

Effect of p53 Status and STAT1 on Chemotherapy-Induced, Fas-Mediated Apoptosis in Colorectal Cancer

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Abstract

We investigated the role of p53 and the signal transducer and activator of transcription 1 (STAT1) in regulating Fas-mediated apoptosis in response to chemotherapies used to treat colorectal cancer. We found that 5-fluorouracil (5-FU) and oxaliplatin only sensitized p53 wild-type (WT) colorectal cancer cell lines to Fas-mediated apoptosis. In contrast, irinotecan (CPT-11) and tomudex sensitized p53 WT, mutant, and null cells to Fas-mediated cell death. Furthermore, CPT-11 and tomudex, but not 5-FU or oxaliplatin, up-regulated Fas cell surface expression in a p53-independent manner. In addition, increased Fas cell surface expression in p53 mutant and null cell lines in response to CPT-11 and tomudex was accompanied by only a slight increase in total Fas mRNA and protein expression, suggesting that these agents trigger p53-independent trafficking of Fas to the plasma membrane. Treatment with CPT-11 or tomudex induced STAT1 phosphorylation (Ser⁷²⁷) in the p53-null HCT116 cell line but not the p53 WT cell line. Furthermore, STAT1-targeted small interfering RNA (siRNA) inhibited up-regulation of Fas cell surface expression in response to CPT-11 and tomudex in these cells. However, we found no evidence of altered *Fas* gene expression following siRNA-mediated down-regulation of STAT1 in drug-treated cells. This suggests that STAT1 regulates expression of gene(s) involved in cell surface trafficking of Fas in response to CPT-11 or tomudex. We conclude that CPT-11 and tomudex may be more effective than 5-FU and oxaliplatin in the treatment of p53 mutant colorectal cancer tumors by sensitizing them to Fas-mediated apoptosis in a STAT1-dependent manner. (Cancer Res 2005; 65(19): 8951-60)

Introduction

The Fas death receptor is a member of the tumor necrosis factor (TNF) receptor superfamily (1). Binding of Fas to its cognate ligand, Fas ligand (FasL) or activating antibodies, results in recruitment of the Fas-associated death domain protein and caspase-8 zymogens to the receptor and the formation of the death-inducing signaling complex (DISC; ref. 2). Caspase-8 is activated at the DISC and in turn activates downstream executioner caspases, including caspase-3, which cleave a cassette of proteins resulting in cell death (3). Caspase-8 also activates the mitochondrial cell death pathway through cleavage and activation of the proapoptotic Bcl-2 family member Bid. A variety of chemotherapeutic agents have been shown

to up-regulate Fas expression in cancer cell lines, including colorectal cancer (4). The ability of chemotherapy drugs to induce the receptor has stimulated interest in targeting Fas with either therapeutic antibodies or peptides to enhance cell death.

Colorectal cancer is the second highest cause of cancer mortality in the Western world. Despite improvements in the efficacy of chemotherapy drugs used in the treatment of colorectal cancer, response rates in the advanced disease setting are of the order of 45% to 50% for the most effective drug combinations, with a median survival of up to 21 months (5). The most frequently used chemotherapeutic agents are the fluoropyrimidine 5-fluorouracil (5-FU), the topoisomerase I inhibitor irinotecan (CPT-11), and the platinum agent oxaliplatin. The antifolate tomudex is also used in the treatment of advanced colorectal cancer. Any strategies that target the Fas receptor must take account of the fact that ~45% to 61% of colon cancers harbor mutations in the tumor suppressor gene *p53* (6, 7). *Fas* is a well-documented p53 target gene (4), and loss of p53 function has been shown to affect the ability of cancer cells to up-regulate the Fas receptor in response to chemotherapy (8). However, there is increasing evidence that not all p53 mutations result in absolute loss of function (9, 10). Functional activities or properties of mutant proteins include retained wild-type (WT) activity (11), loss of function (12), gain of function (13, 14), and dominant-negative effects (15). The high frequency of p53 mutations in colorectal cancer increases the clinical significance of p53-independent mechanisms of apoptosis. IFN- γ has been shown to induce Fas expression in p53 mutant cell lines, and this induction was dependent on the signal transducer and activator of transcription 1 (STAT1) protein (16). IFN- γ activates STAT1 by triggering phosphorylation at two distinct sites: Tyr⁷⁰¹ and the COOH-terminal Ser⁷²⁷. The tyrosine phosphorylation results in STAT1 dimerization, translocation to the nucleus, and activation of target genes, whereas the serine phosphorylation strongly increases the transcriptional activity of STAT1. Recent data have indicated that some STAT1-dependent genes are still activated when Tyr⁷⁰¹ is mutated to a nonphosphorylatable phenylalanine residue (17, 18). The induction of p53 responsive genes, such as *Fas*, *Bax*, and *Noxa*, has been reported to be reduced in STAT1-deficient cells (19). Moreover, STAT1 can interact directly with p53, an association that is enhanced following DNA damage (19).

The aim of the present study was to investigate the roles of p53 and STAT1 in regulating Fas-mediated apoptosis in response to chemotherapy in colorectal cancer.

Materials and Methods

Cell lines and cell culture. The p53 WT HCT116 human colorectal adenocarcinoma cell line (HCT116^{+/+}) and a matched isogenic p53 knockout cell line (HCT116^{-/-}; kindly provided by B. Vogelstein, Johns Hopkins University School of Medicine, Baltimore, MD) were maintained in McCoy's medium (Life Technologies Invitrogen, Paisley, United Kingdom). H630 and RKO colorectal adenocarcinoma cell lines were maintained in

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DMEM. All media were supplemented with 10% dialyzed bovine calf serum. HCT116 cell lines expressing the R175H and R248W p53 mutations were created by dual transfection of HCT116 p53-null cells with plasmids containing the appropriate mutant p53 coding sequence (kind gift of Prof. G. Lozano, M.D. Anderson Cancer Center, Houston, TX) and a plasmid containing a puromycin resistance gene (20:1 ratio; pIRESpuo3, BD Biosciences, San Jose, CA). Colonies were selected and assayed for mutant p53 expression by Western blot as well as by nucleotide sequencing.

Western blotting. Western blots were done as described previously (6). The Fas (Santa Cruz Biotechnology, Santa Cruz, CA), p53 (Santa Cruz Biotechnology), caspase-8 (Oncogene Research Products, Merck Biosciences Ltd., Nottingham, United Kingdom), and poly(ADP-ribose) polymerase (PARP; Pharmingen, BD Bioscience, Franklin Lake, NJ) mouse monoclonal antibodies and STAT1, phospho-STAT1 (Ser⁷²⁷), and phospho-STAT1 (Tyr⁷⁰¹) rabbit polyclonal antibodies (Cell Signaling, Beverly, MA) were used in conjunction with a horseradish peroxidase-conjugated sheep anti-mouse or anti-rabbit secondary antibody (Amersham Biosciences, Bucks, United Kingdom). Equal loading was assessed using a β -tubulin mouse monoclonal primary antibody (Sigma-Aldrich, Dorset, United Kingdom). Densitometry was done using a Chemidoc XRS imager and QuantityOne software (Bio-Rad Laboratories, Hertfordshire, United Kingdom).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assay. Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO). Cells were seeded at 1,800 cells per well on 96-well plates 24 hours before drug treatment and were treated with a range of concentrations of 5-FU (Faulding Pharmaceuticals, Warwickshire, United Kingdom), tomudex (AstraZeneca, Macclesfield, United Kingdom), CPT-11 (Pharmacia and Upjohn, Kalamazoo, MI), and oxaliplatin (Sanofi-Synthelabo, Malvern, PA) for 24 hours, after which the agonistic Fas monoclonal antibody, CH-11 (MBL International, Woburn, MA), was added (25-100 ng/mL) for 48 hours. MTT was added to a final concentration of 500 μ g/mL and the cells incubated at 37°C for a further 3 hours. The culture medium was removed and formazan crystals were reabsorbed in 200 μ L DMSO. Cell viability was determined by reading the absorbance of each well at 570 nm using an ELISA plate reader (Molecular Devices, Wokingham, United Kingdom).

Flow cytometric analysis. Cells were seeded in a six-well tissue culture plate at a density of 1×10^5 cells per well. After 24 hours, 5-FU, tomudex, CPT-11, or oxaliplatin were added to the medium and the cells were cultured for a further 24 hours. CH-11 (10-100 ng/mL) was then added for 24 hours. DNA content of harvested cells was evaluated following propidium iodide staining of cells using the EPICS XL Flow Cytometer (Beckman Coulter UK, High Wycombe, United Kingdom). A FITC-conjugated monoclonal anti-human Fas antibody (DakoCytomation, Cambridgeshire, United Kingdom) was used to determine cell surface expression of the receptor. Cells were collected and washed twice in PBS before incubation with the antibody (1:20 dilution) at 4°C for 30 minutes. A nonreactive FITC-conjugated mouse IgG1 antibody was used as a negative control for each sample. Following incubation, the cells were washed twice with PBS containing 2% bovine serum albumin and fixed in 0.3 mL of 4% paraformaldehyde. Samples were then analyzed on the EPICS XL Flow Cytometer.

Caspase-8 assay. A fluorimetric caspase-8 assay kit was used (Sigma) to measure caspase-8 activity. This assay is based on the release of 7-amino-4-methyl coumarin (AMC) by caspase-8. Cells were seeded in a six-well tissue culture plate at a density of 10^5 cells per well and treated with the indicated doses of 5-FU, tomudex, CPT-11, and oxaliplatin the following day. After 24 hours, 100 ng/mL CH-11 was added for an additional 12 hours and the samples were suspended in 500 μ L of the provided lysis buffer. Lysate (20 μ L) was transferred to a 96-well plate and the fluorimetric assay was carried out according to the manufacturer's instructions. Fluorescence detection was carried out using a Microplate Fluorimeter (CytoFluor; Applied Biosystems, Foster City, CA) using excitation and emission wavelengths of 360 and 440 nm, respectively, at 5-minute intervals. Caspase-8 activity was calculated using an AMC calibration curve analyzed on the same plate. All samples were analyzed in duplicate, and the specificity of the reaction for caspase-8 was confirmed by analyzing a second set of duplicate lysates for each sample in the presence of a caspase-8 inhibitor (Ac-IETD-CHO).

Real-time PCR. Real-time reverse transcription-PCR (RT-PCR) was done using a DNA Engine Opticon 2 (Genetic Research Instrumentation Ltd., Essex, United Kingdom). RNA was collected using the RNA STAT-60 reagent (Tel-Test (Friendswood, TX) according to the manufacturer's instructions, and cDNA was synthesized using 1 μ g RNA and a reverse transcriptase kit (Life Technologies Invitrogen). Gene expression was analyzed using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland). PCR conditions consisted of an initial denaturation step of 95°C for 10 minutes followed by 39 cycles of 94°C for 10 seconds, 55°C for 20 seconds, and 72°C for 20 seconds, with a final extension of 72°C for 10 minutes. A melting curve was included at the end of each run to check the specificity of the amplified product. On completion of the run, the PCR products were

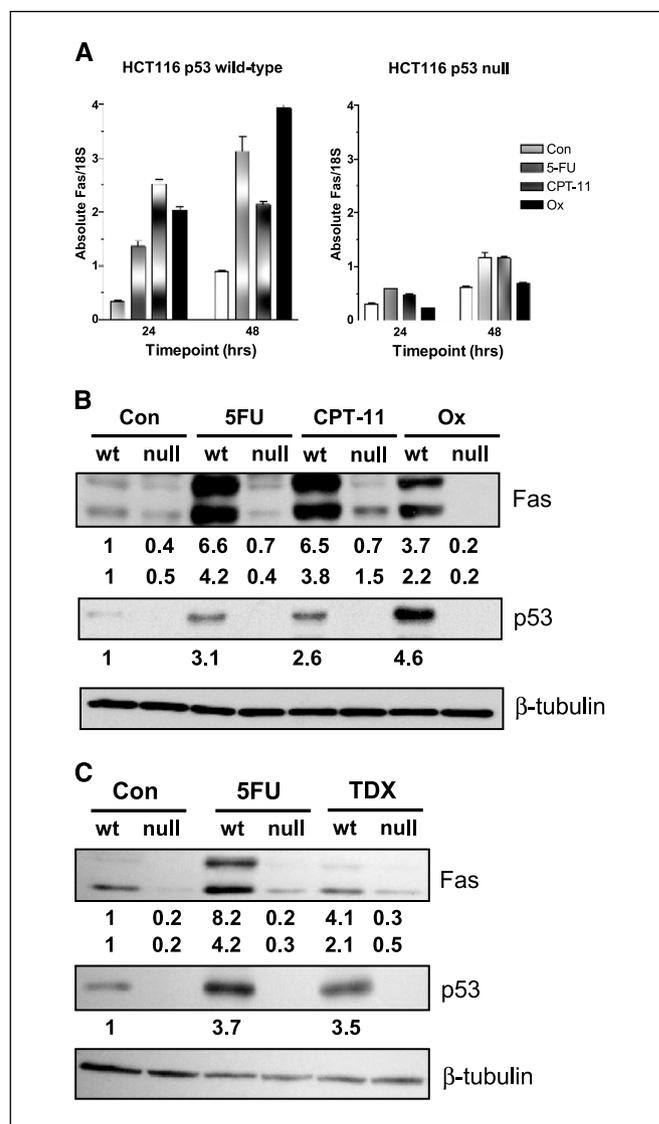


Figure 1. A, expression of Fas mRNA measured by real-time RT-PCR in the isogenic HCT116 p53 WT and null cell lines following treatment with 5-FU (5 μ M), CPT-11 (5 μ M), and oxaliplatin (Ox; 1 μ M) for 24 and 48 hours. Gene expression was calculated at each time point as a ratio of the expression of Fas mRNA to 18S rRNA. The expression of each gene was calculated using standard curves generated for each gene using a serial dilution of a control cDNA. An unpaired *t* test was used to determine whether a treatment effect reached statistical significance. B and C, Western blot analysis of Fas and p53 protein expression in the HCT116 p53 WT and null cell lines following treatment with 5-FU (5 μ M), tomudex (TDX; 100 nmol/L), CPT-11 (5 μ M), and oxaliplatin (1 μ M) for 48 hours. Densitometry values for the 48- and 38-kDa bands and p53 are indicated below the relevant immunoblot and are expressed relative to a control lane.

electrophoresed on a 2% agarose, 0.001% ethidium bromide gel to confirm that their size matched that of the expected amplicon. Primer sequences were as follows: Fas (forward) 5'-AAAGGCTTTGTTCCGAAAG-3', Fas (reverse) 5'-CACTCTAGACCAAGCTTTGG-3', 18S (forward) 5'-CATTCTGATTGC GCCGCTA-3', and 18S (reverse) 5'-CGACGGTATCTGATCGTCT-3'. Experiments were done in triplicate.

STAT1 small interfering RNA. The STAT1-targeted and nonsilencing control sequences used were 5'-AACTAGTGGAGTGGGAAGCGGA-3' and 5'-AAGGGTCGAGAGAGAGCTGTA-3', respectively (Dharmacon RNA Tech, Dallas, TX). The oligonucleotide was diluted in Opti-MEM-1 (Life Technologies Invitrogen) and transfected with Oligofectamine (Life Technologies Invitrogen) according to the manufacturer's instructions.

Statistical analyses. The nature of the interaction between the chemotherapeutic drugs and CH-11 was determined by calculating the R index (RI) as described by Kern (20) and modified by Romanelli (21). The RI is calculated as ratio of the expected cell survival (S_{exp} , defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) to the observed cell survival (S_{obs}) for the combination of A and B ($RI = S_{exp} / S_{obs}$). Synergism is then defined as a RI of greater than unity. This method was selected because treatment with CH-11 alone had little effect on cell viability, which meant that other methods, such as the median effect principle (22) and isobologram methods (23), were not suitable. To further assess the statistical significance of the interactions, we designed a univariate ANOVA using an additive model based on the null hypothesis that there was no interaction between the drugs (SPSS Software, SPSS, Inc., Chicago, IL). The unpaired *t* test was used to determine statistically significant differences between treatment effects. Significance was defined as $P < 0.05$.

Results

Induction of Fas mRNA expression by chemotherapy is p53-dependent. The majority of studies to date have found that induction of the Fas receptor is p53 dependent. A p53-responsive element has been identified within the first intron of the *Fas* gene as well as three putative elements within the promoter (4, 24). In agreement with this, treatment with IC_{60 72 hours} doses of 5-FU (5 μ mol/L), CPT-11 (5 μ mol/L), and oxaliplatin (1 μ mol/L) for 24 and 48 hours resulted in significant induction of Fas mRNA expression in response to these agents in the p53 WT HCT116 cells ($P < 0.001$ for each drug; Fig. 1A). Although the induction of Fas mRNA observed in the isogenic p53-null daughter HCT116 cell line was still significant for 5-FU and CPT-11, it was of a much smaller magnitude than that seen in the p53 WT cells. No significant induction was seen with oxaliplatin (Fig. 1A). These results suggested that induction of Fas mRNA by these chemotherapeutic agents was highly p53 dependent. We next analyzed whether this was reflected in increased protein expression. Treatment of the HCT116 p53 WT cell line with all four chemotherapies for 48 hours resulted in significant induction of Fas protein. Fas was detected as two bands of ~38 and ~48 kDa in agreement with the findings of others (25). The observed induction of Fas protein in this cell line was associated with induction of p53 (Fig. 1B and C). In the p53-null cell line, treatment with 5-FU and oxaliplatin did not significantly induce Fas protein expression; however, a small increase in Fas expression (38-kDa band) was observed in response to both CPT-11 and tomudex (Fig. 1B and C).

The agonistic Fas monoclonal antibody CH-11 synergistically activates apoptosis in response to CPT-11 and tomudex in a p53-independent manner. The agonistic anti-Fas antibody CH-11 has been shown to activate the Fas receptor and cause apoptosis (8). Treatment with 5-FU, tomudex, CPT-11, or oxaliplatin for 24 hours followed by CH-11 (100 ng/mL) for a further 48 hours resulted in a significant synergistic decrease in cell viability for each chemotherapy in the p53 WT HCT116 cell line as

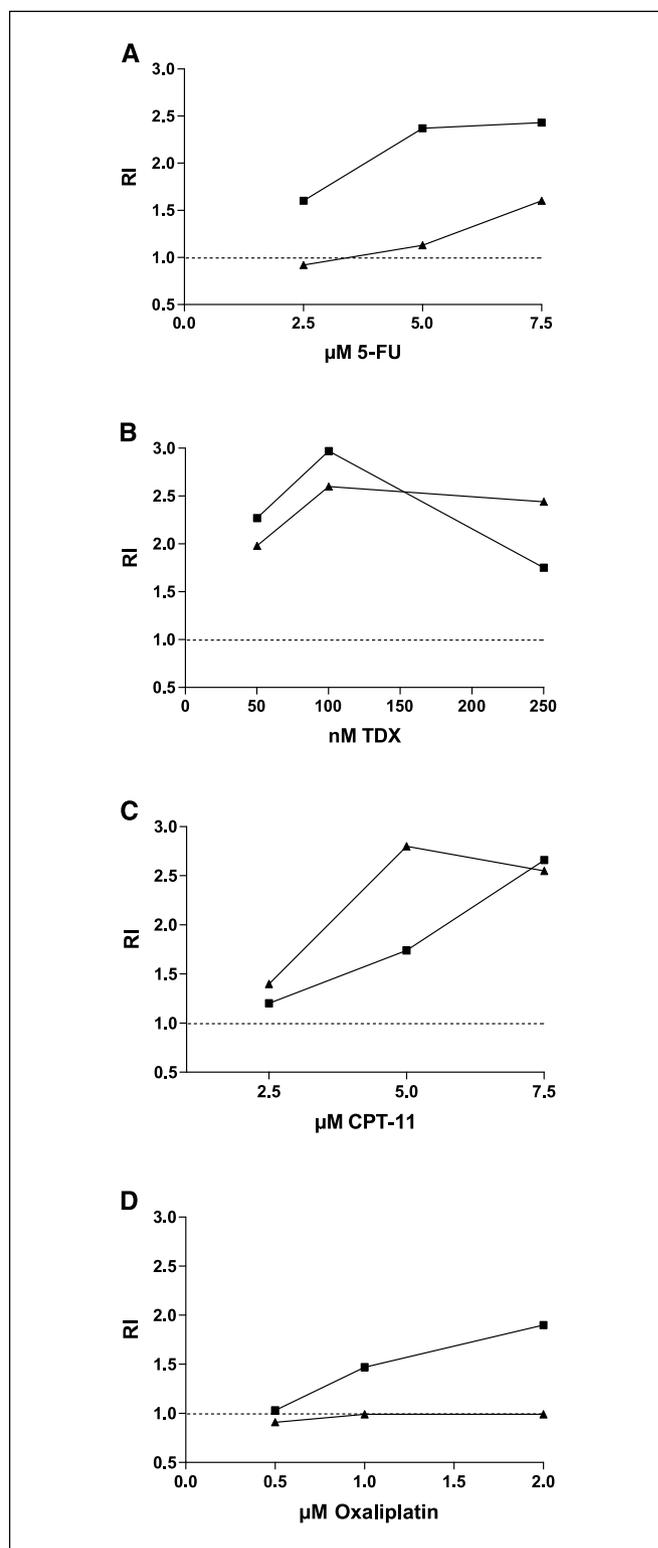
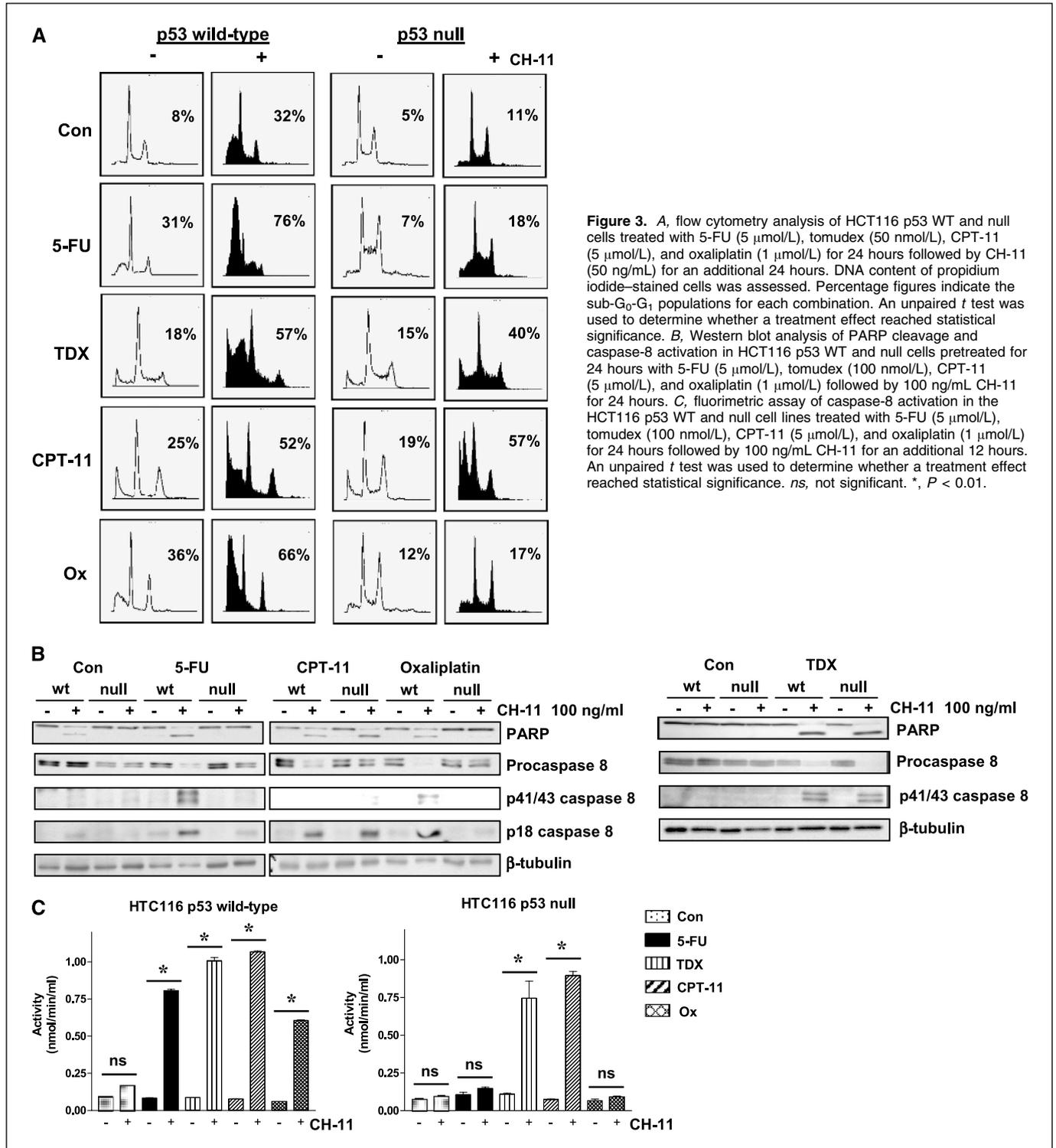


Figure 2. RI values calculated from MTT cell viability assays in response to (A) 5-FU, (B) tomudex, (C) CPT-11, and (D) oxaliplatin used in combination with the agonistic anti-Fas antibody CH-11 (100 ng/mL) in the HCT116 p53 WT (▲) and null cell lines (■). Mean of experiments carried out in triplicate. The RI is calculated as ratio of the expected cell survival (S_{exp} , defined as the product of the survival observed with drug A alone and the survival observed with drug B alone) to the observed cell survival (S_{obs}) for the combination of A and B ($RI = S_{exp} / S_{obs}$). Synergism is defined as $RI > 1$. Representative of at least three separate experiments. Univariate ANOVA was used to determine the statistical significance of the interaction between drug and CH-11.

indicated by RI values >1.5 for the majority of combinations (Fig. 2). In the p53-null cells, synergy between chemotherapy and CH-11 was clearly observed for CPT-11 and tomudex and was highly significant across all drug concentrations ($P < 0.001$; Fig. 2B and C). In contrast, no synergistic interaction was observed between CH-11 and oxaliplatin in the p53-null cells (Fig. 2D), whereas significant synergy between CH-11 and 5-FU was only observed at the highest concentration of 5-FU (7.5 $\mu\text{mol/L}$) with a RI value of ~ 1.5

($P < 0.01$), and the extent of this synergy was significantly less than in the p53 WT cells at this concentration (Fig. 2A).

Cell cycle analysis indicated that the addition of CH-11 (50 ng/mL) to each of the cell lines in the absence of chemotherapy resulted in a higher sub-G₀-G₁ apoptotic population in the HCT116 p53 WT cells compared with the p53-null cells (32% versus 11%; Fig. 3A). This is most likely a consequence of the higher constitutive expression of the Fas receptor in the p53 WT cell line (Fig. 4). Treatment of the p53



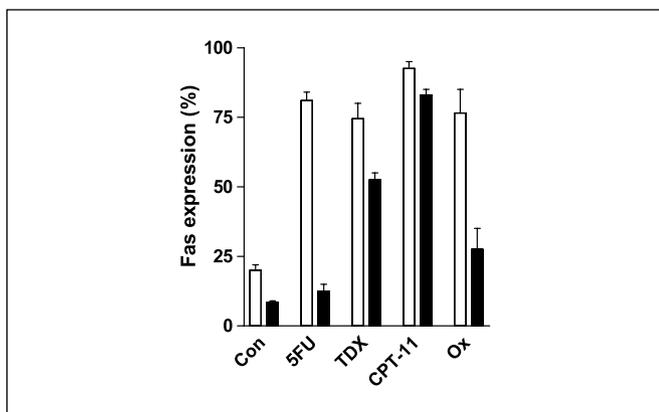


Figure 4. Flow cytometric analysis of Fas cell surface expression in HCT116 p53 WT (white) and null (black) cell lines treated with 5-FU (5 $\mu\text{mol/L}$), tomudex (100 nmol/L), CPT-11 (5 $\mu\text{mol/L}$), and oxaliplatin (1 $\mu\text{mol/L}$) for 48 hours (5,000 cells were examined for each sample). All samples were compared with an isotype-matched negative control.

WT cells with 5-FU alone resulted in a significant increase in the apoptotic fraction to $\sim 31\%$ ($P < 0.001$); however, the increase in apoptosis in response to 5-FU alone in the p53-null setting was not significant. When CH-11 was added to 5-FU-treated p53 WT cells, the induction of apoptosis was dramatic with $\sim 76\%$ of cells in the sub-G₀-G₁ fraction. In contrast, only a modest increase in apoptosis was observed in the p53-null cell line when CH-11 was added to 5-FU pretreated cells (18% compared with 11% and 7% for CH-11 alone and 5-FU alone, respectively). An almost identical pattern was observed in response to oxaliplatin, with the p53-null cells being much more resistant to apoptosis induced by oxaliplatin alone and, more significantly, oxaliplatin in combination with CH-11. In contrast, the levels of apoptosis induced by CPT-11 alone were similar in the p53 WT and null cell lines (25% and 19%, respectively). More importantly, combined treatment with CPT-11 and CH-11 also resulted in similar levels of apoptosis in the p53 WT and null cell lines (52% and 57%, respectively). Results with tomudex were similar to those with CPT-11, with relatively high levels of apoptosis induced in the p53-null cells following tomudex and CH-11 treatment; however, the induction of apoptosis in tomudex/CH-11-treated cells was still significantly higher in the p53 WT cell line (57% compared with 40%; $P = 0.02$).

These results were further confirmed by Western blot analysis of cells, which were treated with 5-FU, tomudex, CPT-11, and oxaliplatin followed by CH-11. In the p53 WT setting, cotreatment with each drug and CH-11 resulted in PARP cleavage and activation of caspase-8, with cleavage of pro-caspase-8 and generation of the active p41/p43 and p18 subunits observed (Fig. 3B). In contrast, in the p53-null cell line, PARP cleavage and pro-caspase-8 activation following the addition of CH-11 was only seen in cells treated with CPT-11 and tomudex (Fig. 3B). Caspase-8 activity was also directly measured using a fluorimetric assay. In the p53 WT cell line, the combination of CH-11 and each chemotherapy resulted in a synergistic increase in caspase-8 activity, which was highly significant ($P < 0.001$). In contrast, in the HCT116 p53-null cell line, increased activity was only observed when CH-11 was combined with either tomudex or CPT-11 (Fig. 3C). Collectively, these results indicate that CH-11 synergistically activated caspase-8 and apoptosis in response to each chemotherapy in the p53 WT setting but only in response to CPT-11 and tomudex in the p53-null setting.

CPT-11 and tomudex cause a p53-independent induction of Fas cell surface expression. Flow cytometric analysis of immunostained cells showed higher constitutive cell surface expression of the Fas receptor in the HCT116 p53 WT cell line compared with the p53-null cell line. This is probably the basis for the greater sensitivity

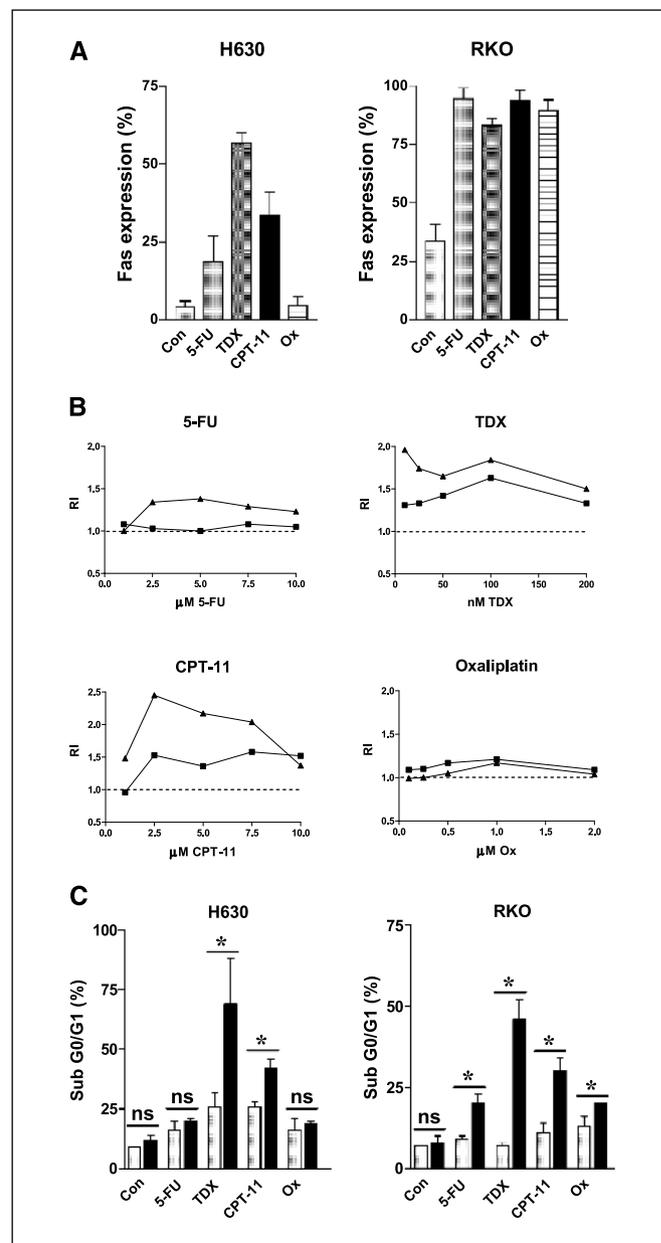


Figure 5. A, flow cytometric analysis of Fas cell surface expression in the H630 and RKO cell lines treated with IC₅₀ doses of 5-FU, tomudex, CPT-11, and oxaliplatin for 48 hours (5,000 cells were examined for each sample). All samples were compared with an isotype-matched negative control. B, RI values calculated from MTT cell viability assays following treatment with 5-FU, tomudex, CPT-11, and oxaliplatin in combination with CH-11 (100 ng/mL) in the H630 (▲) and RKO (■) cell lines. Cells were treated with IC₅₀ doses of each drug for 24 hours before the addition of CH-11 for an additional 48 hours. Univariate ANOVA was used to determine the statistical significance of the interaction between drug and CH-11. C, percentage of sub-G₀-G₁ cells was calculated by flow cytometry in the H630 and RKO cells treated with a combination of each of the chemotherapy drugs for 24 hours followed by CH-11 (100 ng/mL) for a further 24 hours. The DNA content of propidium iodide-stained cells was assessed. An unpaired *t* test was used to determine whether a treatment effect reached statistical significance. *, $P < 0.01$.

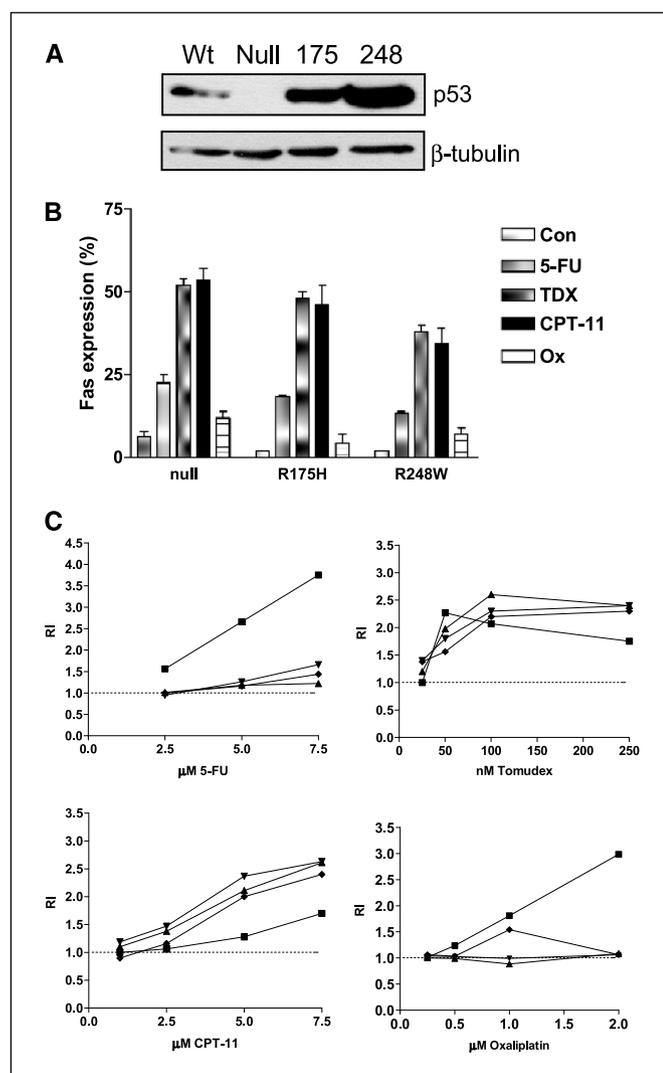


Figure 6. A, p53 protein expression in HCT116 p53 WT, null, and mutant (R175H and R248W) cell lines. B, flow cytometric analysis of Fas cell surface expression in HCT116 p53-null and mutant (R175H and R248W) cell lines treated with 5-FU (5 $\mu\text{mol/L}$), tomudex (100 nmol/L), CPT-11 (5 $\mu\text{mol/L}$), and oxaliplatin (1 $\mu\text{mol/L}$) for 48 hours. The percentage of cells expressing the Fas death receptor is shown (5,000 cells were examined for each sample). All samples were compared with an isotype-matched negative control. C, RI values calculated from MTT cell viability assays following treatment with 5-FU, tomudex, CPT-11, and oxaliplatin in combination with CH-11 (100 ng/mL) in the p53 WT (\blacktriangle), null (\blacksquare), and mutant R175H (\blacktriangledown) and R248W (\blacklozenge) cell lines. Univariate ANOVA was used to determine the statistical significance of the interaction between each drug and CH-11.

of the p53 WT cells to CH-11 in the absence of chemotherapy (Fig. 3A and B). Dramatic increases in Fas cell surface expression were observed in the p53 WT cell line following treatment with each of the chemotherapeutic agents (Fig. 4). However, in the p53-null setting, the induction of cell surface Fas expression following treatment with 5-FU and oxaliplatin was significantly lower than in the p53 WT cell line. In contrast, high levels of cell surface Fas were expressed in response to CPT-11 and tomudex in the p53-null cell line. Of note, the increased cell surface expression of Fas in response to CPT-11 and tomudex in the HCT116 p53-null cell line was accompanied by only a modest increase in total Fas expression (Fig. 1B and C), suggesting that these agents may trigger p53-independent trafficking of Fas to the cell surface. Thus, CPT-11 and tomudex induce p53-independent up-regulation of Fas at the cell surface.

Chemotherapy-induced Fas-mediated apoptosis in other colorectal cancer cell lines. We extended our study to examine whether these effects were observed in other colorectal cancer cell lines. Fas-mediated cell death in response to chemotherapy was examined in the p53 WT RKO and p53 mutant H630 cell lines. Induction of the Fas receptor at the cell surface in the H630 cell line was seen in response to CPT-11 and tomudex and to a lesser extent 5-FU but not oxaliplatin (Fig. 5A). In the p53 WT RKO cell line, there was induction of the Fas receptor at the cell surface in response to IC₆₀ doses of all four chemotherapeutic agents (Fig. 5A). When the H630 cell line was treated with these chemotherapy drugs for 24 hours followed by CH-11 for an additional 48 hours, significant synergy was evident in cells treated with CPT-11 and tomudex ($P < 0.001$) but not 5-FU or oxaliplatin (Fig. 5B). In the RKO cell line, 5-FU, tomudex, and CPT-11 all synergized with CH-11 ($P < 0.001$; Fig. 5B). Surprisingly, oxaliplatin and CH-11 did not synergize in RKO cells despite the high levels of Fas cell surface expression in oxaliplatin-treated RKO cells (Fig. 5A). The synergy (or lack of synergy) between each drug and CH-11 in each cell line was reflected in the extent of apoptosis in chemotherapy and CH-11 cotreated cells as determined by flow cytometry (Fig. 5C). At high concentrations of chemotherapy, lower RI values were sometimes observed than at low concentrations (Fig. 5B). This may be due to more efficient induction of cell death in response to chemotherapy alone at these higher concentrations, with the result that the synergistic effects of CH-11 are less marked.

Effect of specific p53 mutants on chemotherapy-induced Fas-mediated apoptosis. To analyze the effects of specific p53 mutant proteins on chemotherapy and Fas-mediated cell death, we generated HCT116 cell lines expressing the R175H and R248W mutants, which are among the most prevalent p53 mutations in colorectal cancer.¹ Western blot analysis of p53 expression in the two p53 mutant variants showed significant levels of expression of the R175H and R248W mutant p53 protein in the respective cell lines (Fig. 6A). Treatment of the p53 mutant cell lines with 5-FU, tomudex, CPT-11, and oxaliplatin for 48 hours resulted in similar changes in Fas cell surface expression as those observed in the p53-null cell line (Fig. 6B). Of note, tomudex and CPT-11 dramatically up-regulated Fas cell surface expression in both of the p53 mutant cell lines. Furthermore, both of the p53 mutant cell lines behaved in a similar manner to the p53-null cell line following treatment with each chemotherapeutic agent and CH-11, with highly synergistic decreases in cell viability evident when CH-11 was added to tomudex or CPT-11 in these cell lines ($P < 0.001$ for each chemotherapy; Fig. 6C).

Modulation of STAT1 activity and its effect on Fas surface expression. It has been reported previously that STAT1 is an important regulator of Fas expression (16), although a STAT1-responsive element has not been identified in the promoter of *Fas* (26). The induction of p53-responsive genes, such as *Fas*, has been reported to be reduced in STAT1-deficient cells (19). We found no evidence of phosphorylation at Tyr⁷⁰¹ of STAT1 in response to treatment with chemotherapy in either the p53 WT or null HCT116 cell lines (Fig. 7A). However, in the p53-null cell line, treatment with tomudex and CPT-11 resulted in a dramatic increase in phosphorylation of STAT1 on Ser⁷²⁷ after 12 hours (Fig. 7B). Moreover, induction of STAT1 Ser⁷²⁷ phosphorylation in response to 5-FU and oxaliplatin in this cell line was much lower than for CPT-11 and tomudex. Interestingly, in the p53 WT cell line, STAT1 Ser⁷²⁷

¹ <http://www-p53.iarc.fr/index.html>

phosphorylation was not observed in response to CPT-11 and was barely detectable in response to tomudex.

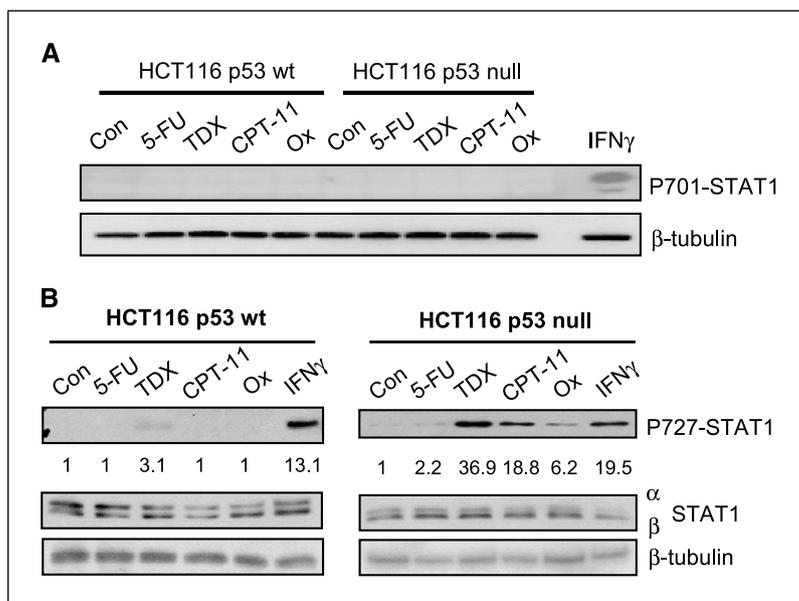
To determine whether STAT1 Ser⁷²⁷ phosphorylation was involved in up-regulating Fas expression in response to chemotherapy, we examined the effect of STAT1 small interfering RNA (siRNA) on Fas expression following treatment with tomudex and CPT-11 in the HCT116 p53 WT and null cell lines. STAT1-targeted siRNA down-regulated expression of both α and β isoforms of STAT1 in the HCT116 cell lines (Fig. 8A). The STAT1 α splice form is the transcriptionally active form containing the Ser⁷²⁷ phosphorylation site; the β splice form lacks this site (27). Interestingly, no changes in chemotherapy-induced up-regulation of Fas mRNA were observed following STAT1 down-regulation in either cell line (Fig. 8B). Despite this, STAT1 down-regulation surprisingly resulted in a significant decrease in Fas cell surface expression in the HCT116 p53-null cell line following treatment with CPT-11 and tomudex for 48 hours (Fig. 8C). Importantly, down-regulation of both the isoforms of STAT1 was still evident at this time point (72 hours after transfection with siRNA; Fig. 8D). In contrast, induction of Fas cell surface expression in response to CPT-11 and tomudex (and also 5-FU and oxaliplatin; data not shown) was not affected by STAT1 knockdown in the p53 WT setting. To determine whether the reduction in Fas expression seen with this siRNA approach abrogated the induction of apoptosis in response to CPT-11 and CH-11, we cotreated STAT1 siRNA-transfected p53-null cells with CPT-11 and CH-11 (Fig. 8E). We found that STAT1 down-regulation inhibited PARP cleavage and caspase-8 activation in response to cotreatment with CPT-11 and CH-11 (10-25 ng/mL). Higher concentrations of CH-11 (>50 ng/mL) still induced apoptosis in STAT1 siRNA-transfected cells, which may reflect the fact that STAT1 siRNA did not completely abrogate the induction of Fas cell surface expression by CPT-11 (Fig. 8C). This suggests that either STAT1 silencing was incomplete or that alternative STAT1-independent signaling pathways operate to regulate CPT-11-induced Fas cell surface expression. Collectively, these results suggest that phosphorylation of STAT1 at Ser⁷²⁷ results in transcription of a cofactor(s) that mediates Fas cell surface trafficking in response to CPT-11 and tomudex in the HCT116 p53-null cell line.

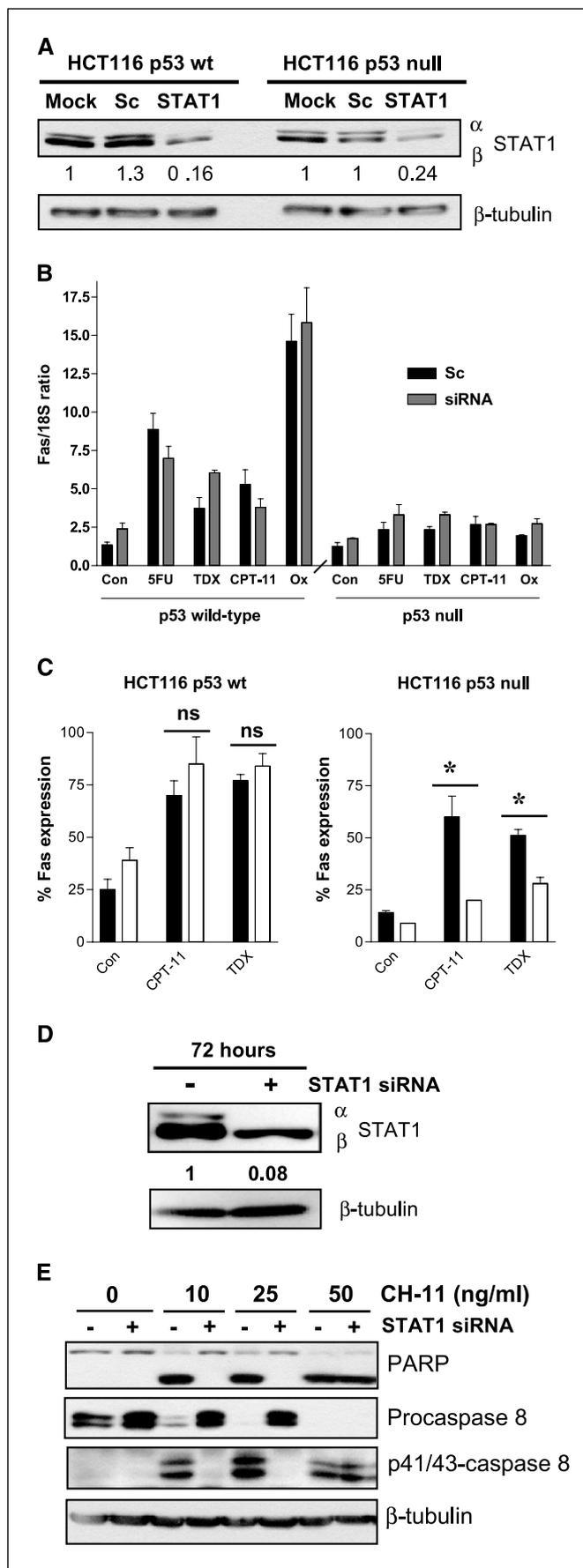
Discussion

The aim of this study was to investigate the role of p53 and STAT1 in regulating Fas-mediated apoptosis in response to chemotherapy in colorectal cancer. The ability of various chemotherapeutic agents to up-regulate expression of the Fas death receptor raises the possibility of targeting the receptor therapeutically to enhance cell death. 5-FU and tomudex as single agents in the treatment of advanced colorectal cancer have shown equivalent efficacy in terms of survival and objective response (16.7% versus 19.3%; ref. 28). Hematologic and gastrointestinal toxicities are frequent for both 5-FU and tomudex (28). The response rates for single agent CPT-11 and oxaliplatin are significantly higher than for 5-FU (24-26%; refs. 29, 30), which led to trials of combinations of these drugs with 5-FU in advanced colorectal cancer. The major adverse reactions with CPT-11 are diarrhea and neutropenia (20-40% of patients), whereas with oxaliplatin neurotoxicity is the major adverse reaction (13% of patients; refs. 29, 30). Combinations of either CPT-11 or oxaliplatin with 5-FU are the most efficacious and frequently used chemotherapy regimens in the treatment of advanced colorectal cancer, yet response rates are still only 40% to 50% and median survival is in the region of 22 months (31). There is therefore considerable scope for improving the therapeutic efficacy of these chemotherapy agents in this disease.

Both agonistic anti-Fas antibodies and Fas-specific peptides have been explored for their potential as targeted therapies against the Fas death receptor. Initial attempts to develop a therapeutic anti-Fas antibody were confounded by severe hepatic toxicity in the three murine models investigated (32). However, a novel agonistic Fas monoclonal antibody has recently been developed, which lacks the severe hepatic toxicity seen previously, suggesting that it may be possible to develop therapeutically useful nontoxic Fas-targeted antibodies (33). Other approaches to activate Fas-mediated apoptosis include local delivery of recombinant FasL and *FasL* gene therapy. In addition, targeting of cell surface Fas constitutes a major mechanism by which immune effector cells, such as circulating T lymphocytes, can eliminate tumor cells, which suggests that clinical studies combining immunotherapy with chemotherapy might be very effective. It is therefore important to

Figure 7. A, Western blot analysis of STAT1 phosphorylation at Tyr⁷⁰¹ in the HCT116 p53 WT and null cell lines 12 hours following treatment with 5-FU (5 μ mol/L), tomudex (100 nmol/L), CPT-11 (5 μ mol/L), and oxaliplatin (1 μ mol/L). An IFN- γ -treated sample was used as a positive control. B, Western blot analysis of STAT1 Ser⁷²⁷ phosphorylation and total STAT1 expression in the HCT116 p53 WT and null cell lines 12 hours following treatment with 5-FU (5 μ mol/L), tomudex (100 nmol/L), CPT-11 (5 μ mol/L), and oxaliplatin (1 μ mol/L). An IFN- γ -treated sample was used as a positive control. Densitometry values are shown for phosphorylated STAT1 Ser⁷²⁷.





understand the mechanisms regulating Fas expression in response to the different chemotherapeutic agents currently used in the treatment of colorectal cancer.

Many genes regulated by p53 have been shown to participate in apoptotic pathways, including *Bax*, *DR5*, *Puma*, *Noxa*, and *Fas*. Owen-Schaub et al. have shown that introduction of WT p53 into the p53 mutant HT29 cell line enhanced cell surface expression of the Fas receptor (34). A strong correlation has also been shown between WT p53 status and induction of Fas receptor expression in cancer cell lines exposed to chemotherapeutic drugs (4, 8, 35). Recent studies have described a p53-responsive element in intron 1 of the human *Fas* gene as well as three putative elements in the promoter (4, 24). Our findings support the concept that for some chemotherapy agents (i.e., 5-FU and oxaliplatin) the ability to induce the Fas receptor is highly dependent on p53-mediated transcriptional up-regulation. However, we have shown for the first time that for other chemotherapies (tomudex and CPT-11) a different p53-independent mechanism can also operate to up-regulate Fas cell surface expression. Furthermore, this mechanism seems to be largely independent of transcriptional up-regulation. Although 5-FU can act as a thymidylate synthase inhibitor, RNA-directed cytotoxicity has been reported to be the more important cytotoxic mechanism in the HCT116 cell line (35). This may be the cause of the differences in the ability of 5-FU and tomudex to induce Fas cell surface expression in the p53-null HCT116 cell line. In support of this, we have recently found that p53 mutant HT29 cells induce Fas cell surface expression in response to both 5-FU and tomudex, and this results in synergistic induction of apoptosis following the addition of CH-11 to both drugs (data not shown). This is notable because the cytotoxic effects of 5-FU in the HT29 cell line have been reported to be predominantly due to thymidylate synthase inhibition (35). The anticancer effects of 5-FU *in vivo* are believed to be predominantly due to thymidylate synthase inhibition; therefore, in this setting, 5-FU treatment may also induce Fas in a p53-independent manner.

Mutations in p53 are present in at least 50% of all colorectal tumors, and specific mutations at codons 175 and 248 account for more than a third of all the p53 mutations in these tumors. We therefore analyzed the effects of these clinically relevant p53 mutations on chemotherapy-induced Fas signaling. Previous reports have found that p53-responsive elements of the *Fas* gene are still capable of being activated by p53 mutants, indicating that some mutant p53 proteins may retain the ability to up-regulate Fas transcription in response to chemotherapy (24, 36). However, we found that cell lines stably expressing the p53 mutations, R175H and R248W, behaved in an almost identical fashion to the p53-null

Figure 8. A, STAT1 α/β expression in the HCT116 p53 wt and null cell lines 24 hours after transfection with 10 nmol/L STAT1 siRNA (STAT1) or a nonsilencing control (Sc) or no siRNA (Mock). Densitometry values are shown for STAT1 α . B, real-time RT-PCR of Fas mRNA expression 24 hours following treatment with 5-FU, tomudex, CPT-11, and oxaliplatin in cells transfected with 10 nmol/L STAT1 siRNA or a nonsilencing control. C, Fas cell surface expression in response to CPT-11 and tomudex treatment for 48 hours in HCT116 p53 WT and null cells transfected with 10 nmol/L STAT1 siRNA (white) or a nonsilencing control (black). All samples were compared with an isotype-matched control. An unpaired *t* test was used to determine whether a treatment effect reached statistical significance. *, *P* < 0.01. D, STAT1 expression in the HCT116 p53-null cell line at 72 hours after transfection with 10 nmol/L STAT1 siRNA or a nonsilencing control. Densitometry values are shown for STAT1 α . E, Western blot analysis of PARP cleavage and caspase-8 activation in STAT1 or control siRNA-transfected HCT116 p53-null cells cotreated with CPT-11 (5 μ mol/L) for 24 hours followed by a range of concentrations of CH-11 for an additional 24 hours.

cell line with respect to drug sensitivity, induction of Fas cell surface expression in response to chemotherapy, and synergy between chemotherapy and CH-11. The ability of tomudex and CPT-11 to induce Fas cell surface expression in the p53-null and mutant cell lines suggests that, in patients with p53 mutant tumors, the use of therapies directed against the Fas receptor would be best combined with either of these two drugs.

IFN- γ has been shown to induce Fas expression in p53 mutant cell lines and this induction was dependent on STAT1 (16). IFN- γ stimulates gene expression through phosphorylation of the Tyr⁷⁰¹ residue of STAT1 by Janus-activated kinases associated with the IFN- γ receptor. Tyr⁷⁰¹ phosphorylation induces dimerization of STAT1, nuclear translocation, and binding to IFN- γ -activated sequence (GAS) elements in the promoters of target genes. The absence of a GAS element in the promoter region of *Fas* makes it possible that additional factors are transcriptionally activated by STAT1 to regulate Fas expression. In addition, STAT1 Ser⁷²⁷ phosphorylation can transactivate genes independently of Tyr⁷⁰¹ phosphorylation (17). Initially, Tyr⁷⁰¹ phosphorylation was thought to be necessary for subsequent Ser⁷²⁷ phosphorylation of STAT1 to occur. However, stress stimuli, such as UV, lipopolysaccharides, TNF- α , and osmotic stress, have been shown to cause phosphorylation of STAT1 at Ser⁷²⁷ without phosphorylation at Tyr⁷⁰¹, and this has been reported to be mediated by p38 mitogen-activated protein kinase (MAPK; ref. 37). The phosphorylation of STAT1 at Ser⁷²⁷ that we observed in response to tomudex and CPT-11 in the p53-null HCT116 cell line suggested that a STAT1-dependent mechanism was responsible for the modulation of Fas cell surface expression, although neither drug activated STAT1 Tyr⁷⁰¹. Intriguingly, we observed that STAT1 siRNA significantly reduced CPT-11- and tomudex-induced Fas cell surface expression in the p53-null cell line despite having no direct effect on Fas mRNA expression. This suggests that STAT1 up-regulates expression of a gene (or genes) involved in trafficking of Fas to the cell surface in response to CPT-11 and tomudex. Furthermore, it would seem that activation of this target gene(s) is dependent on STAT1 phosphorylation at Ser⁷²⁷ but not at Tyr⁷⁰¹.

At present, it is unclear why CPT-11- and tomudex-induced Fas cell surface expression in HCT116 p53 WT cell line is largely STAT1 independent but STAT1 dependent in p53-null cells. p38 MAPK has

been reported to induce STAT1 Ser⁷²⁷ phosphorylation (38), and p38 is negatively regulated by the p53 target gene WIP-1 (39). It is possible that p53 induction in HCT116 p53 WT cells, which we observed in response to each chemotherapy, stimulates induction of WIP-1, which then inhibits p38-mediated Ser⁷²⁷ phosphorylation of STAT1. p53 can also up-regulate PTEN, which inhibits accumulation of phosphatidylinositol-3,4,5-triphosphate (PIP3). Reduced levels of PIP3 may then inhibit phosphoinositide-dependent kinase-1-mediated activation of protein kinase C δ (PKC δ), a serine kinase that has been shown to phosphorylate STAT1 (40, 41). In the absence of p53, p38- and/or PKC δ -mediated STAT1 Ser⁷²⁷ phosphorylation may occur in response to CPT-11 and tomudex resulting in transcription of a gene (or genes) that mediate translocation of Fas to the cell surface. In the p53 WT setting, the increased Fas cell surface expression following chemotherapy may simply reflect the higher levels of total Fas expression resulting from increased *Fas* gene transcription. It is also possible that p53 regulates alternative Fas trafficking mechanisms that are STAT1 independent. These possibilities are currently under investigation.

In conclusion, we have found that of the chemotherapies used to treat colorectal cancer CPT-11 and tomudex up-regulate Fas cell surface expression in a p53-independent, STAT1-dependent manner. Moreover, the mechanism for this up-regulation does not involve increased *Fas* gene expression, suggesting that these agents induce trafficking of Fas to the cell membrane. These findings suggest that tomudex and CPT-11 may be more effective anticancer drugs than 5-FU and oxaliplatin in the treatment of p53 mutant colorectal tumors. Furthermore, our data suggest that Fas-targeted approaches may be most effective in colorectal cancer when used in combination with CPT-11 and tomudex.

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Effect of p53 Status and STAT1 on Chemotherapy-Induced, Fas-Mediated Apoptosis in Colorectal Cancer

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