

REPORT

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p53 directly regulates the glycosidase FUCA1 to promote chemotherapy-induced cell death

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ABSTRACT

p53 is a central factor in tumor suppression as exemplified by its frequent loss in human cancer. p53 exerts its tumor suppressive effects in multiple ways, but the ability to invoke the eradication of damaged cells by programmed cell death is considered a key factor. The ways in which p53 promotes cell death can involve direct activation or engagement of the cell death machinery, or can be via indirect mechanisms, for example through regulation of ER stress and autophagy. We present here another level of control in p53-mediated tumor suppression by showing that p53 activates the glycosidase, FUCA1, a modulator of N-linked glycosylation. We show that p53 transcriptionally activates *FUCA1* and that p53 modulates fucosidase activity via FUCA1 up-regulation. Importantly, we also report that chemotherapeutic drugs induce FUCA1 and fucosidase activity in a p53-dependent manner. In this context, while we found that over-expression of FUCA1 does not induce cell death, RNAi-mediated knockdown of endogenous FUCA1 significantly attenuates p53-dependent, chemotherapy-induced apoptotic death. In summary, these findings add an additional component to p53's tumor suppressive response and highlight another mechanism by which the tumor suppressor controls programmed cell death that could potentially be exploited for cancer therapy.

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Introduction


The removal of damaged cells by programmed cell death is one of the major mechanisms of tumor suppression.¹ Cell death in this context can be achieved by multiple mechanisms, but a key regulator of this response is the tumor suppressor p53.² The importance of this protein in the prevention of tumor development is highlighted by the fact that it is lost in approximately 50% of human cancers and that mice deficient in p53 develop tumors by 6 months of age.^{3,4} While p53 modulates multiple cellular pathways that are important for tumor suppression, studies have shown that its regulation of programmed cell death is of high importance.⁵ The protein primarily functions as a transcription factor and has been shown to activate a spectrum of genes involved in cell death regulation.⁶ These genes include a number that encode pro-apoptotic members of the Bcl-2 family such as Puma and Noxa which directly engage the intrinsic cell death pathway at mitochondria.⁷⁻⁹ In addition, p53 has also been reported to activate genes that stimulate the extrinsic apoptotic pathway.¹⁰ Moreover, in addition to these transactivation-dependent mechanisms, p53 has also been shown to regulate cell death more directly by binding to and repressing anti-apoptotic members of the Bcl-2 family resulting in mitochondrial outer membrane permeabilisation (MOMP) and release of apoptogenic factors into the cytosol.¹¹⁻¹³

In addition to its role in tumor suppression, p53-induced programmed cell death is also an important factor in the response to

chemotherapy.¹⁴ Many chemotherapeutic drugs induce DNA-damage which is a potent activation signal for p53.¹⁵⁻¹⁷ In unstimulated cells, p53 is restrained by its target gene Mdm2 which promotes p53 degradation. However, upon exposure to genotoxic agents, this negative feedback loop is alleviated and the levels of p53 increase and the protein becomes activated.^{16,17} The importance of this response in the effectiveness of chemotherapeutic agents is reflected by the decreased cell death that is observed upon drug exposure of p53-deficient cells.¹⁴ Moreover, the fact that cell death pathways can be perturbed during tumor development - including those caused by loss of p53 - has important implications for resistance to many chemotherapeutic drugs.

The perturbation of cell death pathways is only one facet of tumor progression.¹ For the development of cancer, cells must attain a number of attributes which include for example, deregulated proliferation, evasion of immune surveillance and immortality.¹ One characteristic on many cancers, however, that is relatively under-investigated involves changes in glycosylation. There are 2 principle forms of protein glycosylation and both have been reported to be perturbed in human cancer.¹⁸ O-linked glycosylation involves attachment of sugars to proteins via serine or threonine, whereas N-linked glycosylation involves attachment via asparagine.¹⁸ Interestingly, while increases and changes in glycosylation are considered common events in human cancer,¹⁹ very few connections have been reported between the control of

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glycosylation and the signaling pathways that either promote or repress tumor development. In this regard, we report here that p53 can transcriptionally activate the gene encoding the glycosidase FUCA1 which cleaves fucose linked moieties in N-linked glycans. Moreover, we show that chemotherapeutic drugs activate FUCA1 via p53 and that FUCA1 is a contributing factor to chemotherapy-induced cell death.

Materials and methods

Cell lines, cell culture and treatments

Saos2, RKO and U2OS cell lines were purchased from the American Type Culture Collection and maintained in DMEM (Gibco BRL, Paisley, UK) containing 10% FBS, 4.5 g l⁻¹ glucose, 1 mM L-glutamine, 0.11 g l⁻¹ pyruvate and maintained at 37°C in 5% CO₂ atmosphere. p53wt and mutant 273H inducible cell lines have been previously described.²⁰ Expression of p53 was induced using 1 μg/ml of Doxycycline (Dox). RKO-LMP-Scr, RKO-LMP-p53, U2OS-LMP-SCR, U2OS-LMP-p53 were generated by infection of RKO and U2OS cells with LMP-Scr and LMP-p53 respectively.²¹ RKO-pRS-Scr, RKO-pRS-p53, U2OS-pRS-SCR, U2OS-pRS-p53 were previously described (Crighton 2006 and Crighton 2007). HCT116 p53^{-/-} cells were kind gift from Bert Vogelstein. E1a expressing U2OS were previously described.²² TetOn-TAp73α Saos-2, TetOn-TAp73β Saos-2, TetOn-TAp73γ Saos-2 were kind gifts from Gerry Melino and previously described.¹ Expression of p73 was induced with 1 μg/ml Dox. Where indicated, cells were treated with Actinomycin D (ActD) (Sigma Aldrich St Louis, MI, USA); Adriamycin, Cisplatin or etoposide (Calbiochem).

siRNA, plasmids and transfections

FUCA1 knockdown was performed using pre-designed on target plus siRNAs purchased from Dharmacon (Lafayette, CO, USA). siRNAs directed against FUCA1 were transfected using Oligofectamine (Invitrogen, Life Technologies Paisley, UK) according to the manufacturer's instructions for 48 h before treatment.

pcDNA3-FUCAwt-Myc/His was generated by PCR and cloned in to sites of pcDNA3.1MycHisA (Invitrogen, Life Technologies Paisley, UK). pcDNA3-FUCA1M1-Myc/His was generated by site-directed mutagenesis. pCMV-CD20 has been previously described.²³

Transient transfections into Saos2 cells were undertaken by calcium phosphate precipitation using 5 μg of indicated plasmid together with 1 μg pCMV-CD20.

qRT-PCR

qRT-PCR analysis was undertaken as previously described.²² FUCA1 and FUCA2 primers are QuantiTect primers from QIAGEN. All samples were normalized to 18S rRNA and expressed as relative mRNA expression.

Fuca1 enzymatic activity

The enzymatic activity of alpha-L-fucosidase was assessed as previously described (Rapoport and Pendu 1999). Briefly, cells were lysed in 0.2 M acetate buffer pH5, containing 1% triton-X

100 (TTX), 0.1% SDS and protease inhibitor. Twenty-five μg of protein in 100 μl of 0.2 M acetate buffer pH5 were incubated in a 96 well plate together with 100 μl of 0.2mM 4-methylumbelliferyl alpha-L-fucopyranoside (Sigma Aldrich St Louis, MI, USA) for 90 minutes at 37°C.

Western blotting

Cells were lysed in buffer containing 1% TTX, 0.1 % SDS, 50 mM HEPES pH 7.5, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM Na₄P₂O₇ and protease inhibitors (Roche) as previously described.²⁴ Protein concentrations were determined by BCA assay (Sigma Aldrich St Louis, MI, USA). Cell lysates were separated by SDS-PAGE and transfer into Immobilon[®]-P membranes (Millipore). Membranes were probed with anti-p53, anti-p21 (sc-397, Santa Cruz, CA, USA), anti-HDM2, anti-FUCA1, cleaved caspase-3, anti-PARP (Cell Signaling Technology Beverly, MA, USA), Myc -tag (4A6) (Upstate Biotechnology), β-actin (ab8227, Abcam, Cambridge, UK) or Hsp90 (D-19) (Santa Cruz, CA, USA) antibodies.

Cell death analysis and caspase 3 activity

Cell death was evaluated by flow cytometry (FACScalibur, Becton Dickinson San Jose, CA, USA) as previously described.²⁵ The percentage of cells with sub-G1 DNA content was taken as a measure of apoptotic rate.²⁶

Cells which had been transfected with the pCMV-CD20 were stained with a FITC-conjugated CD20 antibody, sorted for fluorescence isothiocyanate fluorescence, and analyzed for DNA content.²⁵

Clonogenic survival assays were performed on Saos2 cells transfected with the indicated plasmids. 48 hours after transfection, cells were selected with 600 μg/ml G418 (Invitrogen, Life Technologies Paisley, UK) for 2 to 3 weeks and then stained with Giemsa (Sigma).

Caspase 3 activation was assessed by flow cytometry. Cells were fixed and permeabilized with Cytofix/Cytoperm, then incubated for 30 min with anti-active caspase-3-FITC antibody.

Chromatin immunoprecipitation

Chromatin was prepared from Saos2 cells treated with or without Dox. CHIP assays were performed using the CHIP-Assay kit (Merck Millipore) according to manufacturer's instructions. Chromatin was immunoprecipitated with 10 μg of anti-human p53 (clone DO-7, PharMingen) or anti-adenovirus E1A (PharMingen) as a negative control. PCR amplifications of FUCA1 region containing the consensus p53-binding sites, were performed using the specific primers,

FUCA1 (i) GAGAACAAGGGTGCAAAGG

TCGGTTTGCATAGTGGTCTTT

FUCA1 (ii) TGGGACTTCTCAAATCTGC

TCATGTGGTTTTGCTGTCCACAG

Control primers have been previously described²⁷. The amount of coprecipitating DNA was normalized to inputs.

Luciferase reporter assay

Saos-2 cells were co-transfected with luciferase reporter constructs containing potential p53 binding sites and either control vector or a p53-WT-expressing construct. Twenty-four hours post-transfection, cells were lysed in 200 μ l of 1 \times Luciferase Cell Culture Lysis Reagent (Promega, Southampton, UK) and assayed for luciferase activity using the Veritas Microplate Luminometer.

Luciferase constructs containing potential p53 binding site i: AGGCTAGTCTCCAACCTTGTGG within the promoter region (chromosome 1: 23,868, 672) was generated by PCR using the specific primer:

Forward(i): CTGGGATTACAGGCGCACCCCATG

Reverse(i): CCGGAGCCCGCATCGCTACCTCAGCG

Luciferase constructs containing the potential p53 binding site ii: GGGCAAGTTCATGCAAGTTC within intron 1 (chromosome 1: 23,865,667) was generated by PCR using the specified primers

Forward(ii): TCGTTCCTACCAGAAGTGTGAAG

Reverse(ii): CCTGTGGACAGCAAACCATGAGC

Results

The glycosidase *FUCA1* is a p53 target gene

As an approach to understand the mechanisms by which p53 directs programmed cell death, our group has previously performed microarrays using p53-inducible cells to identify novel p53 target genes. These microarray screens lead to the identification of *DRAM1* and *ADORA2B* as genes activated by p53 in response to cellular stress.^{22,28} With the aim of identifying other factors regulated by p53 which contribute to its cell death response, we once again scrutinized these microarray data. Since little is known about the role of glycosylation in cancer, we were drawn to the fact that the mRNA for the glycosidase *FUCA1* had increased levels when p53 was switched on in this inducible system (array data not shown).

To examine the relationship between p53 and *FUCA1* in more detail, we performed qPCR on RNA from cells containing a tetracycline-inducible (TetOn) transgene for either wild-type p53 or a tumor-derived mutant of p53 in which amino acid 273 is changed from arginine to histidine. In confirmation of our microarray data we found that induction of wild-type p53 by treatment with doxycycline (Dox) could induce *FUCA1* as well as 2 previously described target genes, *HDM2* and *p21* (Fig. 1A and B).^{16,17,29} By contrast, the 273H mutant of p53 had no impact on *FUCA1* expression, nor did it affect *p21* and *HDM2* (Fig. 1A and B). Interestingly, p53 (either wild-type or mutant) had no significant impact on the expression of the *FUCA1*-related gene, *FUCA2* (Fig. S1A). Moreover, we also found that *FUCA1* was not induced by isoforms of the p53 family member p73 (Fig. S1B), indicating that unlike many other genes induced by p53, the induction of *FUCA1* is to some level p53-specific.

We considered that *FUCA1* may be a direct target gene of p53 and so we examined the *FUCA1* gene for p53 binding

sites using the previously described MH algorithm.³⁰ This revealed a number of potential p53 binding in the *FUCA1* promoter and one potential site in *FUCA1* Intron 1 (Fig. 1C). Examination of these potential binding sites using chromatin immuno-precipitations with a p53-specific antibody revealed that p53 binds to the site within *FUCA1* Intron 1 (shown as site ii in Fig. 1C), whereas p53 binding could only be detected at a low level at the cluster of potential binding sites identified in the *FUCA1* promoter (Fig. 1D). To test if the binding site in *FUCA1* Intron 1 is functionally significant, we cloned this site into a luciferase reporter construct. This construct was then transiently transfected into p53-null Saos-2 cells either with or without an expression construct for p53. As can be seen in Figure 1E, expression of p53 caused an approximate 6-fold induction of this reporter construct, whereas p53 had no effect on a reporter construct which contained the cluster of potential p53 binding sites from the *FUCA1* promoter (Fig. 1E).

As our data indicated that *FUCA1* was a direct p53 target gene, we next examined if chemotherapeutic drugs which are known to induce p53-dependent responses also lead to induction of *FUCA1*. To test this we treated HCT116, U2OS and RKO cells, each of which contain endogenous wild-type p53, with actinomycin D, Adriamycin (doxorubicin) or etoposide. RNA was then isolated from these cells and the levels of *FUCA1* mRNA determined by qPCR. This revealed that each of the chemotherapeutic agents increased the levels of *FUCA1* mRNA with varying degrees in the cell types (Fig. 2A, C, E). Analysis of a p53-deficient variant of HCT116 cells,³¹ revealed that a large component of *FUCA1* induction in response to Adriamycin and actinomycin D was p53-dependent (Fig. 2A and B). In contrast, knockdown of p53 by RNA interference in RKO cells revealed that induction of *FUCA1* in these cells was also driven by p53, but only in response to Adriamycin and etoposide (Fig. 2C and D). In U2OS cells, similar to RKO cells, *FUCA1* mRNA levels were induced in a p53-dependent manner in response to Adriamycin and etoposide (Fig. 2E and F). In summary, we conclude that *FUCA1* is a direct p53 target gene that is induced by several classes of chemotherapeutic drugs. In many cases this effect involves a marked aspect of p53-dependency, but clearly other factors are also involved the regulation of *FUCA1* in response to these drugs.

p53 and chemotherapeutic drugs induce fucosidase activity

FUCA1 encodes for a glycosidase which cleaves fucose moieties from N-linked glycan.^{32,33} As fucosidase activity is believed to be central to *FUCA1* function,^{34,35} we next sought to determine if p53 could modulate fucosidase activity and whether this was dependent on *FUCA1*. To this end, we adapted an in vitro enzymatic assay using the fluorogenic substrate 4-Methylumbelliferyl- α -L-fucopyranoside (4-MU).³⁶ 4-MU has the property to fluoresce when it is excited at 365nm and this is directly proportional to *FUCA1* activity. Examination of lysates from TetOn-p53 cells that had been incubated in either the absence or presence of Dox (to induce p53) revealed that expression of p53 induces a gradual increase in fucosidase activity over time that is coincident with an increase in *FUCA1* protein levels (Fig. 3A–B). To test if the increase in fucosidase activity

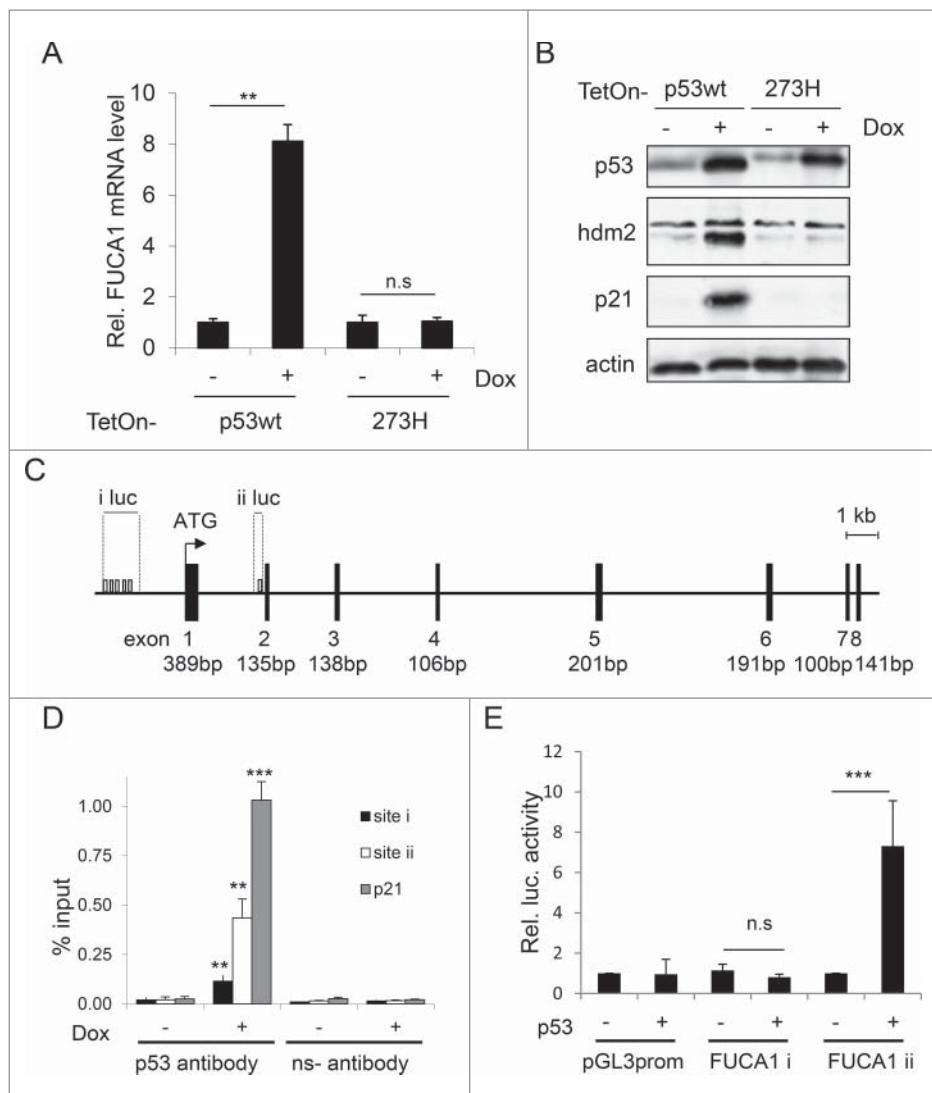


Figure 1. FUCA1 is a direct p53 target gene. (A-B) TetOn-p53wt and TetOn-p53273H Saos2 cells were treated with doxycycline (Dox) for 24 hours. *FUCA1* mRNA levels were quantified by qRT-PCR and expressed as Relative Fuca1 mRNA level and represented as mean \pm SD ($n = 2$ independent experiments, 6 replicates, one way Anova *** $p < 0.0001$; n.s = non significant)(A). p53, Hdm2 and p21 expression were assessed by western blotting. Immunoblot against actin was used as a loading control (B). (C) Identification of 2 potential p53 binding site in the sequence of *FUCA1* gene: one in the promoter region (i) the other one in the first intron (ii). (D) Chromatin immunoprecipitation (ChIP) was performed on TetOn-p53 cells treated with Dox for 24 hr. Immunoprecipitations were carried out with anti-sera against p53 or a non-specific antibody (Ns). The % input of coprecipitating DNAs were calculated by quantitative PCR and presented as mean \pm SD ($n = 3$ independent experiments, one way Anova: Site i ** $p = 0.0053$, Site ii ** $p = 0.0006$, p21 *** $p < 0.0001$). (E) Saos-2 cells were co-transfected with either an empty pcDNA3 vector or a wild-type-p53-expressing construct along with luciferase reporter constructs containing potential p53 binding sites i-ii (pGL3-FUCA1 i-ii) or vector control (pGL3 prom). Luciferase activity was analyzed 24 h after transfection and expressed as the relative fold increase relative to vector control ($n = 4$ independent experiments, one way Anova: Site i n.s $p = 0.6751$, Site ii *** $p < 0.0001$).

induced by p53 is FUCA1-dependent, we induced p53 in Saos-2 cells that had been transfected with 3 different FUCA1-targeting siRNAs. In line with a role for endogenous FUCA1 in this response, each of these siRNAs markedly impaired the ability of p53 to induce both FUCA1 levels and fucosidase activity (Fig. 3C-D).

As we discovered that p53 can induce fucosidase activity, we were interested to know if chemotherapeutic drugs can also induce the activity of this enzyme. We therefore treated RKO cells with either Adriamycin, cisplatin or etoposide for 48h prior to examination of cell lysates for fucosidase activity using our enzymatic assay. This indeed revealed that these 3 chemotherapeutic drugs induce fucosidase activity when administered to cells (Fig. 3E-J). Moreover, using a matched cell line containing a p53-targeting shRNA, we found that this increase in

fucosidase activity was virtually entirely p53 dependent (Fig. 3E-J). We conclude therefore that fucosidase activity may be important not only for the activity of p53 during tumor suppression, but also for the therapeutic response of certain tumors containing wild-type p53.

***FUCA1* expression does not induce cell death, but *FUCA1* is involved in p53's apoptotic response**

As the purpose of our microarray screens was to identify factors involved in p53-mediated programmed cell death, we examined if FUCA1 has a role in the regulation of cell viability. Firstly, we over-expressed FUCA1 in Saos-2 cells which are known to undergo a pronounced apoptotic response following expression of p53. In line with previous reports, expression of p53 in these

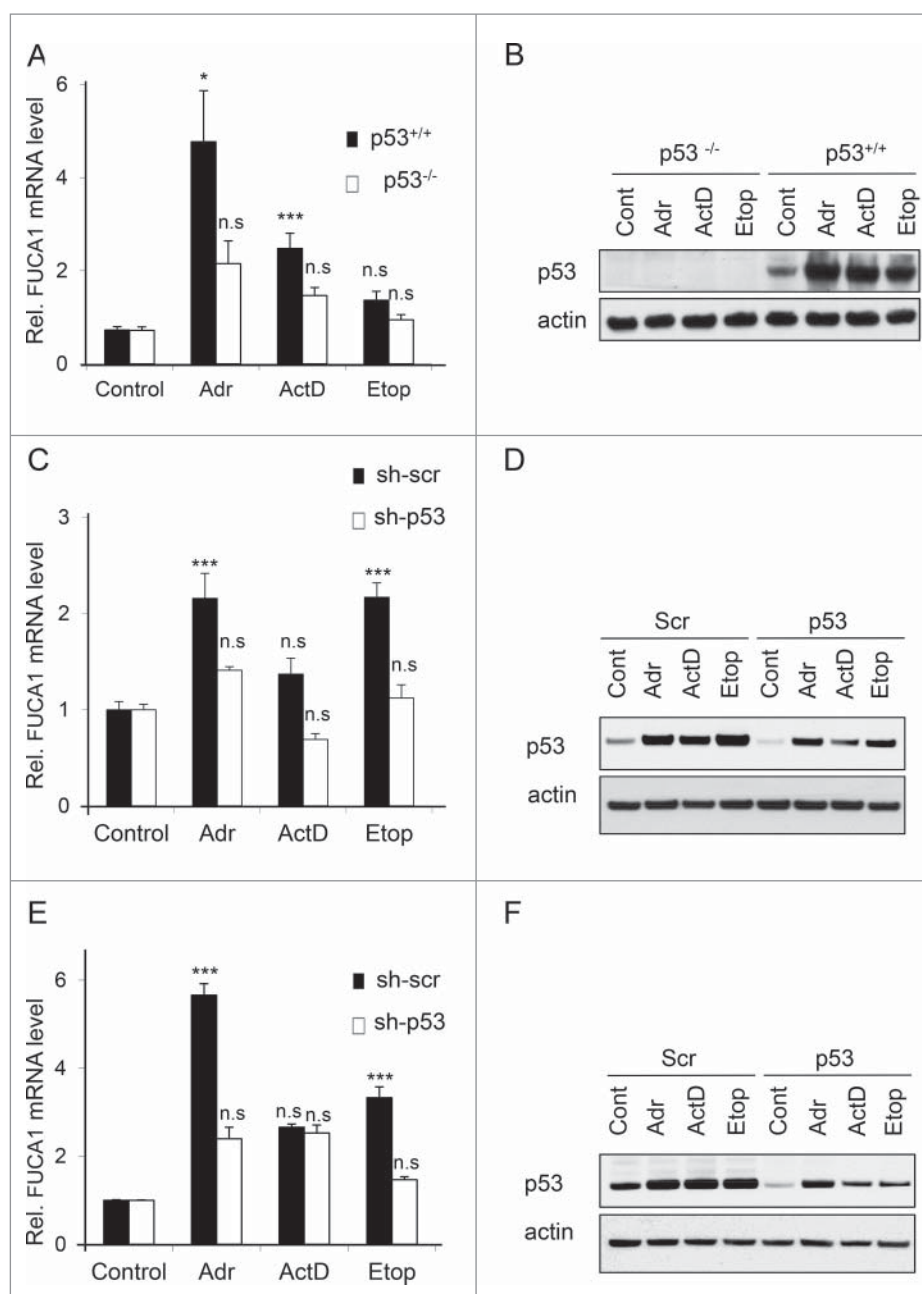


Figure 2. FUCA1 is induced by genotoxic stress in a p53 dependent manner. (A-F) p53 null HCT116 ($-/-$ p53) and WT HCT116 ($+/+$ p53) (A-B), RKO expressing scrambled (LMP-Scr) or p53-specific (LMP-p53) shRNAs (C-D) and U2OS expressing scrambled (LMP-Scr) or p53-specific (LMP-p53) shRNAs (E-F) were treated with either Adriamycin (Adr)(0.5 μ g/ml), ActinomycinD (ActD)(2nM) or Etoposide (Etop) (20 μ m), for 24 hr prior to qRT-PCR quantification of *FUCA1* (A,C,E). Data are expressed as relative *FUCA1* mRNA level and represented as mean \pm SD ($n = 2$ or 3 independent experiments, 5 to 9 replicates, one way Anova: * $p < 0.005$ ** $p < 0.001$, p21 *** $p < 0.0001$). (B,D,F) p53 expression were assessed by western blotting. Immunoblot against actin was used as a loading control.

cells indeed caused a large proportion of cells to undergo apoptosis as measured by analysis of cells with sub-G1 DNA content by flow cytometry (Fig. 4A).²⁶ By contrast, expression of FUCA1 did not seem to affect apoptosis as measured by this assay (Fig. 4A-B). Cell death was also not induced by a mutant of FUCA1 that is associated with the lysosomal storage disorder, fucosidosis.^{37,38}

Since FUCA1 overexpression does not appear to induce apoptosis, we considered that FUCA1 may affect cell viability by other mechanisms. To test this, we performed clonogenic assays which act as readout for all events that can affect cell viability or growth. As would be expected, expression of p53

significantly affected the clonogenic potential of the cells (Fig. 4C). Expression of FUCA1 however, appeared not to have any impact in this assay with FUCA1-expressing cells having a clonogenic potential equivalent to cells transfected with empty plasmid (pcDNA3) as control (Fig. 4C).

Although over-expression of FUCA1 did not affect cell viability, we considered that it may still have an effect on cell death as a part of a p53 response in which other cell death factors are induced or regulated. We therefore utilized FUCA1-targeting siRNAs to knockdown FUCA1 in TetOn-p53 cells and examined the effect this had on cell death following p53 induction. As can be seen in Figure 5A, each of the FUCA1-

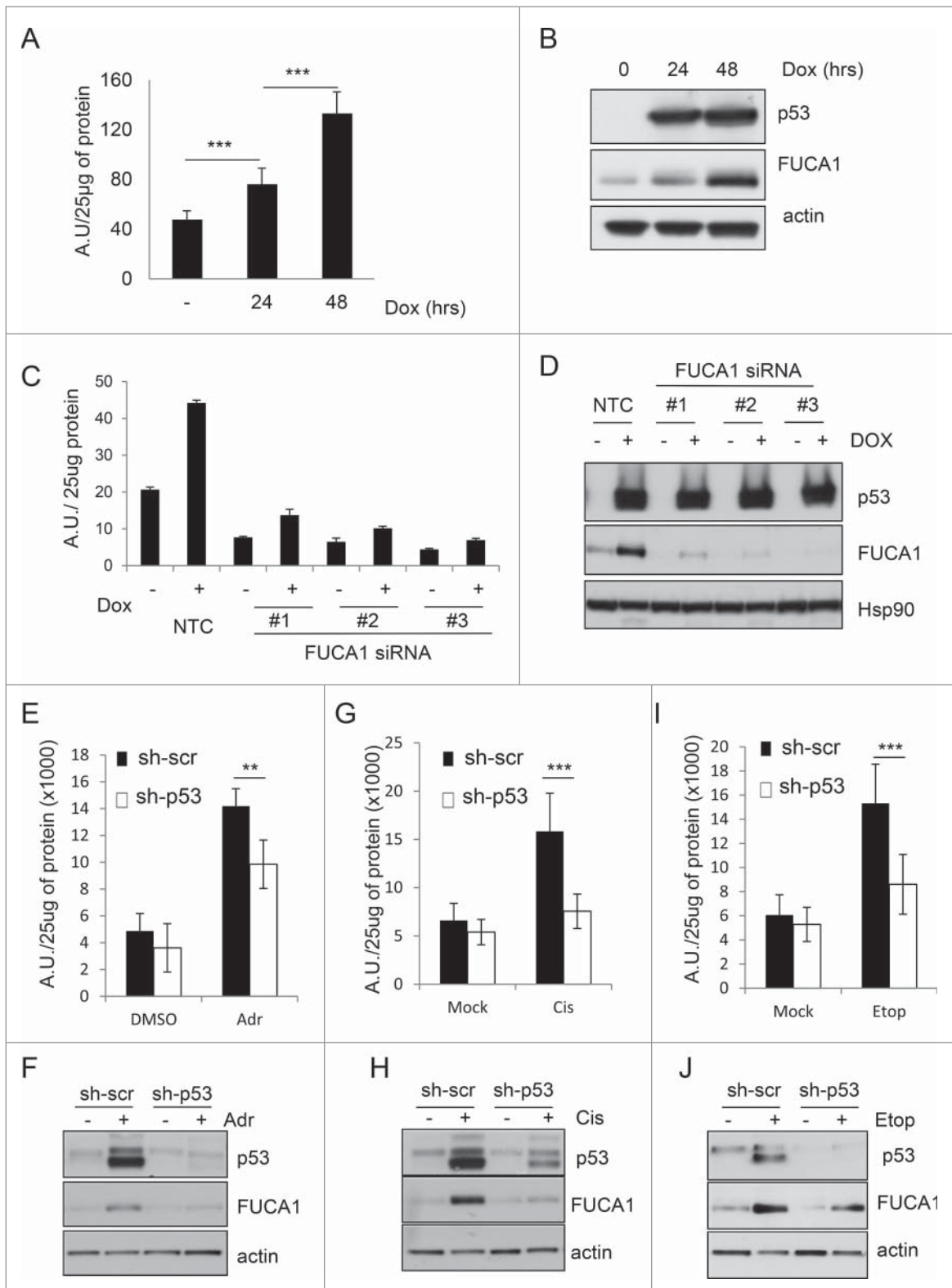


Figure 3. FUCA1 protein level and activity increase following p53 expression. (A-B) TetOn-p53wt Saos2 cells were treated with doxycycline (Dox) for 24 and 48 hours. Fuca1 activity (A) was measured following Dox treatment and expressed as arbitrary units (A.U.) per 25 µg of protein (n = 3 independent experiment, 9 replicates, one way Anova ***p < 0.0001). (B) FUCA1 and p53 expression was assessed by western blotting and immunoblot against actin was used as a loading control. (C-D) TetOn-p53wt Saos2 cells were transfected with 3 different siRNA directed against FUCA1, prior to doxycycline treatment. Fuca1 enzymatic activities were assessed 48 hours after p53 induction, expressed as arbitrary unit (A.U.) per 25 µg of protein and represented as mean ± SD. The graph shown represents data from one representative experiment done with 3 technical replicates. p53 and Fuca1 expression were assessed by Western blotting and immunoblot against Hsp90 was used as a loading control. RKO expressing scrambled (pRS-Scr) or p53-specific (pRS-p53) shRNAs were either Adriamycin (Adr)(0.5 µg/ml) (E-F), Cisplatin (Cis) (20 µM) (G-H) or Etoposide (Etop) (20 µM) (I-J) for 48 hr. FUCA1 activity was assessed and expressed as arbitrary units (A.U.) per 25 µg of protein (n = 3 independent experiment, 9 replicates, one way Anova **p = 0.0008, ***p < 0.0001). (E,G,I). FUCA1 and p53 expression was assessed by western blotting and immunoblot against actin was used as a loading control (F,H,J).

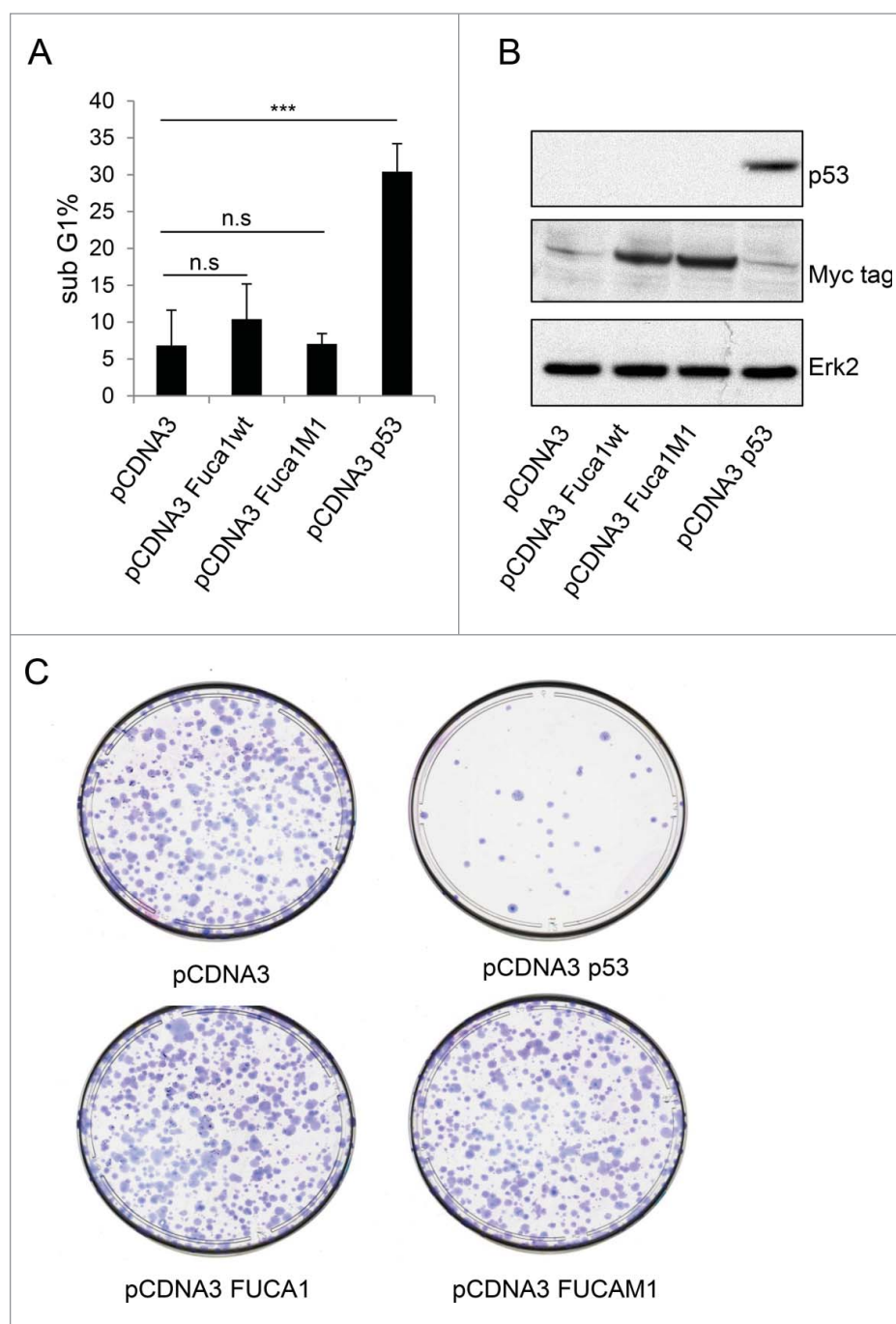


Figure 4. FUCA1 overexpression is not sufficient to induce cell death. Saos 2 cells were transiently co-transfected with either an empty pcDNA3 vector, a wild-type-Fuca1wt (pCDNA3 FUCA1wt), an enzymatically inactive mutant of FUCA1 (pCDNA3 FUCA1M1), or a wild-type-p53-expressing construct together with pCMV-CD20. After 72 h, the transfected cells were identified by staining for CD20 and analyzed for sub-G1 DNA content by flow cytometry (n = 4 independent experiments, one way Anova ***p < 0.0001) (A). (B) 24 hours post transfection p53 and Fuca1 expression were assessed by western blotting using respectively an anti-p53 antibody and an anti-Myc-tag antibody. Immunoblot against actin was used as a loading control. (C) Saos-2 cells were transfected with either an empty pcDNA3 vector, a wild-type-FUCA1wt (pCDNA3 FUCA1wt), an enzymatically inactive mutant of FUCA1 (pCDNA3 FUCA1M1), or a wild-type-p53-expressing construct. Following selection, cells were assessed for clonogenic survival using Giemsa staining (C).

targeting siRNA reduced FUCA1 expression and also reduced the percentage of cells with sub-G1 DNA content following induction of p53 expression by treatment with Dox (Fig. 5A and B). To confirm if the effect on sub-G1 DNA content was via modulation of p53-mediated apoptosis, we also examined the effect of FUCA1 knockdown on the ability of p53 to affect caspase activation and PARP cleavage – a substrate of

caspase activation.³⁹ In both cases, it was clearly evident that knockdown of FUCA1 impaired p53's ability to increase the activity of the effector caspase, caspase 3 and reduced the extent of p53-induced PARP cleavage (Fig. 5C and D). These collective results therefore show that while FUCA1 does not affect cell viability when expressed alone, it has a clear role in p53's apoptotic response.

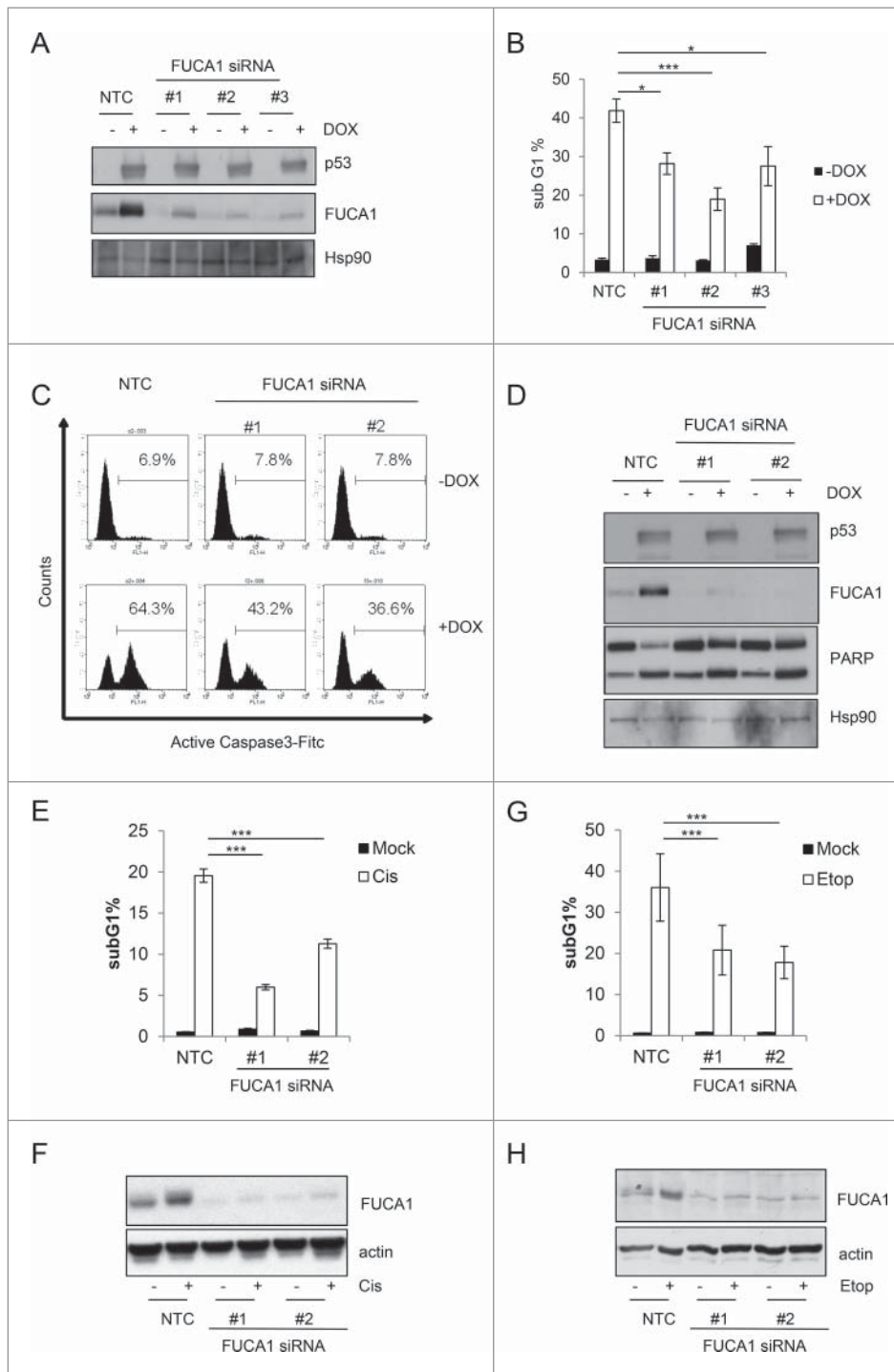


Figure 5. FUCA1 expression contribute to Chemotherapeutic-induced cell death. (A-D). TetOn-p53wt Saos2 cells were transfected with independent siRNA directed against FUCA1 prior to doxycycline treatment (DOX) for 48 hours. (A) P53 and Fuca1 expression were assessed by Western blotting and immunoblot against Hsp90 was used as a loading control. Cell death was analyzed by flow cytometry, measuring the percentage of cells with sub-G1 DNA content ($n = 3$ independent experiment, one way Anova $^{*}p < 0.01$, $^{***}p < 0.0001$) (B), Caspase 3 activation using an anti-active caspase 3 antibody (C), and western blotting assessing PARP cleavage (D). (E-H) U2OS E1a were transfected with 2 different siRNA directed against FUCA1 prior treatment with $20 \mu\text{M}$ Cisplatin (Cis) (E-F) or $20 \mu\text{M}$ Etoposide (Etop) (G-H) for 48 hours. Cells were assessed for cell death by flow cytometry measuring sub-G1 DNA content content ($n = 2$ independent experiment, 6 replicates one way Anova, $^{***}p < 0.0001$) (E-G) and expression of Fuca1 by western blotting. Actin was used as a loading control (F-H).

Since we had discovered that FUCA1 and fucosidase activity is induced by chemotherapeutic drugs in a p53-dependent manner (Figs. 2A, B, and 3E-J), we were naturally intrigued to know if FUCA1 plays a role in the apoptotic response to these agents. To this end, we once again employed FUCA1-targeting siRNAs

to knockdown FUCA1 in RKO and U2OS cells. These cells were then treated with either cisplatin (U2OS) or etoposide (U2OS and RKO) and the levels of FUCA1 and apoptosis were assessed by western blotting and flow cytometry respectively (Fig. 5E-H and Fig. S2). Following treatment with either drug, the extent of

FUCA1 induction was greatly reduced in cells transfected with FUCA1-targeting siRNAs when compared to controls (Fig. 5F, H and Fig. S2B). In addition and in line with a role for FUCA1 in p53-mediated cell death, the extent of apoptosis induced by etoposide or cisplatin was also markedly reduced by knockdown of FUCA1 (Fig. 5E, 5G and Fig. S2A).

Discussion

In this study we report that the gene encoding the fucosidase FUCA1 is induced by the tumor suppressor p53. We show that p53 mediates this effect by direct transcriptional activation through binding of p53 to *FUCA1* at a site within intron 1 of the gene. Moreover, we also report that FUCA1 is induced by different classes of chemotherapeutic drugs in a p53-dependent manner indicating that the induction of FUCA1 is a function of endogenous p53. Interestingly, for a gene that is so widely induced by p53, we were surprised to find that FUCA1 is not induced by the p53 family member, p73. In contrast, many key components of p53-mediated tumor suppression including *p21* and *PUMA* have also been reported to be induced by p73.⁴⁰⁻⁴² So, what does this tell us about the selective activation of FUCA1 by p53? It could be considered that FUCA1 is a relatively 'weak' target gene that is only moderately induced by p53 and as a result not induced by other p53-related proteins. Comparison, however, of the levels of *FUCA1* and other p53 targets studied in this TetOn system or in response to chemotherapeutic drugs, such as *DRAM1* and *ADORA2B*, which are also induced by p73,^{43,44} shows that the induction of *FUCA1* by p53 is at least equivalent. So, does this tell us that the function of p53 is indeed something specific to p53? Since p53 has a greater impact on tumor suppression compared to p73, it is tempting to speculate that the regulation of *FUCA1* represents a component of this p53-specific effect and future studies in this area would certainly be worthwhile.

Although beyond the scope of the present study, another question arising from our findings relates to how FUCA1 contributes to p53-mediated apoptotic death. Intriguingly, we found that expression of FUCA1 alone does not induce cell death indicating that unlike the induction of pro-apoptotic members of the Bcl2 family e.g. PUMA, NOXA and Bax,^{7-9,45} FUCA1 does not contribute to cell death via direct engagement of the cell death machinery. It is therefore clear that p53 must induce other target genes that work in conjunction with FUCA1 in order to execute a cell death response. It seems likely though that this would not be one target gene, but a number of other p53 targets making their identification a considerable challenge. As a result, perhaps future studies should focus on what functions of FUCA1 contribute to this effect. In this regard, we also report in this study that p53 and chemotherapeutic drugs not only induce FUCA1, but they also induce fucosidase activity. When this is coupled with the fact that naturally occurring mutations in FUCA1 that lead to fucosidosis all involve impairment of fucosidase activity,^{37,38} it seems conceivable that FUCA1 also contributes to p53-induced cell death through this enzymatic activity. If this is the case, then FUCA1 would function by the removal of fucose moieties from selective target proteins. As a result, the levels or activities of these proteins would change in a way that together with the action of other p53 target genes would push cells toward elimination by apoptotic death. Currently, however, no substrates of

fucosidase activity have been identified making this a large area for further investigation.

Since p53 and programmed cell death are both important components of tumor suppression, perhaps the biggest question arising from this work regards the role of fucosylation control in cancer. Interestingly, several previous studies have reported that glycosylation and more specifically fucosylation are perturbed during tumor development.¹⁸ Moreover, increased levels of a fucosylated protein are even used as an FDA-approved biomarker for the detection of hepatocellular carcinoma.^{46,47} However, whether these changes in glycosylation actually contribute to tumor development is still an open question. The evidence we provide in this study undoubtedly adds weight to this possibility, but the field is currently lacking appropriate animal models with which to address this issue. The development of mice where FUCA1 and as a result fucosidase activity could be deleted during the genesis of cancer as would be a great step forward in this area. In addition, since we show that FUCA1 and fucosidase activity are induced by chemotherapeutic drugs, the development of mice in which the relative levels of fucosylation could be modulated in established tumors may well lead to the identification of novel targets for treatment of tumors associated with changes in glycosylation.

Disclosure of potential conflicts of interest

The authors do not have any financial, personal or professional interests to declare that could be construed to influence this paper.

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