forbesi*,<sup>19</sup> *Drosophila melanogaster*,<sup>20-22</sup> *Caenorhabditis elegans*<sup>23,24</sup> and *Entamoeba histolytica*.<sup>25</sup> Invertebrate p53 family homologs show variable structural features. Their similarity with vertebrate p53 family members is mostly restricted to the central DNA binding domain, where only the aminoacidic residues which are demonstrated to be critical for the human p53 binding to DNA, are conserved. The *L. forbesi* and *M. arenaria* homologs have a long C-terminal region showing similarity with the SAM domain distinctive of p63 and p73, so they both appear to be more closely related to vertebrate p73 and p63 than to p53, while *E. histolytica*, *D. melanogaster* and *C. elegans* homologs have a C-terminal region of different length and with no similarity with any members of the vertebrate p53 family. The functional role of the p53 family members in invertebrates is still far from being clearly elucidated. In *C. elegans*, it has been reported that the p53 homolog, called CEP-1, is required for the apoptosis of the germ cells following DNA damage.<sup>23,24</sup> Also the *D. melanogaster* p53 homolog seems to be involved in DNA damage-induced apoptosis, thus revealing a conservation of the p53 family function in the apoptotic response from invertebrates to mammals.<sup>20</sup>

In previous papers we studied the evolutionary origin of the p53 gene family members.<sup>26,27</sup> We suggested that the ancestor of the family was a p63/p73-like protein, from which, through a first event of gene duplication in vertebrates, the p53 gene and a p63/p73 precursor gene evolved. Later, a second event of gene duplication gave rise to p63 and p73. On the basis of this evolutionary pattern, we speculated that the ancestral functions of the single invertebrate gene, in the course of evolution, might have been spread out among the three vertebrates genes and each one of them might have acquired a specific role in cell cycle regulation. To validate this hypothesis, it is necessary to identify the ancestral functions of the p53 family homolog in invertebrates and verify whether these functions have been maintained by all p53 family proteins in vertebrates or have been split among the different isoforms. To understand the specific functions of the p53 family members, the identification of their target genes is of crucial importance.

With this aim, by using in silico tools developed in our lab,<sup>28,29</sup> we performed a comparative search of p53 family responsive elements (p53REs) in the genome of two model organisms, the *C. elegans*, for invertebrates and the *H. sapiens*, for vertebrates, in order to identify possible conserved p53 family target genes. Among the selected genes containing p53REs, in this paper we have focused our attention on the human and worm homologous genes (*FASN* and *fasn-1* gene, respectively) coding for the fatty acid synthase (FAS), a key metabolic enzyme which catalyses the de novo synthesis of long-chain fatty acids from small carbon units. While in most normal human tissues FAS is expressed at very low levels, in rapidly proliferating normal cells and tissues, including some fetal tissues, FAS expression is very high. In these tissues, the major role of the fatty acid synthesis is to support membrane synthase, since the endogenously synthesized

fatty acids are incorporated into membrane phospholipids.<sup>30</sup> Recent study demonstrated that the de novo synthesis of fatty acids catalyzed by FAS is of essential importance during the embryonic development, since FAS knockout mice and most FAS heterozygous mutants die in the early stages of their development.<sup>31</sup>

The important role performed by FAS in cellular proliferation and development prompted us to investigate a possible involvement of the p53 family in the regulation of the FAS expression, both in invertebrates and vertebrates. The comparative study performed here demonstrates that FAS is an evolutionary conserved p53 family target gene, from invertebrates to vertebrates. In particular, the *C. elegans* *fasn-1*, is the first direct transcriptional target gene identified for CEP-1. This finding suggests a new additional role of CEP-1 in cellular proliferation and development, besides the one already described in apoptosis of germ cells. In human, we demonstrate that *FASN* gene is a new target of TAp73 $\alpha$  and  $\Delta$ Np63 $\alpha$ , but not of p53, TAp73 $\beta$  and TAp63 $\alpha$ , thus supporting the hypothesis of a differentiation of the p53 family members roles during the evolution. Finally, we present results obtained in normal keratinocytes, UV irradiated, showing a correlation between  $\Delta$ Np63 $\alpha$  and FAS gene expression in cellular proliferation.

## MATERIALS AND METHODS

**Cell culture.** H1299 and MCF-7 and HaCat cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). A2780pcDNA3 and A2780/p73 $\alpha$ .4 cell lines were cultured in the Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) containing G418 (500 $\mu$ g/ml). The T-rex 293- $\Delta$ Np63 $\alpha$  cell line was cultured in the same medium containing blasticidin (15  $\mu$ g/ml) and hygromycin (160  $\mu$ g/ml).

The D. Mel-2 cells were cultured in D-SFM medium (Gibco BRL) plus 90ml/l L-glutamine 200mM and 5ml/l penicillin (100U/ml)-streptomycin (100 $\mu$ g/ml) at 28°C, in a nonhumidified, ambient air-regulated incubator.

Human normal keratinocytes (NHEK) were isolated from neonatal foreskin specimens derived from a normal human Caucasian (PromoCell). Cells were maintained in Keratinocyte Growth Medium 2 (PromoCell) supplemented with 0.4% Bovine Pituitary extract, 0.125 ng/ml Epidermal Growth Factor, 5  $\mu$ g/ml Insulin, 0.33  $\mu$ g/ml Hydrocortison, 10  $\mu$ g/ml Transferrin, 0.39  $\mu$ g/ml Epinephrine, 0.15 mM CaCl<sub>2</sub>. UV irradiation consisted in a 40  $\mu$ J/cm<sup>2</sup> light treatment performed in a CL-1000 Ultraviolet Crosslinker (UVP).

**Establishment of T-Rex-293- $\Delta$ Np63 $\alpha$  cells.** The human embryonic kidney Flp-In T-Rex-293 cell line (Invitrogen) was used for generating stable  $\Delta$ Np63 $\alpha$  overexpressing cells. This system allows to generate stable mammalian cell lines exhibiting tetracycline-inducible expression of a cDNA of interest, integrated in single specific locus by a recombinase-mediated reaction. The Flp-In T-Rex-293 cell line contains two stably, independently integrated plasmids: the pcDNA6/TR plasmid, which stably expresses the Tet repressor gene under the control of the CMV promoter and confers blasticidin resistance; the pFRT/lacZeo plasmid, which stably expresses the lacZ-zeocin fusion gene under the control of the SV40 promoter and introduces a single Flp Recombination Target (FRT) site into the genome for the Flp recombinase. The  $\Delta$ Np63 $\alpha$  cDNA was cloned into the expression vector pcDNA5/FRT/TO, which contains a single FRT site for Flp recombinase-mediated integration and in which the expression of a cDNA is regulated by two copies of the tet operator. The Flp-In T-rex 293 cells were cotransfected with the  $\Delta$ Np63 $\alpha$ -pcDNA5/FRT/TO and the pOG44 plasmid, which encodes a site-specific recombinase, according to the manufacturer's protocol. Integration of the  $\Delta$ Np63 $\alpha$ -pcDNA5/FRT/TO construct, conferred hygromycin resistance and zeocin sensitivity to the T-Rex-293 cells. At 48 h after transfection, the cells were split in medium containing hygromycin (160  $\mu$ g/ml) and blasticidin (15  $\mu$ g/ml). The selective medium was replaced every 3-4 days until hygromycin-resistant foci became visible.

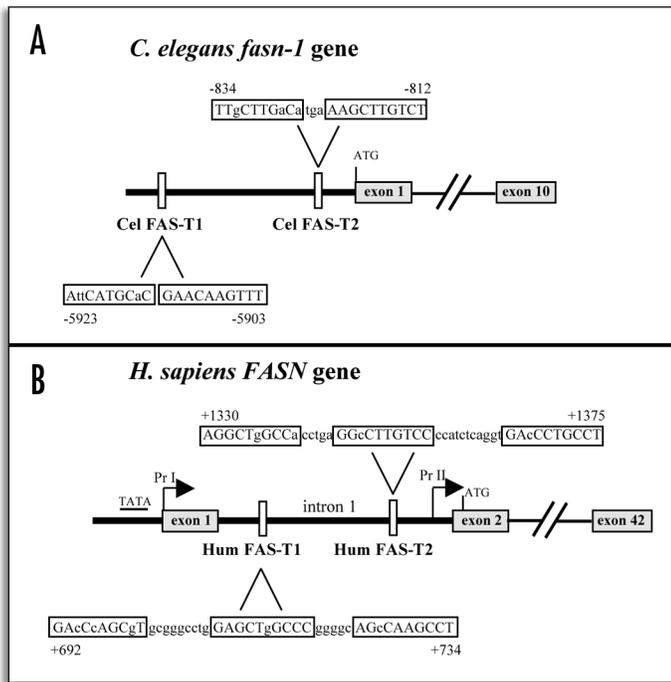


Figure 1. Schematic representation of the genomic region of the *C. elegans fasn-1* and human *FASN* genes, containing the p53 family REs. The decamers are boxed and nucleotides mismatched from the p53 family consensus sequence are in lower case. (A) *C. elegans fasn-1* gene numbering refers to the translation start site. Cel FAS-T1 consists of two decamers, without any spacer and one decamer shows no mismatch. Cel FAS-T2 consists of two decamers spaced by four bases and one decamer shows no mismatch. (B) The human *FASN* gene numbering refers to the transcription start site from the main promoter I. Hum FAS-T1 contains three decamers, with the first spacer of 9 nucleotides and the second spacer of 5 nucleotides. Hum FAS-T2 contains three decamers, with the first spacer of 5 nucleotides and the second spacer of 11 nucleotides. The two *FASN* promoters are indicated by arrows.

Cells were isolated, expanded and characterized for the expression of  $\Delta Np63\alpha$  following tetracycline induction (1  $\mu$ g/ml). The same procedure was followed to generate T-Rex-293 cells overexpressing human p53, Tap73 $\beta$  and Tap63 $\alpha$ .

**C. elegans culture.** Culture and maintenance procedures for *C. elegans* strains were as described.<sup>46</sup> The wild-type Bristol strain N2 and the TJ1 *cep-1(gk138)* mutant strain used in this work were provided by the *Caenorhabditis* Genetics Center, care of T. Stiernagle (University of Minnesota), which is funded by the NIH National Center for Research Resources (NCRR). *cep-1* allele *gk138* was created by the *C. elegans* Reverse Genetics Core Facility at UBC, which is part of the International *C. elegans* Gene Knockout Consortium.

**Construction of recombinant vectors.** The entire cDNA of CEP-1 was amplified from a *C. elegans* embryo Lambda cDNA library (Stratagene) using the Platinum Taq High Fidelity (Invitrogen) and cloned into the pcDNA3 expression vector (Promega) containing the CMV promoter and into the pAc5.1/V5-His expression vector (Invitrogen), containing the *Drosophila* actin 5C promoter for expression of a gene of interest in *Drosophila* cells. The resulting vectors were termed pcDNA3CEP-1wt and pAc5.1CEP-1wt, respectively. The two pAc5.1CEP-1R298H and pAc5.1CEP-1H310N constructs were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions. A 220 bp DNA fragment including Cel FAS-T1 and a 250 bp DNA fragment including Cel FAS-T2 were amplified from *C. elegans* genomic DNA and cloned into the pGL3 basic vector (Promega) digested with KpnI and NheI and with NheI and HindIII, respectively. The resulting

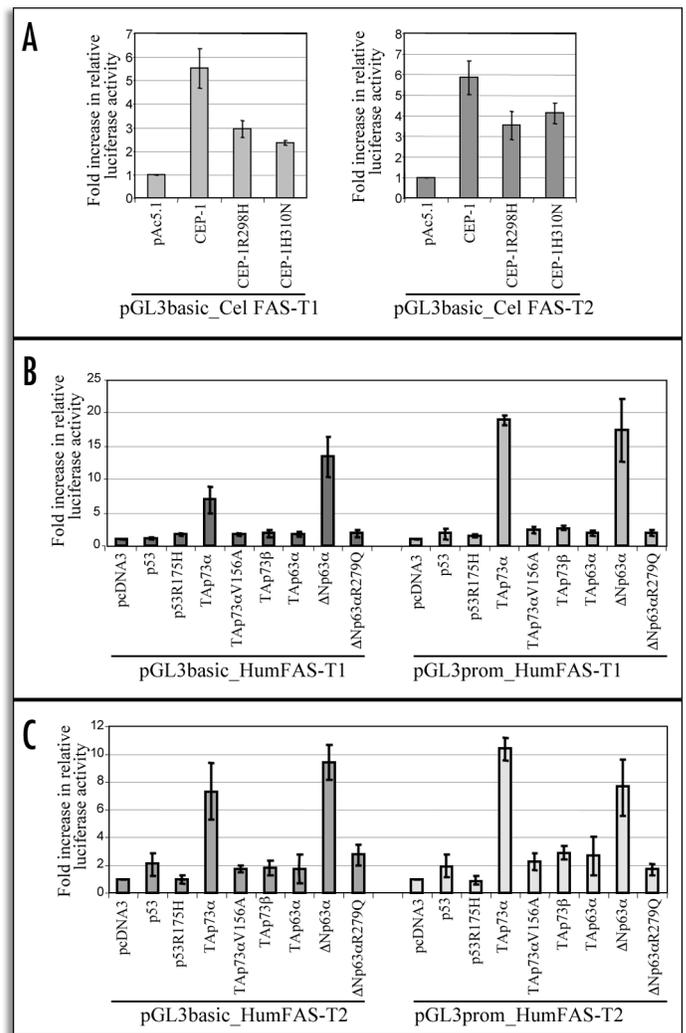


Figure 2. The p53REs in human *FASN* and *C. elegans fasn-1* genes are transcriptionally responsive in luciferase assay. (A) D-mel2 cells were cotransfected with the vector pAc5.1 expressing the wild-type CEP-1 or two CEP-1 mutant versions (CEP-1R298H and CEP-1H310N) and the luciferase reporter vector pGL3 basic containing Cel FAS-T1 (left panel) and Cel FAS-T2 (right panel). The fold increase in relative luciferase activity by CEP-1 was calculated using the empty pAc5.1 vector as control. (B and C) H1299 cells were cotransfected with the pcDNA3 empty vector or expressing various p53 family proteins (p53, Tap73 $\alpha$ , Tap73 $\beta$ , Tap63 $\alpha$  and  $\Delta Np63\alpha$ ), the mutated versions p53R175H, Tap73 $\alpha$ V156A and  $\Delta Np63\alpha$ R279Q and the reporter constructs pGL3basic\_HumFAS-T1 and pGL3prom\_HumFAS-T1 (in B) or pGL3basic\_HumFAS-T2 and pGL3prom\_HumFAS-T2 (in C). The fold increase in relative luciferase activities by the different p53 family proteins was calculated using the empty pcDNA3 vector as control. In (A, B and C), the results represent the average of at least three independent experiments and are shown with their standard deviation.

vectors were designed pGL3\_CelFAS-T1 and pGL3\_CelFAS-T2, respectively. A 300 bp DNA fragment including Hum FAS-T1 and a 395 bp DNA fragment including Hum FAS-T2 were amplified using the "GC Rich PCR System" (Roche) and cloned into the pGL3 basic and pGL3 promoter vectors (Promega) digested respectively, with NheI and HindIII and with NheI and BglII. The resulting vectors were designed pGL3basic\_HumFAS-T1 and pGL3basic\_HumFAS-T2 and pGL3prom\_HumFAS-T1 and pGL3prom\_HumFAS-T2, respectively. The pGL3-HumFAS(-843/+1794), pGL3-HumFAS(+1132/+1794), pGL3-HumFAS(+1574/+1794) and pGL3-HumFAS(+556/+1794) vectors were constructed by cloning the correspondent FAS promoters fragments into the pGL3 basic vector.

**Luciferase assay.** H1299 cells were plated in 35 mm tissue culture dishes ( $1 \times 10^5$  cells/dish) 24 h before transfection. Each well was then cotransfected using FUGENE 6 reagent (Roche), according to the manufacturer's instructions, with either empty pcDNA3 vector or containing various members of the p53 family and their mutated versions (150 ng), the reporter construct (1.5  $\mu$ g) along with *Renilla* pRL-SV40 vector (Promega) (10 ng) for normalization.

The D. Mel-2 cells were plated in 35 mm tissue culture dishes ( $1 \times 10^5$  cells/dish) 24h before transfection. Each well was then cotransfected using FUGENE 6 reagent (Roche) with either empty pAc5.1 vector or pAc5.1 containing CEP-1 wild-type or its mutated versions (500ng), reporter vector (2  $\mu$ g) along with *Renilla* pRL-SV40 vector (100 ng).

Thirty-six hours after transfection, H1299 and D. Mel-2 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 standard deviations.

**Electrophoretic mobility-shift assay.** A 220 bp DNA fragment including Cel FAS-T1 and a 250 bp DNA fragment including Cel FAS-T2 were amplified by PCR from *C. elegans* genomic DNA and labelled with  $\gamma^{32}$ P-dATP at 5' end by T4 Polynucleotide Kinase. The pcDNA3CEP-1wt was in vitro translated using the TNT Quick Coupled Transcription/Translation System (Promega). The in vitro translated CEP-1 protein was incubated with the radiolabeled fragments for 30 min at room temperature in Gel Shift binding 5X buffer (Promega) and with unlabelled specific competitor (100 fold) or unlabelled non-specific competitor (1.75 pmol of AP2 consensus oligo). After incubation, each sample was loaded on a native 4% polyacrylamide gel and electrophoresed in 0.5X TBE buffer. The gels were dried and exposed for autoradiography overnight at  $-80^\circ\text{C}$ .

**Chromatin immunoprecipitation assay.** For ChIP experiments, A2780pcDNA3 and A2780/p73 $\alpha$ .4 cells were cultured in 15 cm culture dishes in presence of G418 (500 $\mu$ g/ml) until they reached a 60–80% of confluency. T-rex 293- $\Delta$ Np63 $\alpha$  cells were cultured in 15 cm culture dishes in presence of blasticidin (15 $\mu$ g/ml) and hygromycin (160  $\mu$ g/ml) until they reached a 60–70% of confluency and then induced with tetracycline (1  $\mu$ g/ml) and harvested at different times (0 h, 6 h, 12 h). Then, proteins and DNA were cross-linked by adding formaldehyde (1% final concentration) directly in the culture medium and incubated for 10 min at  $37^\circ\text{C}$ . Cells were washed with phosphate buffered saline (PBS), scraped on ice, resuspended in hypotonic buffer and passed through a 26-gauge needle. Nuclei were pelleted and resuspended in SDS lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin) and sonicated by 20 sets (pulsed: 20 sec on/20 sec off) at 40 watt to generate DNA fragments about 300–800 bp long. After centrifugation, the supernatant was diluted 10 fold with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin) and 30  $\mu$ l were taken and stored at  $-20^\circ\text{C}$  for the input chromatin sample. The chromatin solution was precleared by incubation for 2 hours at  $4^\circ\text{C}$  with salmon spermDNA/proteinA agarose. The cleared lysates from A2780pcDNA3 and A2780/p73 $\alpha$ .4 were incubated at  $4^\circ\text{C}$  overnight with a mixture of 5  $\mu$ g of anti-p73 antibody (H79 and C20, Santa Cruz) or without antibody; the cleared lysates from T-rex 293- $\Delta$ Np63 $\alpha$  cells were incubated at  $4^\circ\text{C}$  overnight with 5  $\mu$ g of anti-p63 antibody (H137, Santa Cruz) or

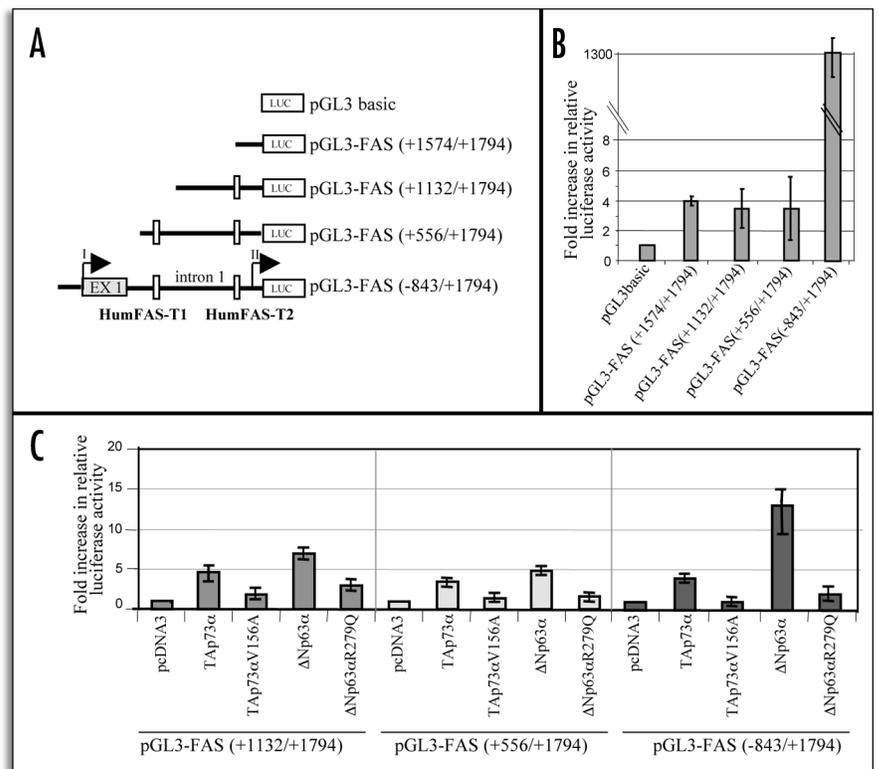


Figure 3. Effects of Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  and of the two p53REs on the activity of the two human FASN gene promoters. (A) Schematic representation of the pGL3 reporter constructs containing different 5' genomic regions of human FASN gene. (B) Basal activity of the two FASN gene promoters analyzed in H1299 cells transfected with the reporter vectors described in (A). (C) Effects of Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  and their mutated versions (Tap73 $\alpha$ V156A and  $\Delta$ Np63 $\alpha$ R279Q) on the basal activity of the two FASN promoters analyzed in H1299 cells cotransfected with the reporter vectors described in (A) and the pcDNA3 vector expressing the wild-type and mutated isoforms. The fold increase in relative luciferase activities was calculated using the empty pcDNA3 vector as control. In (B and C), the results represent the average of at least three independent experiments and are shown with their standard deviations.

without antibody. Immunocomplexes were precipitated with 30 $\mu$ l of salmon spermDNA/proteinA agarose for 30 min at  $4^\circ\text{C}$ . After centrifugation, the beads were washed and the DNA-proteins complexes were eluted with 1% SDS, 0.1 M NaHCO<sub>3</sub>. DNA-protein cross-links were reversed by heating at  $65^\circ\text{C}$  for 4 hours. DNA was phenol-extracted and ethanol-precipitated. DNA fragments were analyzed by PCR using specific primer pairs for Hum FAS-T1, Hum FAS-T2 and Interleukin-10 promoter, used as negative control.

**Semiquantitative RT-PCR analysis.** Total *C. elegans* RNA was prepared by using TRIREAGENT (Sigma), following the manufacturer's procedure either from N2 and *cep-1(gk138)* unsynchronized animals (mixed stages) or eggs. Eggs were collected by hypochlorite treatment of gravid hermaphrodites.

Total RNA was extracted from A2780pcDNA3, A2780/p73 $\alpha$ .4 and T-rex 293- $\Delta$ Np63 $\alpha$  cells using RNeasy spin-column kits (Qiagen) according to the manufacturer's instructions.

cDNAs were synthesized from 1.5  $\mu$ g of total mRNAs using the Superscript First-Strand System (Invitrogen). 5% of the cDNAs obtained were used as template for the amplification with the gene specific primers set. The sequences of all primers are available upon request. PCR was run in the exponential region to allow semi-quantitative comparisons among cDNAs developed from identical reactions.

**Western blot analysis.** For protein analysis, cells were washed once in cold PBS and then lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholat, 0.1% SDS, protease inhibitors cocktail tablets (Roche)) for 1 h on ice. Lysates were then cleared by centrifugation at 13,000 rpm for 10 min at  $4^\circ\text{C}$ , aliquoted and stored at  $-20^\circ\text{C}$ . 20  $\mu$ g of the total proteins, in 2X SDS-PAGE sample buffer, were

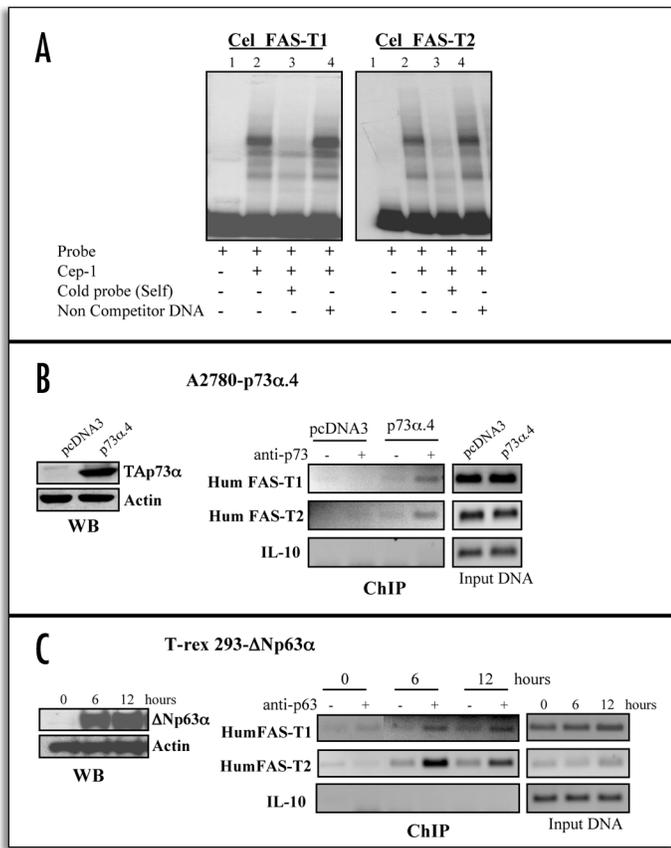


Figure 4. CEP-1, TAp73α and ΔNp63α bind the p53REs present in the homologous FAS genes. (A) Electrophoresis mobility shift assay using the in vitro translated *C. elegans* CEP-1 protein and the Cel FAS-T1 and Cel FAS-T2 containing fragments, labelled with  $\gamma^{32}$ P-dATP at their 5' ends. For competition, 100-fold excess of cold specific (self) and unspecific (non competitor) probes were added, in lanes 3 and 4, respectively. (B) Chromatin immunoprecipitation assay (ChIP) performed on human A2780 cells stably transfected by pcDNA3 (pcDNA3 clone) and by TAp73α (α.4 clone) using an anti-p73 antibody. Immunoprecipitated DNA was amplified using specific primer pairs for Hum FAS-T1 and Hum FAS-T2 and for the unrelated interleukin-10 promoter. A2780 clones were controlled for the TAp73α expression by Western blot (WB, left panel). (C) Chromatin immunoprecipitation assay (ChIP) performed on human T-rex 293 cells, stably transfected with ΔNp63α, at 6 and 12 hours after induction of ΔNp63α expression, using an anti-p63 antibody. Immunoprecipitated DNA was amplified using specific primer pairs for Hum FAS-T1 and Hum FAS-T2 and for the unrelated interleukin-10 promoter. T-rex 293-ΔNp63α cells were controlled for the ΔNp63α expression at 6 and 12 hours after induction by Western blot (WB, left panel).

heated at 95°C for 2 min and separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (PROTRANR BA85, Schleicher & Schuell). Membranes were then blocked for 1 h in a PBS solution containing 3% non fat milk powder and 0.1% Tween-20 and then probed for 60 min at room temperature with the following primary antibodies: Abp73 (IMGENEX), Abp63 (4A4, Santa Cruz), Ab-actin (Ab-1, Oncogene) in 3% milk, 0.1% Tween-20 PBS. After washing, membranes were incubated with horseradish peroxidase-linked secondary antibody in 3% milk, 0.1% Tween-20 PBS. After two 10 min washes in 0.1% Tween-20 PBS and one 10 min wash in PBS proteins were visualized using ECL Western Blotting detection reagents (Amersham Pharmacia biotech).

**RNA interference assay.** Silencing of p73 and p63 genes was performed by using the SMARTpool Reagent technology (Dharmacon) which combines four selected designed siRNAs into a single pool, thus enhancing the reduction of the target mRNA levels. siCONTROL Nontargeting siRNA pool

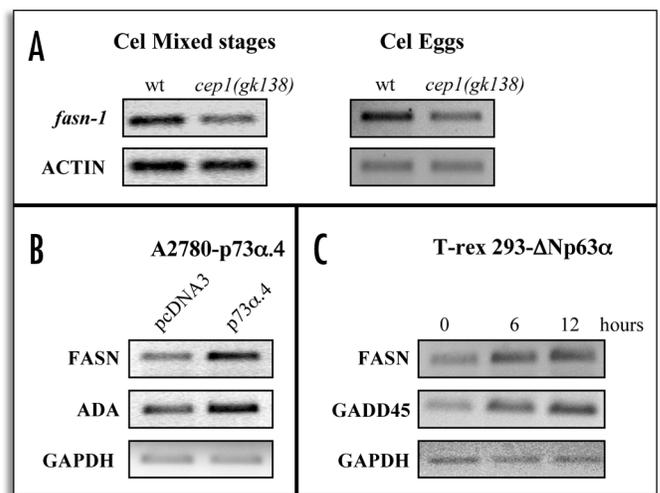


Figure 5. *C. elegans* CEP-1 and human TAp73α and ΔNp63α regulate FAS gene expression. (A) *C. elegans* *fasn-1* gene expression in mixed stages of worms (left panel) and in eggs (right panel) from wild-type and mutant *cep-1(gk138)* strains determined by RT-PCR analyses. Expression of actin was used as quantity control. (B) Human *FASN* gene expression in A2780 cells, stably transfected by pcDNA3 and by TAp73α (clone α.4) determined by RT-PCR analyses. ADA and GAPDH mRNA levels were analyzed as control. (C) Human *FASN* gene expression in the T-rex 293-ΔNp63α cells at 6 and 12 hours after induction of ΔNp63α determined by RT-PCR analyses. GADD45 and GAPDH mRNA levels were analyzed as controls.

(Dharmacon) was used as RNAi control.  $2 \times 10^6$  MCF-7 and HaCat cells were plated in 10 cm culture dishes without antibiotics 24 h before transfection (about 60–80% confluency). 160 pmol of control-siRNA, p73-siRNA or p63-siRNA were diluted in 100 μl D-MEM without serum plus 16 μl of PlusTM Reagent (Invitrogen) and incubated at room temperature for 15 min. 6 μl of LipofectamineTM Reagent (Invitrogen) were diluted in 100 μl D-MEM without serum in a second tube. Precomplexed DNA and diluted LipofectamineTM Reagent were mixed and incubated for 15 min at room temperature and subsequently added to the cell cultures. Then the cells were cultured in DMEM plus serum without antibiotics for 24 h. After, fresh complete medium was added and the cells were cultured for additional 24 h.

## RESULTS

**Identification of p53 family responsive elements in *C. elegans* and human homologous genes coding for the fatty acid synthase.** We used PatSearch and DNafan algorithms<sup>28,29</sup> to automatically search in silico for p53 family responsive elements (p53REs) in intronic and promoter regions of the human genes present in the EMBL database. The p53RE consists of at least a double decamer, 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', with a maximum 3 nucleotide substitutions and spaced by 0–13 nucleotides.<sup>32,33</sup> Among the human genes containing the p53REs, we searched for p53REs in their *C. elegans* homologous genes. We chose for this study the human and worm homologous genes (*FASN* and *fasn-1*, respectively) coding for the fatty acid synthase (FAS). Despite the difference in the gene organization, the human *FASN* and the *C. elegans* *fasn-1* (F32H2.5) genes code for a protein of about 250 kDa, well conserved in the course of evolution (45.72% of identity). In the *C. elegans* *fasn-1* locus, we identified two putative p53 REs, upstream the coding region, which we called Cel FAS-T1 and Cel FAS-T2 (Fig. 1A). Also in the human *FASN* gene we identified two putative p53 family REs, both localized in intron 1, which we called Hum FAS-T1 and Hum FAS-T2 (Fig. 1B). In the human *FASN* gene, two promoters have been identified: the main promoter (promoter I), which contains TATA and CAAT boxes upstream the untranslated exon 1 and a second, weaker TATA-less promoter (promoter II) mapped in intron 1, upstream of the translation initiation site in exon 2 (Fig. 1B).<sup>34</sup>

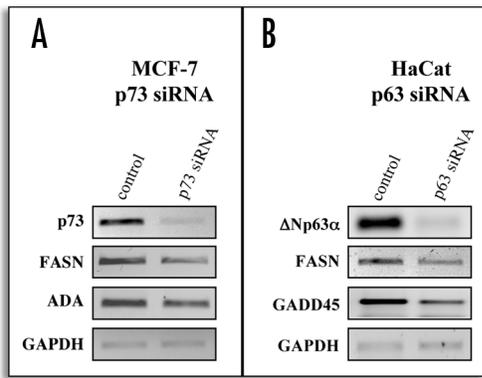


Figure 6. Disruption of human p73 and  $\Delta$ Np63 $\alpha$  expression leads to a reduction of *FASN* gene expression. (A) RT-PCR analyses of *FASN* mRNA levels in MCF-7 cells transfected with p73-siRNA and nontargeting siRNA (control). ADA and GAPDH mRNA levels were analyzed as controls. (B) RT-PCR analyses of *FASN* mRNA levels in HaCat cells transfected with p63-siRNA and nontargeting siRNA (control). GADD45 and GAPDH mRNA levels were analyzed as controls.

In order to verify whether *C. elegans* CEP-1 was able to directly activate transcription from reporter plasmids containing Cel FAS-T1 and Cel FAS-T2, we started our study by performing luciferase assays. Previous data reported that CEP-1 is able to activate transcription, although no CEP-1 direct target was identified, but only reporter plasmids containing human p53 REs were analyzed.<sup>23,24</sup> To this purpose, a fragment of 220 bp containing Cel FAS-T1 and a fragment of 250 bp containing Cel FAS-T2 were cloned in the promoter-less pGL3 basic luciferase vector. The resulting constructs were designed pGL3basic\_CelFAS-T1 and pGL3basic\_CelFAS-T2, respectively. D-mel 2 cells, derived from *D. melanogaster* embryos, were transiently cotransfected with these reporter constructs, with either an empty pAc5.1 vector as control or a pAc5.1 vector containing the wild-type CEP-1 or two mutant versions (CEP-1R298H and CEP-1H310N). We chose to mutagenize two CEP-1 aminoacids (R298 and H310) which correspond to conserved residues in human p53 central domain, crucial for the DNA binding (R175 and R179). As shown in Figure 2A, the luciferase activities of both pGL3basic\_CelFAS-T1 and pGL3basic\_CelFAS-T2 were increased of about 6 fold when cotransfected with the wild-type CEP-1, compared to the control, while a reduction was observed with the two mutated forms of CEP-1 (CEP-1R298H and CEP-1H310N). Interestingly, we do not observe a complete decrease of the luciferase activities of the two constructs with these two mutated forms of CEP-1, suggesting that other still undefined residues of the central domain are important for an efficient binding of CEP-1 to DNA. Cel FAS-T1 and Cel FAS-T2 fragments were also cloned in the pGL3promoter vector, containing the minimal SV40 promoter, but they showed no enhancer activity for CEP-1 (data not shown).

In human, luciferase assays were performed in p53-null H1299 cells (human nonsmall-cell lung carcinoma) to determine whether the human p53 family members were able to transactivate the human *FASN* gene expression via the two putative p53REs (Hum FAS-T1 and Hum FAS-T2). A fragment of 300 bp containing the Hum FAS-T1 and a fragment of 395 bp containing the Hum FAS-T2 were cloned either in the promoter-less vector, pGL3 basic (pGL3basic\_HumFAS-T1 and pGL3basic\_HumFAS-T2) or in the pGL3 promoter vector (pGL3prom\_HumFAS-T1 and pGL3prom\_HumFAS-T2) to evaluate the possible promoter and enhancer activity of the two REs. The resulting constructs were cotransfected into the H1299 cells either with a pcDNA3 empty vector as control or with the pcDNA3 vector expressing various members of the human p53 family (p53, Tap73 $\alpha$ , Tap73 $\beta$ , Tap63 $\alpha$ ,  $\Delta$ Np63 $\alpha$ ) and the mutated versions of p53, Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  (p53R175H, Tap73 $\alpha$ V156A,  $\Delta$ Np63 $\alpha$ R279Q) (Figs. 2B and C). These mutations are known to annul the capacity of the wild-type proteins to bind the p53REs. Interestingly, we found that both

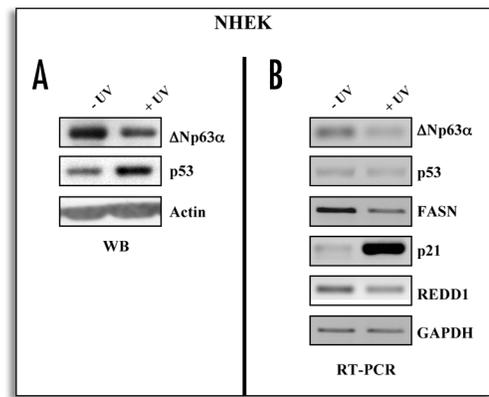


Figure 7. Effect of UV irradiation on human  $\Delta$ Np63 $\alpha$  and *FASN* gene expression in human keratinocytes. NHEK cells were exposed to 40 J/cm<sup>2</sup> UV light and proteins were extracted after 12 hours and analyzed by (A) western blot for  $\Delta$ Np63 $\alpha$  and p53 protein levels and by (B) RT-PCR analyses for  $\Delta$ Np63 $\alpha$ , p53 and *FASN* mRNA levels. p21, REDD1 and GAPDH mRNA were analyzed as controls.

Hum FAS-T1 and Hum FAS-T2 have a promoter and enhancer activity only for Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$ , but not for p53, Tap63 $\alpha$  and Tap73 $\beta$ . Indeed, the luciferase activities of pGL3basic\_Hum-T1 and pGL3prom\_Hum-T1 were increased of about 7 and 20 fold with respect to the control, when cotransfected with Tap73 $\alpha$  and of about 12 and 18 fold when cotransfected with  $\Delta$ Np63 $\alpha$  (Fig. 2B). Similarly, the luciferase activities of pGL3basic\_Hum-T2 and pGL3prom\_Hum-T2 were increased of about 7 and 10 fold when cotransfected with Tap73 $\alpha$  and of about 9 and 8 fold when cotransfected with  $\Delta$ Np63 $\alpha$ , compared to the control (Fig. 2C). The activation was dependent on Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  since the mutated version of Tap73 $\alpha$  (Tap73 $\alpha$ V156A) and of  $\Delta$ Np63 $\alpha$  ( $\Delta$ Np63 $\alpha$  R279Q) were not able to induce a significant transcription from the same four reporter vectors (Figs. 2B and C). p53, p53R175H, Tap73 $\beta$  and Tap63 $\alpha$  produced no significant increase in the luciferase activities of all four reporter vectors (Fig. 2B and C).

Having demonstrated that Hum FAS-T1 and Hum FAS-T2 responded per se to Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  we analyzed the effects of the two p53REs on the activity of the two human *FASN* promoters. We prepared four pGL3 reporter constructs containing the two *FASN* promoters: pGL3-HumFAS(-843/+1794), containing promoter I and complete intron 1; pGL3-HumFAS(+556/+1794), containing a portion of intron 1, which includes promoter II and both HumFAS-T1 and HumFAS-T2; pGL3-HumFAS(+1132/+1794) containing a portion of intron 1, which includes promoter II and HumFAS-T2; pGL3-HumFAS(+1574/+1794) containing a portion of intron 1, corresponding to promoter II (Fig. 3A). First, in H1299 cells, we analyzed the basal activity of the two *FASN* gene promoters. We found that promoter II has a lower activity compared to promoter I (about 4 fold increase in luciferase expression of promoter II compared to 1300 fold increase of promoter I) (Fig. 3B), confirming previously reported data.<sup>34</sup> Interestingly, when we cotransfected pcDNA3 vector expressing Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  with the pGL3-HumFAS(+1132/+1794) the basal activity of the weak promoter II was increased of about 5 and 8 fold, respectively, compared to the control and when we cotransfected pcDNA3 vector expressing Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  with the pGL3-HumFAS(+556/+1794) an increase of about 3 and 5 fold respectively, compared to the control was observed (Fig. 3C). On the contrary, no relevant increase of the basal activity of the promoter II in the two constructs was produced by the mutated forms Tap73 $\alpha$ V156A and  $\Delta$ Np63 $\alpha$  R279Q. The basal activity of the strong promoter I present in the pGL3-HumFAS(-843/+1794) construct was also increased of about 4 and 13 fold by Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$ , respectively but not by their mutated versions (Fig. 3C). For both promoters, no relevant increase of the basal activity was

produced by p53, Tap63 $\alpha$  and Tap73 $\beta$  (data not shown). On the whole, the reporter assays suggest that CEP-1 is involved in the transcription of the *fasn-1* gene in *C. elegans* and that in human only Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  are involved in the transcriptional activity of the *FASN* gene. Moreover, Hum FAS-T1 and Hum FAS-T2 enhance the basal transcriptional activity of the two *FASN* promoters through the interaction with Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$ .

**CEP-1, Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  are able to bind the p53REs present in the homologous FAS genes.** In order to determine whether CEP-1 directly interacts with the two putative *fasn-1* REs (Cel FAS-T1 and Cel FAS-T2), we performed gel mobility-shift assays, using the in vitro translated CEP-1 protein and fragments containing Cel FAS-T1 and Cel FAS-T2 as labeled probes. As shown in Figure 4A, the addition of CEP-1 produced a shifted band for both Cel FAS-T1 and Cel FAS-T2 p53REs. The specificity of the binding was supported by the addition of an excess (100 fold) of unlabelled specific competitor DNA which reduces the complex formation and by the addition of an unlabelled nonspecific competitor which does not modify the shifted band. These results suggest that CEP-1 has the potential to form a protein/DNA complex with both Cel FAS-T1 and Cel FAS-T2.

In human, since the luciferase experiments indicated that, among the p53 family members, only Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  were able to interact with Hum FAS-T1 and Hum FAS-T2 sites, we performed chromatin immunoprecipitation (ChIP) assay, in order to verify the in vivo binding of these two isoforms to these sites. For studying the Tap73 $\alpha$  binding, we used the A2780/p73 $\alpha$ .4 clone, obtained by stably transfecting Tap73 $\alpha$  in the human A2780 ovarian cancer cells.<sup>35</sup> The A2780/p73 $\alpha$ .4 clone has a level of Tap73 $\alpha$  protein about 10 times higher than the clone obtained transfecting the empty vector pcDNA3 (A2780/pcDNA3) (Fig. 4B, left panel). Cross-linked chromatin from A2780/p73 $\alpha$ .4 and A2780/pcDNA3 clones were immunoprecipitated with an anti-p73 antibody and without antibody as negative control. As shown in Figure 4B, right panel, the amplification of a 180bp DNA fragment containing Hum FAS-T1 and of a 170bp DNA fragment containing Hum FAS-T2 from the immunoprecipitated DNA-protein complexes, demonstrated the direct in vivo binding of Tap73 $\alpha$  to both Hum FAS-T1 and Hum FAS-T2, in the A2780/p73 $\alpha$ .4 clone.

For the analysis of the  $\Delta$ Np63 $\alpha$  binding, we stably transfected the human 293 embryonic kidney cells with  $\Delta$ Np63 $\alpha$  by using the human Flp-in 293 T-rex<sup>TM</sup> system, in which the expression of a cDNA of interest is induced by tetracycline. The 293-T-rex- $\Delta$ Np63 $\alpha$  cells were harvested at 6 and 12 hours after tetracycline-induction of  $\Delta$ Np63 $\alpha$  expression and characterized by western blot analyses (Fig. 4C, left panel). Flow cytometric analyses showed that  $\Delta$ Np63 $\alpha$  overexpression did not affect the cell cycle phases distribution of the 293-T-rex cells (data not shown). Cross-linked chromatin from the T-rex-293- $\Delta$ Np63 $\alpha$  cells, at 0 h, 6 h and 12 h after tetracycline induction, was immunoprecipitated with an anti-p63 antibody and without antibody as negative control. As shown in Figure 4C, right panel, the amplification of a 180bp DNA fragment containing Hum FAS-T1 and of a 170bp DNA fragment containing Hum FAS-T2 from the immunoprecipitated DNA-protein complexes demonstrated that  $\Delta$ Np63 $\alpha$  is able to bind both Hum FAS-T1 and Hum FAS-T2 p53REs, in vivo.

For all ChIP experiments, specific primers for the amplification of the interleukin-10 promoter (IL-10), which does not contain any p53 family RE, were used as negative control, and indeed Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  were not recruited to this promoter (Fig. 4B and C, right panels). Taken together, these results demonstrate that human Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  are able to directly bind in vivo both Hum FAS-T1 and Hum FAS-T2 p53REs.

**FAS gene expression is regulated by CEP-1 in *C. elegans* and by Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  in human cells.** As the luciferase and binding assays provided indications that the worm CEP-1 and the human Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  can activate their respective homologous FAS genes, we tested whether the endogenous FAS gene expression was regulated by these proteins.

To study the *C. elegans fasn-1* expression, total mRNA was extracted from mixed stages of worms and from eggs isolated from gravid hermaphrodites belonging to a *C. elegans* wild-type strain and to a mutant strain, homozygous for the deletion allele *cep-1(gk138)* and analyzed by semi-quantitative RT-PCR assay. We found that *fasn-1* mRNA levels were

reduced in the *cep-1(gk138)* strain compared to the wild-type strain, either in the mixed stages or in the eggs (Fig. 5A). These results suggest that CEP-1 supports the transcriptional activity of the *fasn-1* gene. Therefore, this is the first CEP-1 direct target gene identified so far.

To analyze the human *FASN* gene expression, we first tested the effect of the ectopic expression of Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  on *FASN* endogenous mRNA levels. To investigate the effect of Tap73 $\alpha$  overexpression, total mRNA was isolated from the A2780/p73 $\alpha$ .4 and A2780/pcDNA3 clones and analyzed by semi-quantitative RT-PCR analyses. As shown in Figure 5B, *FASN* mRNA levels increased in A2780/p73 $\alpha$ .4 cells, compared to the control clone A2780/pcDNA3, suggesting that the ectopic expression of Tap73 $\alpha$  markedly induced *FASN* gene expression. We analyzed ADA mRNA levels as positive control, since we had previously demonstrated that ADA is a p73 and not a p53 target gene.<sup>36</sup>

To investigate the effect of  $\Delta$ Np63 $\alpha$  overexpression on *FASN* transcription, total mRNA was isolated from the 293-T-rex- $\Delta$ Np63 $\alpha$  cells at 0h, 6h and 12h after  $\Delta$ Np63 $\alpha$  tetracycline- induction and analyzed for *FASN* expression by semi-quantitative RT-PCR analyses. As shown in Figure 5C, at 6h and 12h *FASN* mRNA levels increased, with respect to the non-induced sample. As positive control, we analyzed the levels of GADD45 mRNA, whose expression was demonstrated to be activated by  $\Delta$ Np63 $\alpha$ .<sup>8</sup>

We also analyzed the effects of the ectopic expression of p53, Tap73 $\beta$  and Tap63 $\alpha$  on *FASN* expression, by stably transfecting the 293-T-rex cells with these proteins. We did not observe any modification of the *FASN* mRNA levels in these cells at 6 and 12 hours after induction compared to the non induced sample (data not shown).

Subsequently, to further confirm the above-described results, we used the RNA interference approach to silence p73 and  $\Delta$ Np63 expression and analyze the effect on *FASN* mRNA levels. With this aim, breast cancer MCF-7 cells, which express high levels of p73,<sup>37</sup> were transfected with siRNA-targeting p73 mRNA, whereas HaCat keratinocyte cells, which express high levels of  $\Delta$ Np63 $\alpha$  isoform,<sup>38</sup> were transfected with siRNA-targeting p63 mRNA. To control the specificity of RNAi, both cell lines were transfected with a nontargeting siRNA pool. The silencing of p73 in MCF-7 cells and of  $\Delta$ Np63 in HaCat cells produced a reduction of p73 and of  $\Delta$ Np63 $\alpha$ , respectively, at mRNA (Fig. 6A and B) and protein levels (data not shown), after 48h. In particular, in MCF-7 cells, the reduction of p73 was associated with a reduction of *FASN* mRNA levels, as well as of ADA mRNA levels, used as control (Fig. 6A); in HaCat cells, the decrease of  $\Delta$ Np63 was correlated to a decrease of *FASN* mRNA levels, as well as of GADD45 mRNA levels, used as control (Fig. 6B).

On the whole, our results demonstrate that, in human cells, Tap73 $\alpha$  and  $\Delta$ Np63 $\alpha$  overexpression has positive effects on endogenous *FASN* transcription while the silencing of the two proteins reduces *FASN* mRNA levels.

**UV irradiation decreases  $\Delta$ Np63 $\alpha$  and FASN expression in normal human keratinocytes.**  $\Delta$ Np63 $\alpha$  is the principal p63 splice variant expressed in the basal layer of the mature epidermis, involved in maintaining the proliferative potential of basal cells.<sup>39</sup> It was reported that in normal keratinocytes after ultraviolet irradiation,  $\Delta$ Np63 $\alpha$  decreases dramatically at both protein and mRNA levels, while p53 increases only at protein level.<sup>40</sup> On the contrary, p73 protein is not induced after UV irradiation in several cell types.<sup>41</sup> Since the three proteins show different responses, we considered this physiological stress to be an ideal condition to investigate the effect of the  $\Delta$ Np63 $\alpha$  downregulation on *FASN* gene expression. With this aim, normal human keratinocytes (NHEK) were exposed to 40 J/m<sup>2</sup> UV light and proteins and RNA were extracted after 12 hours. Western blot and RT-PCR analyses confirmed that the UV treatment produced a decrease of  $\Delta$ Np63 $\alpha$  at protein and mRNA levels and an increase of p53 protein levels (Fig. 7A and B). Cytofluorimetric analyses indicated that, after the UV treatment, cells were arrested in G<sub>1</sub> phase (data not shown). This arrest of cell proliferation was mediated by p53, as RT-PCR analyses showed the increase of the cyclin dependent kinase inhibitor, p21, a specific p53 target gene involved in G<sub>1</sub> cell cycle arrest (Fig. 7B). Interestingly, the RT-PCR analyses demonstrated that the decrease of  $\Delta$ Np63 $\alpha$  was correlated with a decrease of *FASN* mRNA levels, as well as of REDD1 mRNAs levels, used as control.

Taken together, these results demonstrate that in normal keratinocytes, arrested in G<sub>1</sub> following UV irradiation, the decrease of  $\Delta$ Np63 $\alpha$  is consistent with a decrease of *FASN* gene expression, thus suggesting that *FASN* is a specific  $\Delta$ Np63 $\alpha$  target gene during cellular proliferation.

## DISCUSSION

Soon after the identification of p73 and p63 as homologues of p53, the three proteins seemed to play overlapping functions, raising the question of why three copies of functionally similar genes should have been maintained during the evolution. However, rapidly, significant functional differences among the three proteins emerged, suggesting that, while p53 is crucial for preservation of tumorigenesis, p73 and p63 are mostly involved in development and differentiation. More recently, the discovery that the three p53 family members in vertebrates correspond to a single homolog in invertebrates has raised the problem of understanding the functional roles of the family ancestor and of determining whether these functions have been retained by all vertebrate members or spread out among them. The identification of the specific target genes conserved throughout the evolution is crucial to understand the specific biological functions the p53 gene family members acquire in the regulation of cellular pathways along the evolution.

With the aim to provide an insight in understanding the evolution of the p53 family members functional roles, we performed an in silico comparative search of p53REs in the genome of two model organisms, the *C. elegans* for invertebrates and *H. sapiens* for vertebrates, in order to identify evolutionary conserved target genes of the p53 family. We identified p53REs in *C. elegans* and human homologous genes (*fasn-1* and *FASN* genes, respectively), encoding for the fatty acid synthase (FAS) (Fig. 1). Since FAS is a crucial metabolic enzyme, involved in biogenesis of cellular membranes in rapidly dividing cells,<sup>30</sup> this gene appeared to be a good candidate as target of the p53 family members. Our data demonstrate that *C. elegans fasn-1* is a direct target gene of CEP-1, the worm p53 homolog, and that the human *FASN* gene is only a target of TAp73 $\alpha$  and  $\Delta$ Np63 $\alpha$ , and not of p53, TAp73 $\beta$  and TAp63 $\alpha$ .

We show that the two *fasn-1* p53REs, localized upstream the coding region, have a promoter activity for CEP-1 in luciferase assay (Fig. 2A) and EMSA experiments (Fig. 4A) demonstrate that CEP-1 is able to bind the two p53REs. In the luciferase assays, we did not observe a complete reduction of the reporter constructs activities by cotransfecting the two mutated versions of CEP-1 protein (CEP-1R298H and CEP-1H310N), both carrying an aminoacidic substitution in one residue which is conserved in CEP-1 and human p53 central domains (Fig. 2A). We interpret these data considering that relevant differences exist between the three-dimensional structure of CEP-1 and the human p53 DNA binding domain.<sup>42</sup> These results open to further investigation on the efficiency of other residues of the CEP-1 central domain in the binding to the REs.

Moreover, we demonstrate that, in vivo, *fasn-1* expression seems to be modulated by CEP-1 since a decrease of *fasn-1* mRNA levels were observed in the worms homozygous for the deletion allele *cep-1(gk138)* both in mixed stages of worms and in eggs isolated from gravid hermaphrodites (Fig. 5A). In particular, the expression of *fasn-1* mRNA is higher and its modulation appears more evident in eggs, a rapidly proliferation and differentiation worm stage. We did not observe a complete decrease of *fasn-1* expression in the *cep-1(gk138)* worms, with respect to the wild-type strain, suggesting that *fasn-1* expression is regulated also by other transcriptional factors. Moreover, FAS essential role is supported by previous data which

demonstrated that in *C. elegans*, the silencing of *fasn-1* expression produces embryonic lethality in genome wide RNAi screening.<sup>43</sup>

It is noteworthy that *fasn-1* is the first direct CEP-1 target gene in *C. elegans*, identified so far. Previous studies demonstrated the transactivation activity of CEP-1 using only reporter plasmids containing the human p53 RE.<sup>23,24</sup> The other important aspect that arises from this study concerns the functional roles of CEP-1. Until now, it was reported that CEP-1 is involved in germ line apoptosis induced by genotoxic treatments in adult worms, but not in the physiological apoptosis occurring during worm development.<sup>23,24</sup> Our results, showing that *fasn-1* is a CEP-1 target gene, suggest a novel additional CEP-1 functional role in the regulation of the cellular proliferation and development. It will be interesting to carry out further studies to investigate CEP-1 involvement during the worm development and in the adult state.

For the human *FASN* gene, reporter assays revealed that the two human *FASN* REs show either striking promoter and transcription enhancer activity for TAp73 $\alpha$  and  $\Delta$ Np63 $\alpha$ , but not for p53, TAp73 $\beta$  and TAp63 $\alpha$  (Fig. 2B and C). The presence in intron 1 of the two REs, Hum FAS-T1 and Hum FAS-T2, may explain their promoter and enhancer activities, with respect to the two *FASN* promoters, the main one localized upstream the untranslated exon I, and the second one mapped in intron I. We also showed that TAp73 $\alpha$  and  $\Delta$ Np63 $\alpha$  but not p53, TAp73 $\beta$  and TAp63 $\alpha$ , increase the basal activity of the two *FASN* promoters when the two p53 family REs are present within their genomic context (Fig. 3). Chromatin immunoprecipitation assays confirmed the specific interaction of TAp73 $\alpha$  e  $\Delta$ Np63 $\alpha$  in vivo with the two *FASN* REs (Fig. 4B and C). Moreover, we presented data which demonstrate an increase of *FASN* mRNA levels when TAp73 $\alpha$  e  $\Delta$ Np63 $\alpha$  are ectopically overexpressed (Fig. 5B and C) and a decrease of the *FASN* mRNA levels when TAp73 $\alpha$  e  $\Delta$ Np63 $\alpha$  are silenced by RNAi (Fig. 6A and B), thus supporting the idea that *FASN* gene expression is regulated by these two proteins.

Our data showing that the human *FASN* is a new target gene of TAp73 $\alpha$  and  $\Delta$ Np63 $\alpha$ , proteins involved in development and cellular proliferation, is in accordance to the key FAS role in the biogenesis of cellular membranes in rapidly proliferating cells and during embryonic development.

TAp73 activity has been demonstrated to be regulated along the cell cycle, with an accumulation of the TAp73 protein in S phase cells.<sup>44</sup> During S phase, the membrane phospholipids content doubles in preparation for cell division.<sup>45</sup> Therefore, *FASN* may represent one of the unknown S phase specific target genes regulated by p73. Furthermore, several lines of evidence showed that TAp73 may play a role in the nervous and immune system development.<sup>7</sup> It would be interesting to investigate on the physiological conditions in which FAS expression is modulated by TAp73 $\alpha$ .

With regards to  $\Delta$ Np63 $\alpha$ , several studies reported that this is the principal p63 splice variant expressed in the basal layer of the mature epidermis, where its expression was demonstrated to maintain the proliferative capability of the basal cells.<sup>39</sup> We report data showing that in human keratinocytes, which express high levels of  $\Delta$ Np63 $\alpha$ , *FASN* mRNA levels decrease when  $\Delta$ Np63 $\alpha$  expression is disrupted by RNAi (Fig. 6B). Moreover, we demonstrate, in a physiological context, that *FASN* is a specific  $\Delta$ Np63 $\alpha$  target gene during cellular proliferation, since in normal keratinocytes, arrested in G<sub>1</sub> following UV irradiation, the reduction of  $\Delta$ Np63 $\alpha$  is related to a decrease of *FASN* gene expression (Fig. 7B).

Our comparative study demonstrates that FAS gene can be considered an evolutionary conserved target of the p53 gene family,

in worm and human. The evidence obtained in this work leads us to point out that the ancestor of the p53 family in invertebrates is involved in development and cellular proliferation. In vertebrates, these functions might have been covered by p63 and p73, and later on, p53 may have acquired a new specific role, in tumour suppression. In other words, in the course of evolution, the ancestral functions of the single invertebrate gene may have been spread out among the three genes present in vertebrates and each of them might have acquired a specific role in cell cycle regulation.

This study underlines the relevant importance of the comparative approach in the functional genomics and suggests that the genetic redundancy is non neutral in evolution. Indeed, starting from one ancestor gene, the different products generated by gene duplications and alternative splicing mechanisms, can acquire new and not overlapped functions in the different lineages.

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