Novel p53 target gene FUCA1 encodes a fucosidase and regulates growth and survival of cancer cells

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The tumor suppressor p53 functions by inducing the transcription of a collection of target genes. We previously attempted to identify p53 target genes by microarray expression and ChIP-sequencing analyses. In this study, we describe a novel p53 target gene, FUCA1, which encodes a fucosidase. Although fucosidase, \( \alpha-L-1 \) (FUCA1) has been reported to be a lysosomal protein, we detected it outside of lysosomes and observed that its activity is highest at physiological pH. As there is a reported association between fucosylation and tumorigenesis, we investigated the potential role of FUCA1 in cancer. We found that overexpression of FUCA1, but not a mutant defective in enzyme activity, suppressed the growth of cancer cells and induced cell death. Furthermore, we showed that FUCA1 reduced fucosylation and activation of epidermal growth factor receptor, and concomitantly suppressed epidermal growth factor signaling pathways. FUCA1 loss-of-function mutations are found in several cancers, its expression is reduced in cancers of the large intestine, and low FUCA1 expression is associated with poorer prognosis in several cancers. These results show that protein defucosylation mediated by FUCA1 is involved in tumor suppression.

The tumor suppressor gene p53 encodes a transcription factor and is the most frequently mutated tumor suppressor gene in human cancer.\(^{1,4}\) It has been called “the guardian of the genome” and it exerts its tumor suppression function by inducing a collection of target genes. The high incidence of p53 mutations in human cancers illustrates its importance in maintaining normal cell proliferation. In order to discover potentially novel, cancer-associated genes, we previously undertook a comprehensive search for p53 target genes, and analyzed several target genes whose functions had been unknown.\(^{5,10}\) This study focuses on one of these newly identified genes, FUCA1.

FUCA1 encodes an \( \alpha-L-1 \) fucosidase that removes terminal \( \alpha-L-1 \) fucose residues present in glycoproteins.\(^{11}\) The function of FUCA1 in human metabolism is well known, due to its involvement in a malignant, genetic disease called fucosidosis, which is caused by mutation of the FUCA1 gene.\(^{12,13}\) Fucosidosis patients have symptoms of neurodegeneration with progressive mental and motor deterioration. These symptoms are caused by a lack of fucosidase activity in cells, which leads to the accumulation of fucosyl-glycopeptides in various tissues. However, the function of FUCA1 in tumorigenesis is not well understood, although there are several studies that indicate a link between fucosylation and tumorigenesis. For example, abnormal fucosylation is known to occur during tumor development, and several well-known tumor markers such as CA19-9, \( \alpha-L-1 \) fetoprotein-L3 fraction, and haptoglobin are fucosylated glycoproteins that are over-represented in tumors.\(^{14,15}\) In addition, a number of signaling proteins, such as EGFR, and the transforming growth factor-\( \beta \)1 receptors, E-cadherin and integrin, are fucosylated, and this modification plays a key role in the regulation of their functions.\(^{16–20}\) Furthermore, there are reports that enhanced protein fucosylation is associated with breast and colorectal cancers.\(^{21,22}\)

Our study shows that FUCA1 functions downstream of p53, and is the first report showing that the p53 pathway can modulate protein glycosylation. We also show that FUCA1 removes fucose
from EGFR and contributes to the repression of EGFR signaling. Furthermore, we show that various cancers carry FUCA1 loss-of-function mutations, that FUCA1 expression is decreased in breast and colorectal cancers, and that low expression of FUCA1 is associated with poorer prognosis in these cancer patients.

Materials and Methods

Cell culture and transfection. Cell culture was carried out as previously described.(60) COS7, 293T, Saos2, HCT116, H1299, T98G, HeLa, HepG2, Huh7, and MRC5 cells were cultured in DMEM supplemented with 10% FBS. H1648 and HCC2935 cells were cultured in RPMI-1640 medium supplemented with 10% FBS. Epidermal growth factor was added at 100 ng/mL. Transient transfection assays were carried out using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA).

Northern blot analysis and microarray expression analysis. RNA was prepared using a RNeasy Midi kit (Qiagen, Hilden, Germany). Northern blot analysis and microarray expression analysis were carried out as previously described.(60) Probes were prepared using a BcaBEST labeling kit (Takara Bio, Shiga, Japan), and purified using a Probe Quant G-50 MicroColumn (Amersham, Little Chalfont, UK) following the manufacturer’s instructions. Total RNA (0.2–1.0 μg) was used for RT. Reverse-transcribed cDNAs were subjected to real-time PCR, which was carried out with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). For the detection of FUCA1, PHLDA3, and GAPDH, custom-designed TagMan Dual-labeled Probes from Applied Biosystems (Foster City, CA, USA) (FUCA1: Hs00609173_m1) or from Sigma-Aldrich (St. Louis, MO, USA) (PHLDA3 and GAPDH) were used. Data are shown as the mean fold expression ±SD.

Chromatin immunoprecipitation-chip assay and ChIP-seq assay. The ChIP-chip assays were carried out as previously reported.(23) For p53 induction, cells were treated with 5-FU (0.375 mM for 9 h) or UV (10 J or 45 J, harvested 9 h after irradiation). Antibodies against p53 (FL393; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and trimethyl H3 Lys4 (Abcam, Cambridge, UK) were used to precipitate immune complexes. The p53-binding consensus regions were computationally determined using the TRANSFAC database.

Chromatin immunoprecipitation assay. The ChIP assay was carried out as previously described.(23) Control or ts-FL-p53-expressing Saos2 cell lines were tested for p53 binding to FUCA1 enhancer after a shift to the permissive temperature. Cells were collected 6 h after temperature shift. Prepared cell lysates were immunoprecipitated using EZview Red ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich), and used for subsequent analyses. Both input and bound (p53-IP) fractions were analyzed for FUCA1 DNA content; forward, 5′-GTGACGTCGACGGCTCTCCTGATA-3′, and reverse, 5′-GTGGACAGCAAAAACACCATGA-3′.

Luciferase reporter assay. For the luciferase reporter assay, Saos2 cells were seeded in 96-well dishes and cotransfected with 60 ng firefly luciferase reporter DNA and 1 ng of each p53 gene cloned into the pcDNA3 vector, together with 15 ng Renilla luciferase expression vector (pGL474 [hRluc/TK] vector; Promega, Tokyo, Japan) as an internal control for transfection efficiency. Cells were harvested 24 h post-transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega). All of the luciferase reporter assay data are the mean-±SD of three independent experiments.

Promoter-reporter constructs. A 131-bp fragment of intron 1 of the FUCA1 gene was amplified by PCR using the primers shown below and cloned between the KpnI and Smal sites of the pGL3-promotor vector; forward, 5′-CTAGCAATAAGCCTGTCCCG-3′ and reverse, 5′-GTGGGACGGTACACTGGCAT-GAACCTTGGCAAGCAT-3′.

Polymerase chain reaction primers. Plasmids carrying one copy of FUCA1-p53RE or FUCA1-p53RE mut were obtained by cloning double-stranded oligonucleotides into the PicaGene basic vector (Wako, Osaka, Japan) containing a minimal promoter. Oligonucleotide sequences: FUCA1-p53RE, 5′-AGGCA TGTGGGCAAGTTC-3′; and FUCA1-p53RE mut, 5′-AGGCA TGCTGGGAAATTTC-3′.

Human wild-type FUCA1, mutant FUCA1 (Q422X; 422–466, terminal three amino acids of human FUCA1 deleted, N329Y; a.a. 329 of human FUCA1 mutated to tyrosine) and EGFR were tagged with BamHI and Xhol sites at the 5′- and 3′-ends, respectively, and cloned into the BamHI/Xhol site of the pcDNA3 vector.

Western blot analysis. Cells were lysed in lysis buffer containing 0.1 M Tris–HCl (pH 6.8), 2% SDS, 10% (v/v) glycerol, 0.01% boronphenol blue (BPB), and 0.1 M DTT. Whole cell lysates were analyzed by Western blotting. Antibodies used in this study were as follows: anti-FUCA1 mouse mAb (sc-365496), anti-EGFR rabbit polyclonal antibody (sc-03) (Santa Cruz Biotechnology), anti-phospho-EGFR rabbit polyclonal antibody (2220), anti-cleaved caspase-3 rabbit mAb (9664), anti-p21 rabbit mAb (2947) (Cell Signaling Technology, Danvers, MA, USA), anti-phospho-EGFR rabbit polyclonal antibody (9664), and anti-p21 rabbit mAb (A2228; Sigma-Aldrich). After washing with TBS-Tween (0.05%), membranes were incubated with mouse (NA931V) or rabbit (NA934V) secondary antibodies (GE Healthcare Life Sciences, Piscataway, NJ, USA) for 6 h at room temperature and were visualized with an LAS4000 imaging system (GE Healthcare Life Sciences).

Immunoprecipitation. Cells were lysis buffer containing 50 mM HEPES (pH 7.0), 150 mM NaCl, 1.5 mM MgCl2, 10% glycerol, 1% Triton-X, 1 mM EGTA (pH 8.0), 100 mM NaF, 1 mM NaVO4, and a protease inhibitor mix. For immunoprecipitation of FLAG-tagged proteins, cell lysates were immunoprecipitated with M2 agarose beads (Sigma-Aldrich) for 15 h at 4°C. For elution of FLAG-tagged proteins, FLAG-tagged protein and M2 agarose beads mix solution were incubated with 3 x FLAG peptide for 3 h at 4°C.

Fucosidase enzyme activity assay. Activity of ω-1-fucosidase was assayed as described previously(24) using 1 mmol/L 4-nitrophenyl ω-1-fucopyranoside (Sigma-Aldrich) as substrate in PBS (Fig. 3b,g,i) or in 0.1 M citrate/0.2 M sodium phosphate buffer at the indicated pH (Fig. 3c,d). Absorbance was read on a Tecan plate reader (Wako, Tokyo, Japan) using wavelengths of 405 nm. Absorbance from control wells containing no substrate were taken as background and subtracted from the test wells. Deoxyfluconojirimycin, a potent, specific, and competitive inhibitor of ω-1-fucosidase, was used to inhibit fucosidase activity (25).

Lectin blot analysis. Cells were lysed in lysis buffer containing 0.1 M Tris–HCl (pH 6.8), 2% SDS, 10% (v/v) glycerol,
0.01% bromophenol blue, and 0.1 M DTT. Whole cell lysates were analyzed by lectin blotting with the following biotinylated lectins (J-Oil Mills, Tokyo, Japan): PhoSL, recognizing α-1,6-fucosylation; AAL, recognizing α-fucosylation; and UEA-1, recognizing α-1,2-fucosylation. Membranes were incubated with each lectin overnight at 4°C. Membranes were washed with TBS-Tween (0.05%), incubated with avidin-HRP (Vectorstain; Vector Laboratories, Burlingame, CA, USA) for 6 h at room temperature and these lectins were visualized with an LAS4000 imaging system (GE Healthcare Life Sciences).

Construction of recombinant adenovirus expressing p53 and FUCA1. Recombinant adenovirus was constructed as reported. The Adenovirus Dual Expression Vector Kit (Takara Bio, Shiga, Japan) and Adenovirus Genome DNA-TPC (Takara Bio) were used to obtain adenovirus. The full ORF of p53 and FUCA1 were inserted into the Smal site of the pAxCW-Aw-tit2 vector. Infectious titer and optimal m.o.i. were determined by the 50% tissue culture infectious dose method using 293 cells, the E1-complementing helper cell line, according to the manufacturer’s instructions.

Immunostaining. H1299 cells were infected with control LacZ or FUCA1-expressing adenoviruses (m.o.i., 0.2). Cells were incubated with LysoTracker Red (Thermo Fisher Scientific) for 40 min at 36 h post-infection. At 37 h post-infection, the cells were incubated with LysoTracker Red (Thermo Fisher Scientific) for 40 min at 36 h post-infection. The cells were fixed with 70% ethanol overnight, then washed with PBS and incubated with 25 μg/mL BSA, and 50 mM glycine for 1 h at room temperature. The cells were sequentially incubated with anti-FUCA1 mouse mAb (sc-365496) for 1 h at room temperature, then Alexa Fluor 488-labeled secondary antibody (Molecular Probes; Thermo Fisher Scientific). Images were obtained with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Colony formation assay. T98G, HCT116 p53−/−, HepG2, and H1299 cells were transfected with control lentivirus or lentivirus expressing FUCA1 or mutant FUCA1 (Q422X). Colony formation assay was carried out using cells selected in blastidicin-containing medium for 6–10 days in 6-well plates (2 × 104 cells per well). Subsequently, cells were fixed with 100% methanol for 10 min and stained with Giemsa for 40 min. Images were obtained with a GT-X980 scanner (Epson, Nagano, Japan). Colonies were analyzed using ImageJ software. Briefly, the images were converted into binary format using ImageJ’s binary convert function, and analyzed by ImageJ’s batch measure function.

Quantitation of cell death by flow cytometry. Cells were collected and fixed with 70% ethanol overnight, then washed with PBS and incubated with 25 μg/mL propidium iodide and 20 μg/mL RNase A. Flow cytometry analysis was carried out using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA), and the proportion of cells in sub-G1 (chromosome fragmentation) was calculated.

Analysis of FUCA1 mutations. FUCA1 mutations found in human cancers were analyzed using the COSMIC database (http://cancer.sanger.ac.uk/cosmic). Expression of FUCA1 (NM_000147) was analyzed using the ONCOMINE database (https://www.oncomine.org/).

Expression of FUCA1 and correlation to cancer patient prognosis were analyzed using the PrognoScan database (http://www.abren.net/PrognoScan/), a large collection of publicly available cancer microarray datasets with clinical annotations and a tool for assessing the biological relationships between gene expression and prognosis.

Statistical analysis. Data were calculated and shown as mean ± SD. Significance of differences was determined by Student’s t-test (Figs 1c,g,S4c), one-way ANOVA (Fig. 4a), two-way ANOVA (Figs 1f,3b,d-g,i) or Mann–Whitney U-test (Fig. 1h). Statistical significance was defined as P < 0.05.

Results

FUCA1 is a p53 target gene. We first identified the FUCA1 gene as a p53-inducible gene by comprehensive microarray expression analysis of a cell line that expresses ts-p53. As shown in Figure 1(a), FUCA1 mRNA is induced by ts-p53 at the permissive temperature. We further confirmed that FUCA1 mRNA is induced by DNA damage caused by γ-ray irradiation, adriamycin, UV, etoposide, or 5-FU in cell lines that have wild-type p53 (Fig. 1b–f). Induction of FUCA1 mRNA was not observed in cell lines that do not have functional p53 (Fig. 1d–f). We further analyzed whether FUCA1 mRNA is upregulated by exogenous 5-FU expression. As shown in Figure 1(g), expression of p53 strongly induced mRNA expression of both FUCA1 and a representative p53 target gene, PHLDA3, in p53-null Saos2 cells. In addition, as shown in Figure 2(a), both the control and ts-p53 expressed FUCA1 mRNA expression in breast cancer tissues. As shown in Figure 1(h), FUCA1 expression was significantly lower in cancers with mutant p53 compared to p53 wild-type cancer tissues. Collectively, these results indicate that FUCA1 expression is regulated by p53.

The FUCA1 genomic region was analyzed by ChIP-seq analysis of HCT116 p53+/+ and HCT116 p53−/− cells that were treated with 5-FU or untreated (Fig. 2a). This analysis identified a p53-binding site in intron 1 of the FUCA1 gene. After 5-FU treatment, H3K27 acetylation was detected around this p53-binding region in Saos2 cells (carrying ts-p53) at the permissive temperature. We further confirmed that the positive and negative controls of p53 binding to the p53-responsive region in Saos2 cells (carrying ts-p53) at the permissive temperature. The positive and negative controls of p53 binding to the p53-responsive region in Saos2 cells (carrying ts-p53) at the permissive temperature.

As a negative control, we also analyzed the binding of p53 to the p53-responsive region in Saos2 cells (carrying ts-p53) at the permissive temperature. As a negative control, we also analyzed the binding of p53 to the p53-responsive region in Saos2 cells (carrying ts-p53) at the permissive temperature.
(a) Bar graph showing the signal intensity of FUCA1 with Time (h) and Treatment groups. (b) Western blot analysis of FUCA1 and 28S under different conditions: HCT116 (wt p53) treated with IR (Gy) 0, 10, 30. (c) Graph illustrating the relative induction of FUCA1 with ADR concentration (0.0, 1.0). (d) Western blot analysis of FUCA1 and 28S under different UV and cell lines. (e) Western blot analysis of FUCA1 and 28S under different conditions: HCT116 p53 +/- treated with Etoposide and 5-FU. (f) Graph showing the relative expression of FUCA1 with 5-FU and cell lines. (g) Graphs showing the relative induction of FUCA1 and PHLDA3 with ad-LacZ and ad-p53. (h) Box plot showing the expression of FUCA1 with wt-p53 and mt-p53.
**Fig. 1.** *FUCA1* is a p53-inducible gene. (a) *FUCA1* expression was analyzed by microarray analysis. Temperature-sensitive, p53-expressing Saos2 cells (ts-p53) were tested for *FUCA1* induction after temperature shift to the permissive temperature with or without γ-ray irradiation (IR). Cells were subjected to γ-ray irradiation (30 Gy) 2 h after temperature shift to 32°C. Cells were collected 5 or 14 h post-temperature shift. (b) HCT116 p53+/+ cells were subjected to γ-ray irradiation (10 Gy or 30 Gy). Cells were collected 18 h post-temperature shift. *FUCA1* expression was analyzed by Northern blotting. (c) MRC5 cells were subjected to adriamycin (ADR; 1 μM) treatment. Cells were harvested 24 h post-treatment. *FUCA1* expression was analyzed by quantitative RT-PCR. The mRNA levels of *FUCA1* were normalized to *GAPDH* mRNA levels. (d) HepG2 or Huh7 cells were subjected to UV treatment (45 J/m²). Cells were collected at the indicated time points post-treatment. *FUCA1* expression was analyzed by Northern blotting. (e) HCT116 p53+/+ or p53−/− cells were subjected to etoposide (20 mM) or 5-fluorouracil (5-FU) (0.13 or 0.38 mM) treatment. Cells were collected 21 h post-treatment. *FUCA1* expression was analyzed by Northern blotting. (f) HCT116 p53+/+ or p53−/− cells were subjected to 5-FU (0.38 or 0.76 mM) treatment for 18 h. *FUCA1* expression was analyzed by quantitative RT-PCR. mRNA levels of *FUCA1* were normalized to *GAPDH* mRNA levels. (g) HeLa cells were infected with an adenovirus expressing p53 (ad-p53) or control LacZ (ad-LacZ). Cells were harvested 48 h post-infection. *FUCA1* and *PHLDA3* expression was analyzed by quantitative RT-PCR. mRNA levels of *FUCA1* and *PHLDA3* were normalized to *GAPDH* mRNA levels. (h) Using the cBioPortal database, *FUCA1* expression and the p53 status of breast invasive carcinoma were obtained. In total, 971 samples were analyzed. P-values were calculated using the Mann–Whitney U-test (*P* < 0.0001). mt, mutant.

**Fig. 2.** *FUCA1* is a direct target gene of p53. (a) HCT116 p53+/+ or p53−/− cells were treated with or without 5-fluorouracil (5-FU), and p53 ChIP sequencing analysis was carried out. Genomic locus of *FUCA1* is shown together with the results obtained. ChIP sequencing analyses were carried out using antibodies against p53, H3K27ac, H3K4me1, H3K4me3, and phospho-RNAP II. A p53 binding site (p53RE) was identified within intron 1 of the *FUCA1* gene. (b) ChIP assay was carried out for *FUCA1* intron 1, which contains the p53RE. A Saos2 cell line that stably expresses a temperature-sensitive p53 (ts-p53) was used to analyze p53 binding to the *FUCA1* promoter. p53 binding to p53RE was analyzed at the non-permissive (38°C) and permissive (32°C) temperatures. The positions amplified by PCR (131-bp fragment was amplified) are shown. (c) The 131-bp fragments within intron 1 containing wild-type or mutant p53RE were cloned upstream of a firefly luciferase reporter gene with a minimal promoter, and a luciferase reporter assay was carried out. Constructs were tested for transactivation by WT p53 and p53-V143A. The assay was undertaken 24 h post-transfection. mt, mutant.
These data suggest that the p53-binding region contains a p53-responsive element that may regulate \textit{FUCA1}. Examination of the sequences within this region of intron 1 revealed the presence of sequences highly similar to the p53 consensus binding sequence (p53RE; TRANSFAC match score, 0.61) at the center of the region to which p53 was shown to bind. We generated oligonucleotides containing this sequence (p53RE), as well as a version mutated in the p53 consensus sequence (p53RE mut), and cloned each upstream of a luciferase reporter gene containing a minimal promoter (the sequences of wild-type and mutant p53RE are shown in Fig. 2c). As shown in Figure 2(c), p53 strongly activated the reporter containing the wild-type p53RE but not p53RE mut. These results collectively demonstrate that p53RE is a p53-responsive element in the \textit{FUCA1} gene, and confirms that \textit{FUCA1} is a p53 target gene.

We also compared the p53RE sequence with the consensus sequence recognized by different p53 family proteins. As shown in Figure S3, p53RE showed high identity with the consensus sequences for p53, p63, and p73 (27–29). In addition, p53RE has been identified as a p63 binding site in a genome-wide comprehensive analysis of p63 binding sites using cervical carcinoma cells. (28) Thus, the \textit{FUCA1} gene may be a common target of various p53 family proteins, and it would be interesting to ask in a future study if \textit{FUCA1} is regulated by p53 or p73.

\textbf{\textit{FUCA1} encodes a fucosidase that removes α-L-fucose.} \textit{FUCA1} has been reported to be a fucosidase that localizes in the lysosome. (30) In addition, fucosidase activity is reported to be lost in fucosidosis patients carrying mutations in the \textit{FUCA1} gene.\textsuperscript{(13)} We therefore analyzed the fucosidase activity of the wild-type \textit{FUCA1} protein, as well as two mutants (N329Y and Q422X) that are found in fucosidosis patients and which are believed to be defective in fucosidase activity. We expressed each of these in COS7 cells and analyzed their enzymatic activities. As shown in Figure 3(a,b), similar amounts of each protein were expressed, but only wild-type \textit{FUCA1} showed fucosidase activity. This activity was efficiently inhibited by deoxynojirimycin, a potent, specific, and competitive inhibitor of α-L-fucosidase.\textsuperscript{(25)} We next analyzed the optimum pH for \textit{FUCA1} enzymatic activity and found it to be high, between pH 6.4 and 7.0, and the highest at pH 6.7 (Fig. 3c,d). \textit{FUCA1} has been reported to reside in the lysosome,\textsuperscript{(30)} which has a pH in the 4.5–5.0 range; however, we observed that at pH 5.0 \textit{FUCA1} enzymatic activity was very low (Fig. 3c).

This suggests that \textit{FUCA1} may be optimized for activity in the cytoplasm or another organelle, where the pH is closer to 6.7. This observation prompted us to examine the subcellular localization of \textit{FUCA1} protein by immunofluorescence analysis. We expressed \textit{FUCA1} ectopically in H1299 cells and observed that \textit{FUCA1} was mainly detected in areas that did not overlap with the lysosomotropic dye LysoTracker Red (Fig. 3e). Rather, \textit{FUCA1} appeared as spots in the perinuclear region, which suggests that it may be localized in some organelle surrounding the nucleus.

Fucosylation may be divided into several types according to the linkages it produces, including α1,2-, α1,3-, α1,4-, and α1,6-fucosylation.\textsuperscript{(31)} We analyzed the specificity of \textit{FUCA1}-mediated fucosylation by probing blots of fractionated cellular proteins with lectins that can detect and distinguish some of these different forms of fucosylation. Lectins PhoSL, and UEA-1 are specific for α1,6- and α1,2-fucosylation, respectively,\textsuperscript{(32,33)} whereas AAL is a lectin that detects all types of fucosyl linkages.\textsuperscript{(33)} As shown in Figure 3(c), PhoSL and AAL staining revealed significant differences between cells expressing vector versus \textit{FUCA1}, whereas no obvious differences were seen by UEA-1 staining. These data indicate that \textit{FUCA1} efficiently removes α1,6-fucosylation but not α1,2-fucosylation.

We note that AAL staining was significantly decreased in the \textit{FUCA1}-expressing sample, suggesting that \textit{FUCA1} may also remove other types of common fucosyl linkages such as α1,3- and α1,4-fucosylation, which can be detected by AAL.

As \textit{FUCA1} is a p53 target gene, we also analyzed whether fucosidase activity is induced by p53. As shown in Figure 3(g), DNA damage by adriamycin treatment resulted in increased fucosidase activity and decreased fucosylation levels in normal human fibroblasts that have wild-type p53. In addition, fucosidase activity was induced by exogenous p53 expression and this activity was further enhanced by DNA damage (Fig. 3i). Therefore, p53-regulated \textit{FUCA1} expression leads to increases in fucosidase activity and results in a decrease in fucosylated proteins.

\textbf{Cell death induced by \textit{FUCA1} in a manner dependent on its glycosidase activity.} As our data indicates that \textit{FUCA1} is a p53 target gene, we next asked if \textit{FUCA1} has a tumor suppressive function. As shown in Figure 4(a), stable expression of wild-type \textit{FUCA1} significantly inhibited cell proliferation in both p53-proficient (HepG2) and p53-deficient (H1299, T98G, and HCT116 p53\textsuperscript{−/−}) cell lines. In contrast, expression of the fucosidase-deficient mutant \textit{FUCA1} had a smaller effect on...
proliferation in all of these cell lines. We next used flow cytometry to analyze cell cycle and cell death in cells transiently expressing FUCA1. As shown in Figure 4(b), there was no significant difference in the cell cycle distribution between samples. However, the fraction of cells in sub-G1, indicative of chromosomal fragmentation and cell death, increased significantly in cells expressing wild-type FUCA1 but not in cells expressing vector or mutant FUCA1. These data show that, while fucosidase activity is required for the induction of cell death and suppression of cell proliferation, the p53 status of...
the cells does not affect these functions of FUCA1. This is consistent with the notion that FUCA1 is a downstream mediator of p53 action. We also analyzed whether the cell death induced by FUCA1 may be classified as apoptosis. As shown in Figure S4(a), we could detect cleaved caspase-3 in cells dying as a result of FUCA1 overexpression, indicating that FUCA1 expression results in the induction of apoptosis. Furthermore, we analyzed the effect of FUCA1 knockdown in H1648 cells that express relatively high levels of FUCA1 (Fig. S4b). As shown in Figs. S4(c), FUCA1 knockdown enhanced the proliferation of H1648 cells. We are very much interested in the effect of loss of FUCA1 function, and plan to analyze this further using FUCA1 knockout mice in future studies.

α1,6-Fucosyl linkages on EGFR cleaved by FUCA1 and EGF signaling inhibited. Several reports have described enhanced fucosylation of proteins in cancers (i.e., CA19-9, α-fetoprotein-L3 fraction, haptoglobin, EGFR, the TGF-β1 receptors, and E-cadherin). As p53 is frequently mutated in various cancers, which should lead to a decrease in FUCA1 expression, we next turned our attention to proteins that are highly fucosylated in cancers as potential targets of FUCA1. We considered the possibility that FUCA1 may function outside of the lysosome, as its glycosidase activity has an optimal pH of 6.7. We also noted the effect of FUCA1 expression in suppressing proliferation and inducing cell death. One candidate target that would be consistent with these criteria is EGFR, which has been reported to be a α1,6-fucosylated protein that plays an important role in cell growth and survival. We immunoprecipitated EGFR from control or FUCA1-expressing cells and analyzed EGFR fucosylation by lectin blotting. As shown in Figure 4(c), FUCA1 expression resulted in reduced α1,6-fucosylation of EGFR, as revealed by PhoSL blotting. In addition, FUCA1 expression resulted in reduced phosphorylation of EGFR, an indicator of EGFR activity. We next asked whether FUCA1 inhibits EGFR downstream signaling by examining phosphorylation of Akt, which is essential for Akt activation. As shown in Figure 4(d), wild-type but not mutant, FUCA1 efficiently repressed phosphorylation of Akt. These results show that FUCA1 inhibits EGFR signaling by removing α1,6-fucosyl linkages on EGFR.

Tumor suppressive function of FUCA1. As shown above, FUCA1 can repress EGFR signaling. In addition, the FUCA1 gene is located at 1p36, a chromosome locus frequently deleted in various cancers. We therefore asked if FUCA1 has any function as a tumor suppressor. We first searched the publicly available COSMIC database, which listed a total of 57 FUCA1 mutations found in various cancers (Figs S5a, S5b). It has been reported that several frame shift and nonsense mutations found in fucosidosis patients generating a stop codon before a.a. Q422 result in the loss of FUCA1 function. We also showed above that the Q422X mutant does not have fucosidase activity. Although we have not examined the function of other FUCA1 mutants, we expect that the frame shift mutants (G753fs*60, L185F*1, and T211fs*16) and nonsense mutants (Q267* and E318*), which are found in cancers, result in the loss of FUCA1 function. To understand how these other mutations may affect FUCA1 function, we examined the reported crystal structure for α1-fucosidase from Thermotoga maritima. This fucosidase is the closest bacterial relative to human α1-fucosidase, sharing 38% identity with its human counterpart. As shown in Figure S6, 9 out of 10 amino acids in the catalytic pocket are conserved between human FUCA1 and the T. maritima α1-fucosidase. Among the FUCA1 genes mutated in human cancers, several are mutated in the catalytic pocket (n = 3), and one of them, S229P, is located within the catalytic nucleophile residues of FUCA1. Furthermore, 10 amino acids that are conserved between human FUCA1 and T. maritima α1-fucosidase were mutated in human cancers, and one of them, L288W, corresponds to a.a. residues that are highly conserved among vertebrates, invertebrates, and T. maritima (Fig. 5a). Interestingly, 20 out of 57 mutations were found in cancers of the large intestine, suggesting the possibility that FUCA1 is particularly important for suppressing cancers of the large intestine (Fig. S5). We further queried the ONCOMINE database to obtain information about FUCA1 expression levels in normal and cancer tissues. As shown in Figure 5b(c), FUCA1 expression is significantly lower in cancers of the large intestine compared to normal large intestine tissue. We next searched the PrognoScan database, a large collection of publicly available cancer microarray datasets that includes clinical annotations and a tool for assessing the biological relationships between gene expression and prognosis. As shown in Figure 5d–j, lower FUCA1 expression is associated with poorer prognosis in cancer patients. This association was especially strong in colorectal and breast cancers (Fig. 5e–j). These results suggest that loss of FUCA1 expression may be involved in cancer progression, especially in cancers of the large intestine and breast.

Discussion

FUCA1 is a p53 target gene. In this study, we have identified FUCA1 as a novel p53 target gene. FUCA1 mRNA is induced under various DNA-damaging conditions, and in a p53-dependent manner. It is also induced by ectopic expression of p53. In addition, we have identified a p53-responsive element within intron 1 of the FUCA1 gene. This p53-binding site acts as an active enhancer and several chromatin modifications that are associated with active enhancers, such as H3K27 acetylation and H3K4 mono-methylation, are found at this site and are enhanced following DNA damage. This is the first report showing that p53 is involved in protein glycosylation and suggests a novel pathway by which p53 might exert its effects on cell growth and death.

FUCA1 encodes a fucosidase that shows highest activity at physiological pH. Previously, FUCA1 was reported to be a lysosomal protein. However, the subcellular localization and the optimal pH of FUCA1 suggest that FUCA1 functions not only in the lysosome but in other components within the cell. In this report, we have identified EGFR as one FUCA1 substrate, and shown that FUCA1-mediated removal of fucose from EGFR leads to decreased activation of the EGFR signaling pathway. However it can be assumed that FUCA1 may have other target proteins both inside and outside of the lysosome. As FUCA1 expression induces cell death and inhibits growth, identification of other FUCA1 target proteins may reveal novel pathways of tumor suppression that involve the removal of protein fucosyl linkages.

Tumor-suppressive activity of FUCA1. Although the link between enhanced fucosylation and tumorigenesis has been previously reported, a direct link between the removal of fucosyl linkages and tumor suppression has not. Our data suggest that FUCA1, a fucosidase, has tumor-suppressing activity. We further observed that loss-of-function mutations in the FUCA1 gene occur in various human cancers.
Fig. 5. Fucosidase, α-1 (FUCA1) function is lost/decreased in human cancers and low FUCA1 expression is related to poorer prognosis of cancer patients. (a) FUCA1 mutations found in human cancers are shown. Data were obtained from the COSMIC database. (b,c) FUCA1 (NM_000147) expression in colorectal and rectal cancer was analyzed using the ONCOMINE database. Left and right boxes show the results obtained with normal and cancer tissues, respectively. (b) FUCA1 (probe A_23_P11543) expression in Gaedcke colorectal adenocarcinoma versus normal colorectal tissue was analyzed. FUCA1 was positioned 896th in a ranking of underexpressed genes (in top 5%). P-value, 7.29E–22; fold-change, –2.676. (c) FUCA1 (probe 202838_at) expression in Skrzypczak colorectal carcinoma versus normal rectal tissue was analyzed. FUCA1 was positioned 6th in a ranking of underexpressed genes (in top 1%). P-value, 4.98E–20; fold-change, –2.976. (d–f) FUCA1 prognostic analysis was carried out using the PrognoScan database. (d) Using the PrognoScan database, FUCA1 expression was analyzed using two probes, 202838_at and 229137_at. In total, 101 datasets were available for FUCA1 expression. Pie charts are shown for each end point. The datasets were categorized into seven categories according to Cox P-values and hazard ratios (HR) (d, right). Lower FUCA1 expression showed significant associations with poorer prognosis in patients with cancers of the large intestine, breast, or lung. (e,f) Datasets for colorectal (e) and breast (f) cancers are shown. (g–j) Representative survival curves showing the prognosis of colorectal (g,h) and breast (i,j) cancer patients with high or low FUCA1 expression. (g,h) Overall survival of colorectal cancer patients of datasets GSE17536 (n = 177; HR [95% CI] = 0.15 [0.03–0.68], P = 0.01) and GSE17537 (n = 55; HR [95% CI] = 0.04 [0.00–0.61], P = 0.02). (i) Overall survival of breast cancer patients of dataset GSE1456GPL96 (n = 159; HR [95% CI] = 0.42 [0.20–0.89], P = 0.02). (j) Distant metastasis-free survival of breast cancer patients of dataset GSE11121 (n = 200; HR [95% CI] = 0.29 [0.14–0.60], P = 0.0007).

| Type                                      | count | AA mutation                  |
|-------------------------------------------|-------|------------------------------|
| Frame shift mutation                      | 5     | G73fs’60, L185fs’1, T211fs’16 |
| Nonsense mutation                         | 3     | Q267*, E318* (2)             |
| Missense mutation at amino acids at the catalytic nucleophile | 1     | S229F                        |
| Missense mutation at amino acids that contact fucose | 2     | E75Q, R264Q                  |
| Missense mutation at amino acids within the consensus sequence, PxxK,xxxKWEExC | 1     | L288W                        |
| Missense mutation at amino acids that are conserved | 9     | T135A, T135K, P160S, L164M, D225H (2), P254H, R308C, L440V |
| Other missense mutation                   | 29    |                              |
| Silent mutation                           | 7     |                              |
| Total numbers                             | 57    |                              |
In addition, \textit{FUCA1} expression is significantly reduced in cancers of the large intestine, and low \textit{FUCA1} expression is related to poorer prognosis in patients with cancer of the large intestine or breast. It has previously been reported that \textit{FUCA1} mRNA expression is decreased in colorectal cancers.\(^{33}\) In addition, it was reported that fucosylation promotes cancer development and malignant progression in breast cancers.\(^{38}\) These reports are in agreement with our results, and suggest that \textit{FUCA1} may have tumor-suppressive activity, and could be a therapeutic target in the treatment of cancers in the clinic. To understand the importance of protein fucosylation and defucosylation in cancers, it will be important to study the function of \textit{FUCA1} in vivo using genetically modified mice.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

- a.a. amino acid
- AAL \textit{Aleuria aurantia} lectin
- Akt protein kinase B
- CA19-9 carbohydrate antigen 19-9
- ChIP-chip ChIP with DNA microarray chip
- ChIP-seq ChIP sequencing
- EGF epidermal growth factor
- EGFR epidermal growth factor receptor
- 5-FU 5-fluorouracil
- FUCA1 fucosidase
- PhoSL \textit{Pholiota squarrosa} lectin
- ts-p53 temperature-sensitive p53 mutant
- UEA-1 \textit{Ulex europaeus} lectin

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. p53 Binds to the first intron of the FUCA1 gene.

Fig. S2. Chromatin immunoprecipitation assay carried out using the p53 responsive element of the p53 target gene IER5.

Fig. S3. Alignment of the FUCA1 p53RE with p53, p63, and p73 consensus response elements.

Fig. S4. Analysis of the effect of FUCA1 ectopic expression or FUCA1 knockdown in cancer cells.

Fig. S5. Mutations of FUCA1 found in various cancers.

Fig. S6. Alignment of Thermotoga maritima α-L-fucosidase and human FUCA1 protein sequences.