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

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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 TAp73α and ΔNp63α, but not p53, TAp73β and TAp63α bind the two p53 REs of the human FASN gene. We show that the ectopic expression of TAp73α and ΔNp63α 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 ΔNp63α 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 having acquired specific roles 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.1,2 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.3,4 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.5 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 ΔNp73, ΔNp63 and Δ133p53, respectively.6,7 The ΔNp63 and ΔNp73 isoforms, contain a second activation domain responsible for the transactivation activity of these proteins.8,9 Moreover, p53 and p73 produce other isoforms lacking the TA domain (the ΔN40 isoform for p53 and Ex2p73, Ex2/3p73 and ΔNp73 for p73) through N-terminal alternative splicing mechanisms.5,10 p53, p73 and p63 undergo alternative splicing mechanisms also at the 3’ end of their primary transcripts, producing different C-terminal isoforms (α, β, γ, for p53, α, β, γ, δ, ε and ζ for p73 and α, β, and γ for p63).4,5,11-13 Several structural and functional differences between p53, p63 and p73 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.14 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, phenomonal, inflammatory and homeostasis defects whereas p63-null mice show striking defects in epithelia development. 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.

A single p53 family homologous gene was described in invertebrates: *Mya arenaria*, *Loligo forbesi*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Entamoeba histolytica*. 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 aminocacidic 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 C-terminal region showing similarity with the SAM domain distinct to 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. 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.

In previous papers we studied the evolutionary origin of the p53 family gene members. 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 isofoms. 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, 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. 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.

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* *fas-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, but not of p53, TAp73β and TAp63α, 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 ΔNp63α 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). A2780pDNA3 and A2780/p73α-4 cell lines were cultured in the Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) containing G418 (500 µg/ml). The T-rex 293-ΔNp63α cell line was cultured in the same medium containing blasticidin (15 µg/ml) and hygromycin (150 µg/ml).

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

Human normal keratinocytes (NHKE) were isolated from neonatal foreskin specimens derived from a normal human Caucasian (PromCell). Cells were maintained in Keratinocyte Growth Medium 2 (PromoCell) supplemented with 0.4% Bovine Pituitary extract, 0.125 ng/ml Epidermal Growth Factor, 5 µg/ml Insulin, 0.33 µg/ml Hydrocortisone, 10 µg/ml Transferrin, 0.39 µg/ml Epinephrine, 0.15 mM CaCl2. UV irradiation consisted in a 40 µJ/cm2 light treatment performed in a CL-1000 Ultra violet Crosslinker (UVP).

**Establishment of T-Rex-293-ΔNp63α cells.** The human embryonic kidney Flp-In T-Rex-293 cell line (Invitrogen) was used for generating stable ΔNp63α 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, and 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 ΔNp63α-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 ΔNp63α-pcDNA5/FRT/TO and the pOG44 plasmid, which encodes a site-specific recombinase, according to the manufacturer’s protocol. Integration of the ΔNp63α-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 µg/ml) and blasticidin (15 µg/ml). The selective medium was replaced every 3-4 days until hygromycin-resistant foci became visible.
Cells were isolated, expanded and characterized for the expression of ∆Np63α following tetracycline induction (1 µg/ml). The same procedure was followed to generate T-Rex-293 cells overexpressing human p53, TAp73β and TAp63α.

C. elegans culture. Culture and maintenance procedures for C. elegans strains were as described. 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 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 BglII. The resulting vectors were designed 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.
Luciferase assay. H1299 cells were plated in 35 mm tissue culture dishes (1 x 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 µg) 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 x 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 µg) 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 γ32P-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°C.

Chromatin immunoprecipitation assay. For ChIP experiments, A2780pcDNA3 and A2780/p73α.4 cells were cultured in 15 cm culture dishes in presence of G418 (500µg/ml) until they reached a 60–80% of confluency. T-rex 293-293-Anp63α cells were cultured in 15 cm culture dishes in presence of blasticidin (15µg/ml) and hygromycin (160 µg/ml) until they reached a 60–70% of confluency and then induced with tetracycline (1 µg/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°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 µg/ml leupeptin, 1 µg/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 µg/ml leupeptin, 1 µg/ml aprotinin) and 30 µl were taken and stored at -20°C for the input chromatin sample. The chromatin solution was preincubated by incubation for 2 hours at 4°C with salmon spermDNA/proteinA agarose. The cleared lysates from A2780pcDNA3 and A2780/p73α.4 were incubated at 4°C overnight with a mixture of 5 µg of anti-p73 antibody (H79 and C20, Santa Cruz) or without antibody; the cleared lysates from T-rex 293-293-Anp63α cells were incubated at 4°C overnight with 5 µg of anti-p63 antibody (H137, Santa Cruz) or without antibody. Immunocomplexes were precipitated with 30µl of salmon spermDNA/proteinA agarose for 30 min at 4°C. After centrifugation, the beads were washed and the DNA-proteins complexes were eluted with 1% SDS, 0.1 M NaHCO3. DNA-protein cross-links were reversed by heating at 65°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.

Semi-quantitative RT-PCR analysis. Total C. elegans RNA was prepared by using TRIReAGENT (Sigma), following the manufacturer’s procedure either from N2 and cep-1(pk138) unsynchronized animals (mixed stages) or eggs. Eggs were collected by hypochlorite treatment of gravid hermaphrodites.

Total RNA was extracted from A2780pcDNA3, A2780/p73α.4 and T-rex 293-293-Anp63α cells using RNeasy spin-column kits (Qiagen) according to the manufacturer’s instructions.

cDNAs were synthesized from 1.5 µg 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 deoxycholate, 0.1% SDS, protease inhibitors cocktail tablets (Roché)) for 1 h on ice. Lysates were then cleaned by centrifugation at 13,000 rpm for 10 min at 4°C, aliquoted and stored at -20°C. 20 µg of the total proteins, in 2X SDS-PAGE sample buffer, were
Silencing of p73 and p63 genes was performed to bind the p53REs present in the FAS promoter. A2780 expression at 6 and 12 hours after induction by Western blot (WB, left panel). (C) Chromatin immunoprecipitation assay (ChIP) performed on human T-rex 293 cells, stably transfected by pcDNA3 and by TAp73α (clone α.4) 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 pcDNA3 and by TAp73α (clone α.4) 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. T-rex 293-ΔNp63α cells were controlled for the ΔNp63α expression at 6 and 12 hours after induction by Western blot (WB, left panel).

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 γ32P-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 B85, 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 horsedessin 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 (Dharmacon) was used as RNAi control. 2 × 106 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 algorithms28,29 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′-PuPuPuPuC(A/T)(T/A)GYpPyPy-3′, with a maximum 3 nucleotide substitutions and spaced by 0–13 nucleotides. 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, in their C. elegans homologous genes, 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).34

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(ek138) 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 controls. (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.
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.\(^{23,24}\) 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 designated 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 amino acids (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 mutant 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 pGL3prom 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 as escape constructs in the reporter-promotless vector, pGL3basic (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, TAp73α, TAp73β, TAp63α, ΔNp63α) and the mutated versions of p53, TAp73α and ΔNp63α (p53R175H, TAp73αV156A, ΔNp63αR279Q) (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 Hum FAS-T1 and Hum FAS-T2 have a promoter and enhancer activity only for TAp73α and ΔNp63α, but not for p53, TAp63α and TAp73β. 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 TAp73α and of about 12 and 18 fold when cotransfected with ΔNp63α (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 TAp73α and of about 9 and 8 fold when cotransfected with ΔNp63α, compared to the control (Fig. 2C). The activation was dependent on TAp73α and ΔNp63α since the mutated version of TAp73α (TAp73αV156A) and of ΔNp63α (ΔNp63αR279Q) were not able to induce a significant transcription from the same four reporter vectors (Figs. 2B and C). p53, p53R175H, TAp73β and TAp63α produced no significant increase in the luciferase activities of all four reporter vectors (Figs. 2B and C).

Having demonstrated that Hum FAS-T1 and Hum FAS-T2 responded per se to TAp73α and ΔNp63α 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. 3C). For both promoters, no relevant increase of the basal activity was produced when cotransfected with TAp73α or ΔNp63α, or with the mutated versions of TAp73α (TAp73αV156A) and ΔNp63α (ΔNp63αR279Q) (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 Hum FAS-T1 and Hum FAS-T2 have a promoter and enhancer activity only for TAp73α and ΔNp63α, but not for p53, TAp63α and TAp73β. 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 TAp73α and of about 12 and 18 fold when cotransfected with ΔNp63α (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 TAp73α and of about 9 and 8 fold when cotransfected with ΔNp63α, compared to the control (Fig. 2C). The activation was dependent on TAp73α and ΔNp63α since the mutated version of TAp73α (TAp73αV156A) and of ΔNp63α (ΔNp63αR279Q) were not able to induce a significant transcription from the same four reporter vectors (Figs. 2B and C). p53, p53R175H, TAp73β and TAp63α produced no significant increase in the luciferase activities of all four reporter vectors (Figs. 2B and C).

Having demonstrated that Hum FAS-T1 and Hum FAS-T2 responded per se to TAp73α and ΔNp63α 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.\(^ {24}\) Interestingly, when we cotransfected pcDNA3 vector expressing TAp73α and ΔNp63α 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 TAp73α and ΔNp63α 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 TAp73αV156A and ΔNp63αR279Q. 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 TAp73α and ΔNp63α, respectively but not by their mutated versions (Fig. 3C). For both promoters, no relevant increase of the basal activity was...
produced by p53, TAp63α and TAp73β (data not shown). On the whole, the reporter assays suggest that CEP-1 is involved in the transcription of the "fas-l" gene in C. elegans and that in human only TAp73α and ΔNp63α 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 TAp73α and ΔNp63α.

CEP-1, TAp73α and ΔNp63α 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 "fas-l" 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 do 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 TAp73α and ΔNp63α 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 TAp73α binding, we used the A2780/p73α.4 clone, obtained by stably transfecting TAp73α in the human A2780 ovarian cancer cells. The A2780/p73α.4 clone has a level of Np63α mRNA levels, as well as of ADA mRNA. We analyzed ADA mRNA levels as positive control, since we had previously demonstrated that ADA is a p73 and not a p53 target gene.

CEP-1, TAp63α and ΔNp63α is induced by tetracycline. The 293-T-rex-Flp-in 293 T-rex TM system, in which the expression of a cDNA of interest is governed by a tetracycline-inducible promoter, can be used to express the target gene of interest in the presence of tetracycline. The expression of TAp73α and ΔNp63α was correlated with a decrease of CEP-1 and ΔNp63α expression and gene. Therefore, this is the first CEP-1 direct target gene identified so far.

To analyze the human FASl gene expression, we first tested the effect of the ectopic expression of TAp73α and ΔNp63α on FASN endogenous mRNA levels. To investigate the effect of TAp73α overexpression, total mRNA was isolated from the A2780/p73α.4 and A2780/pDNA3 clones and analyzed by semi-quantitative RT-PCR analyses. As shown in Figure 5B, FASN mRNA levels increased in A2780/p73α.4 cells, compared to the control clone A2780/pDNA3, suggesting that the ectopic expression of TAp73α markedly induced FASN gene expression. We analyzed ADA mRNA levels as control, since we had previously demonstrated that ADA is a p73 and not a p53 target gene.

To investigate the effect of ΔNp63α overexpression on FASN transcription, total mRNA was isolated from the 293-T-rex-ΔNp63α cells at 0h, 6h and 12h after ΔNp63α 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 ΔNp63α.

We also analyzed the effects of the ectopic expression of p53, TAp73β and TAp63α 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 ΔNp63α expression and analyze the effect on FASN mRNA levels. With this aim, breast cancer MCF-7 cells, which express high levels of p73, were transfected with siRNA-targeting p73 mRNA, whereas HaCat keratinocyte cells, which express high levels of ΔNp63α isoform, were transfected with siRNA-targeting Δp63 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 ΔNp63α in HaCat cells produced a reduction of p73 and of ΔNp63α, 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 FASV mRNA levels, as well as of ADA mRNA levels, used as control (Fig. 6A); in HaCat cells, the decrease of ΔNp63α was correlated to a decrease of FASV mRNA levels, as well as of GADD45 mRNA levels, used as control (Fig. 6B).

On the whole, our results demonstrate that, in human cells, TAp73α and ΔNp63α overexpression has positive effects on endogenous FASN transcription while the silencing of the two proteins reduces FASN mRNA levels.

UV irradiation decreases ΔNp63α and FASN expression in normal human keratinocytes. ΔNp63α is the principal p63 splice variant expressed in the basal layer of the mature epidermis, involved in maintaining the proliferative potential of basal cells. It was reported that in normal keratinocytes after ultraviolet irradiation, ΔNp63α decreases dramatically at both protein and mRNA levels, while p53 increases only at protein level.

On the contrary, p73 protein is not induced after UV irradiation in several cell types. Since the three proteins show different responses, we considered this physiological stress to be an ideal condition to investigate the effect of the ΔNp63α downregulation on FASN gene expression. With this aim, normal human keratinocytes (NHEK) were exposed to 40 J/m2 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 ΔNp63α 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 G1 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 G1 cell cycle arrest (Fig. 7B). Interestingly, the RT-PCR analyses demonstrated that the decrease of ΔNp63α was correlated with a decrease of FASV mRNA levels, as well as of REDD1 mRNA levels, used as control.
Taken together, these results demonstrate that in normal keratinocytes, arrested in G1 following UV irradiation, the decrease of ΔNp63α is consistent with a decrease of FASN gene expression, thus suggesting that FASN is a specific ΔNp63α 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 (*fam-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, this gene appeared to be a good candidate as target of the p53 family members. Our data demonstrate that *C. elegans fam-1* is a direct target gene of CEP-1, the worm p53 homolog, and that the *FASN* gene is only a target of TAp73α and ΔNp63α, and not of p53, TAp73β and TAp63α.

We show that the two *fam-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. 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, *fam-1* expression seems to be modulated by CEP-1 since a decrease of *fam-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 *fam-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 *fam-1* expression in the *cep-1*(gk138) worms, with respect to the wild-type strain, suggesting that *fam-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.

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. 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. 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 TAp73α and ΔNp63α, but not for p53, TAp73β and TAp63α (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 TAp73α and ΔNp63α, but not p53, TAp73β and TAp63α, 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 TAp73α e ΔNp63α in vivo with the two *FASN* REs (Fig. 4B and C). Moreover, we presented data which demonstrate an increase of *FASN* mRNA levels when TAp73α e ΔNp63α are ectopically overexpressed (Fig. 5B and C) and a decrease of the *FASN* mRNA levels when TAp73α e ΔNp63α 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 TAp73α and ΔNp63α, 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. During S phase, the membrane phospholipids content doubles in preparation for cell division. 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. It would be interesting to investigate on the physiological conditions in which FAS expression is modulated by TAp73α.

With regards to ΔNp63α, 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. We report data showing that in human keratinocytes, which express high levels of ΔNp63α, *FASN* mRNA levels decrease when ΔNp63α expression is disrupted by RNAi (Fig. 6B). Moreover, we demonstrate, in a physiological context, that *FASN* is a specific ΔNp63α target gene during cellular proliferation, since in normal keratinocytes, arrested in G1 following UV irradiation, the reduction of ΔNp63α 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.

References