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Modulation of p53β and p53γ expression by regulating the alternative splicing of *TP53* gene modifies cellular response

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In addition to the tumor suppressor p53 protein, also termed p53α, the *TP53* gene produces p53β and p53γ through alternative splicing of exons 9β and 9γ located within *TP53* intron 9. Here we report that both TG003, a specific inhibitor of Cdc2-like kinases (Clk) that regulates the alternative splicing pre-mRNA pathway, and knockdown of SFRS1 increase expression of endogenous p53β and p53γ at mRNA and protein levels. Development of a *TP53* intron 9 minigene shows that TG003 treatment and knockdown of SFRS1 promote inclusion of *TP53* exons 9β/9γ. In a series of 85 primary breast tumors, a significant association was observed between expression of SFRS1 and α variant, supporting our experimental data. Using siRNA specifically targeting exons 9β/9γ, we demonstrate that cell growth can be driven by modulating p53β and p53γ expression in an opposite manner, depending on the cellular context. In MCF7 cells, p53β and p53γ promote apoptosis, thus inhibiting cell growth. By transient transfection, we show that p53β enhanced p53α transcriptional activity on the *p21* and *Bax* promoters, while p53γ increased p53α transcriptional activity on the *Bax* promoter only. Moreover, p53β and p53γ co-immunoprecipitate with p53α only in the presence of p53-responsive promoter. Interestingly, although p53β and p53γ promote apoptosis in MCF7 cells, p53β and p53γ maintain cell growth in response to TG003 in a p53α-dependent manner. The dual activities of p53β and p53γ isoforms observed in non-treated and TG003-treated cells may result from the impact of TG003 on both expression and activities of p53 isoforms. Overall, our data suggest that p53β and p53γ regulate cellular response to modulation of alternative splicing pre-mRNA pathway by a small drug inhibitor. The development of novel drugs targeting alternative splicing process could be used as a novel therapeutic approach in human cancers.

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Similar to 95% of human genes,[1,2] the tumor suppressor *TP53* gene expresses several p53 protein isoforms,[3,4] including C-terminal isoforms produced by alternative splicing of intron 9[3] (Figure 1a). Exclusion of the entire intron 9 generates the canonical full-length p53 protein (or p53α), a transcription factor activated in response to diverse intracellular and extracellular signals (Figure 1b). Hence, p53 regulates gene expression to modulate cell-fate outcome by regulating biological processes, including apoptosis and cell cycle progression.[5] Inclusion of alternative exons 9β and 9γ contained in intron 9 gives rise to p53β and p53γ protein isoforms that present new residues in place of the usual oligomerization domain present in p53α. Early studies reported that p53β and p53γ retain characteristics of a tumor suppressor.[4] Indeed, p53β modulates p53α transcriptional activity in a promoter-dependent manner in response to stress[3] and promotes apoptosis and senescence, indicating that p53β can modulate p53α suppressive activity.[3,6] The biological significance of p53β and p53γ isoforms is emphasized by clinical data. Both p53β and p53γ expression is lost in $\sim$60% of breast cancer tumors.[3,7] Furthermore, breast cancer patients expressing both mutant p53 and p53γ had disease outcome as good as patients carrying wild-type p53 tumors, whereas breast cancer patients expressing mutant p53 but not p53γ had a poorer prognosis.[7]

Little is known about the mechanisms leading to deregulation of p53β and p53γ expression in cancers. We have shown that p53β and p53γ proteins are ubiquitinated and degraded by the proteasome.[8] Recently, the serine/arginine-rich (SR) protein SFRS3 has been identified to promote the inclusion of *TP53* intron 9β.[9] SR proteins are essential in spliceosome assembly and are specifically and finely regulated by splicing-related kinases, such as Clk (Cdc2-like kinases) family.[10,11] Small drugs have been shown to specifically inhibit members of the Clk family.[12] In particular, TG003 inhibits Clk1/4 activity, resulting in SFRS1 dephosphorylation, which induced SFRS1 subcellular re-localization and inhibited the SFRS1-dependent splicing of globin mRNA. Moreover, TG003 has been shown to modulate alternative splicing *in vitro* and *in vivo*,[12,13] and to activate p53 inducing a p53-mediated cell response.[14] Here we investigated the effects of TG003 and SFRS1 on splicing of *TP53* intron 9 and characterized the biological consequences.

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Abbreviations: SR, serine/arginine-rich protein; Clk, Cdc2-like kinase; siCT, non-relevant siRNA
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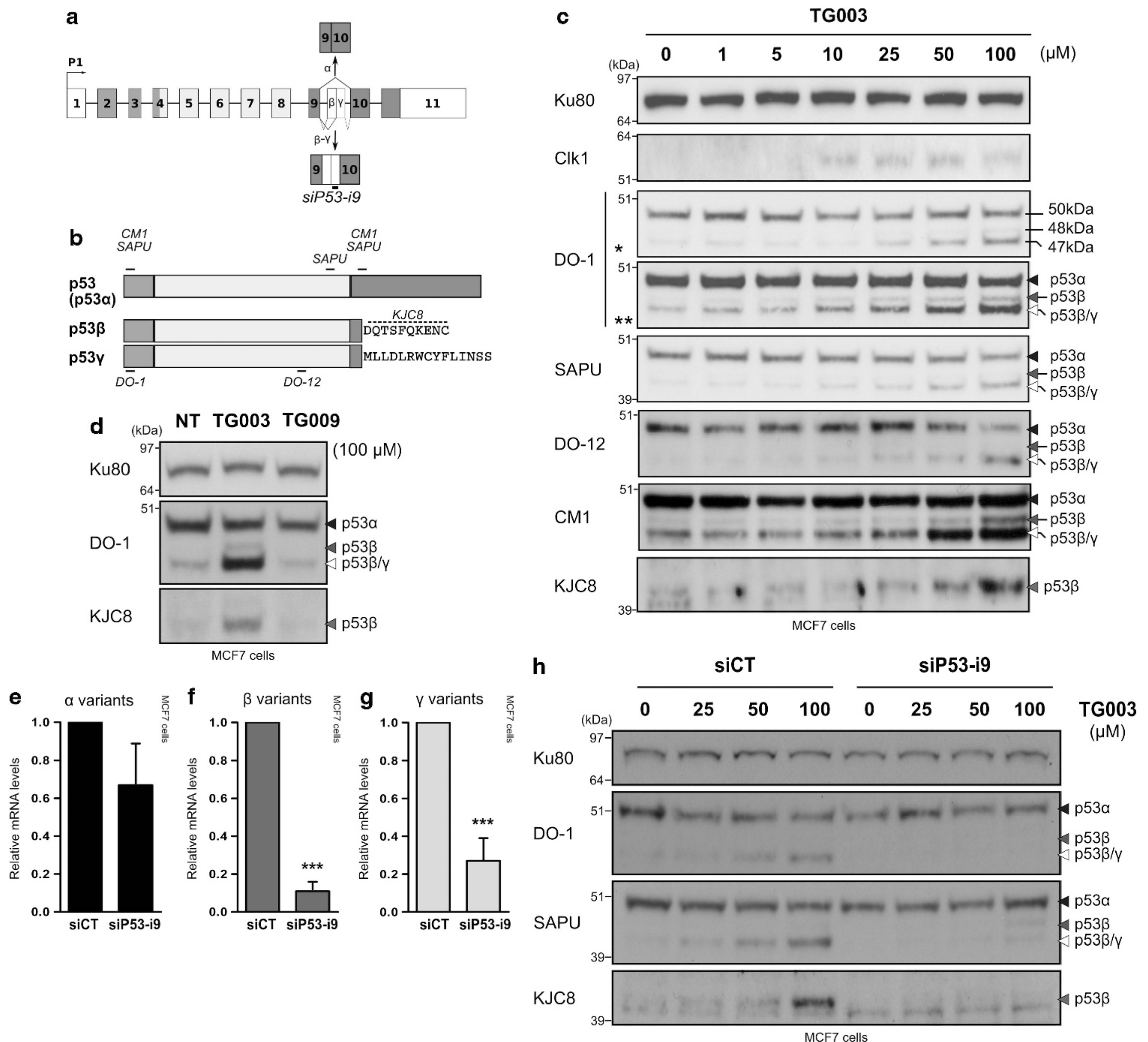

Figure 1 The small drug TG003 modulates expression of endogenous p53 protein isoforms. (**a**) Schematic representation of *TP53* gene, which produces three C-terminal domains (α, β and γ) through alternative splicing of intron 9. P1: proximal promoter; ^: alternative splicing; siP53-i9: siRNA specifically targeting p53β and p53γ protein isoforms. (**b**) Schematic representation of p53 isoforms carrying common transactivation (TAD, gray) and DNA-binding (DBD, white) domains but distinct oligomerization domains (OD, light gray). DO-1 (epitope 21–25) (–), specific of p53α, p53β and p53γ (unable to detect either Δ40 or Δ133 forms); DO-12 (epitope 256–267 shown as ' – '), SAPU (epitopes 11–64, 291–304, 366–393 shown as ' – '), CM1 (epitopes 1–64, 366–393, shown as ' – '), specific of all p53 isoforms; KJC8 (...), specific of β p53 isoforms raised against synthetic β peptide. (**c**) Impact of TG003 treatment on expression of endogenous p53 isoforms. In MCF7 cells, increasing concentrations of TG003 resulted in a dose-dependent increased expression of two bands of 47 and 48 kDa, corresponding to p53β and p53γ, and p53β isoforms, respectively. *Short exposure; **long exposure. Clk1: positive control of TG003 response. (**d**) Impact of TG009, an inactive analog of TG003, on p53 protein expression. TG003 but not TG009 treatment increased expression of p53β and p53γ isoforms. (**e**–**g**) Quantification of spliced p53 mRNA levels by qPCR in response to siP53-i9 transfection. Introduction of siP53-i9 targeting *TP53* alternative exons 9β and 9γ does not alter levels of α mRNA variants (**e**), while it significantly reduces the expression of β and γ p53 mRNA variants (**f** and **g**) compared with cells transfected with a nonspecific siRNA (siCT). ***$P < 0.001$. (**h**) Introduction of siP53-i9 prevented accumulation of p53β and p53γ isoforms in response to TG003 treatment. Ku80: loading control

Results

The small drug TG003 increases endogenous expression of p53β and p53γ protein isoforms. We first analyzed characteristic alterations of TG003 treatment in MCF7 cell line that expresses wild-type *TP53* gene and p53 isoforms,[15] to verify that it constitutes a suitable cellular model for analyzing the impact of TG003 on expression of p53 isoforms. As previously reported,[12] TG003 treatment increased Clk1 protein levels in a dose-dependent manner (Figure 1c) and changed SFRS1 subcellular localization, from a speckled to a more diffuse nuclear signal (Supplementary Figure 1a).

Expression of endogenous p53 protein isoforms in response to increasing concentrations of TG003 was analyzed using a panel of four anti-p53 antibodies.[16] DO-1 recognizes p53α, p53β and p53γ but not Δ40 and Δ133 forms, while SAPU, DO-12 and CM1 recognize all p53 isoforms with different affinities (Figure 1b and Supplementary Figure 1b).[4,16] In the absence of TG003, a 50-kDa band was accompanied with two bands of lower and different intensities, at 47 and 48 kDa (Figure 1c and Supplementary Figure 1c). In response to increasing concentrations of TG003, no variation of the 50 kDa-band was observed. However the intensity of the 47- and 48-kDa bands increased in a dose-dependent manner from 10 μM, which corresponds to the minimal dose of TG003 at which the full-length inactive Clk1 protein was detected (Figure 1c). The 47- and 48-kDa bands were not accumulated in response to TG009, an inactive analog of TG003[12] (Figure 1d). This suggests that TG003 treatment changes p53 isoform expression. A similar p53 band pattern was observed in a panel of cancer cell lines expressing wild-type p53, suggesting a conserved effect of TG003 on p53 isoform expression (Supplementary Figure 2). We confirmed that the 50-, 47- and 48-kDa bands corresponded to p53-related products by transfecting MCF7 cells with siRNA targeting all p53 isoforms (siP53-tot) (Supplementary Figure 3a and b). Based on migration molecular weights and siP53-tot data, we deduced that the 50-kDa band corresponds to the canonical p53α protein.

To identify the 47- and 48-kDa bands, we performed western blotting using KJC8 antibody, which has been developed using a synthetic and non-modified β-peptide corresponding to the peptide sequence encoded by exon 9β[3,4] (Figure 1b). On TG003 treatment, KJC8 antibody detected a dose-dependent increase of a 47-kDa band that was undetectable in response to TG009 treatment (Figures 1c and d, and Supplementary Figure 1c). This indicates that the 47-kDa band contains the p53β protein isoform. MCF7 cells were also transfected with an siRNA targeting the sequence of exons 9β/9γ (siP53-i9, Figure 1a) and siRNA efficiency was assessed by quantitative PCR (qPCR).[17] No significant variation of α mRNA variants was observed in cells transfected with siP53-i9 compared with cells transfected with a non-relevant siRNA (siCT) (Figure 1e). However, a significant reduction of $\sim$80% was observed for both β and γ mRNA variants (Figures 1f and g), indicating that siP53-i9 is specific of β and γ mRNA variants. siRNA-transfected MCF7 cells were then incubated with increasing concentrations of TG003 (Figure 1h). In response to TG003 treatment, accumulation of the 47- and 48-kDa bands was reduced in siP53-i9 compared with siCT-transfected cells, suggesting that these two bands correspond to p53 isoforms encoded by mRNA containing intron 9. As in response to TG003, the detection of the 47-kDa band decreased in siP53-i9-transfected cells (Supplementary Figure 3c), the 47-kDa band corresponds to both p53β and p53γ protein. In contrast, the 48-kDa band detection was reduced in cells transfected with either siP53-i9 or siP53-i9β but not siP53-i9γ, suggesting that the 48-kDa band corresponds also to p53β protein. The p53β of 48 kDa may correspond to a posttranslationally modified p53β not detected by the KJC8 antibody recognizing only synthetic β-peptide. Altogether, usage of specific siRNAs and KJC8 antibody demonstrated that TG003 increased the expression of p53β and p53γ protein isoforms in a dose-dependent manner.

TG003 increases endogenous expression of p53β and p53γ mRNAs by modulation of *TP53* intron 9 alternative splicing. Expression levels of endogenous p53 mRNAs in MCF7 cells were quantified in response to TG003 treatment by qPCR. TG003 treatment significantly reduced the α mRNA levels at 25 and 100 μM (Figure 2a). In contrast, TG003 treatment resulted in a significant induction of both β and γ mRNA variants (Figure 2b) that was abolished by transfecting siP53-i9 (Figure 2c). As α, β and γ primers also amplified shorter p53 mRNA isoforms (Δ133p53α, Δ133p53β and Δ133p53γ[3,17]), we verified that expression levels of Δ133p53 mRNAs and proteins was not significantly altered by TG003 treatment (Supplementary Figure 1c and 4a). Altogether, these data suggest that increased expression of endogenous p53β and p53γ protein isoforms resulted from a dose-dependent increase in p53β and p53γ transcript levels in response to TG003 treatment.

Owing to the specific role of TG003 in inhibiting Clk family involved in SR protein phosphorylation,[12,13] we investigated whether TG003 modulates alternative splicing of *TP53* intron 9. Indeed, no significant variation in the global expression of TAp53 mRNA variants (p53α, p53β and p53γ) generated by the proximal promoter was observed in response to TG003 treatment, suggesting that TG003 increased p53β and p53γ transcripts through a mechanism not affecting *TP53* promoter activity (Supplementary Figure 4b). We developed a *TP53*-i9 minigene that mimics intron 9 splicing (Figure 2d). After transfection in H1299 p53-null cells, all RNA products generated by the *TP53*-i9 minigene were amplified by RT-PCR, separated on polyacrylamide gels and sequenced (Figure 2e). The *TP53*-i9 minigene generated three products corresponding to α, β and γ spliced variants, the α product being the most abundant.[3] *TP53*-i9-transfected cells were then exposed to TG003 and spliced RNA products were analyzed by qPCR and normalized to *Neomycin* expression, which reflects variation in transfection efficiency. *TP53* RNA products were detected in *TP53*-i9-transfected cells but not in cells transfected with an empty vector, demonstrating the primer specificity (Supplementary Figure 4c). TG003 treatment significantly reduced the production of α RNA products in a dose-dependent manner (Figure 2f). However, it increased significantly and in a dose-dependent manner the generation of β and γ spliced RNA products. The inactive analogue TG009 did not affect production of *TP53* spliced RNAs (Figure 2g). Thus, the increased expression of p53β and p53γ mRNA and protein levels on TG003 treatment involved at least alternative splicing.

SFRS1 splicing factor favors exclusion of entire *TP53* intron 9 leading to α variants. We investigated whether SFRS1, an SR splicing factor targeted by Clk family,[12,18] regulates alternative splicing of *TP53* intron 9 in MCF7 cells. Knockdown of SFRS1 was induced using siSFRS1 that significantly reduced SFRS1 mRNA and protein levels (Figures 3a and b). Compared with siCT, siSFRS1 promoted

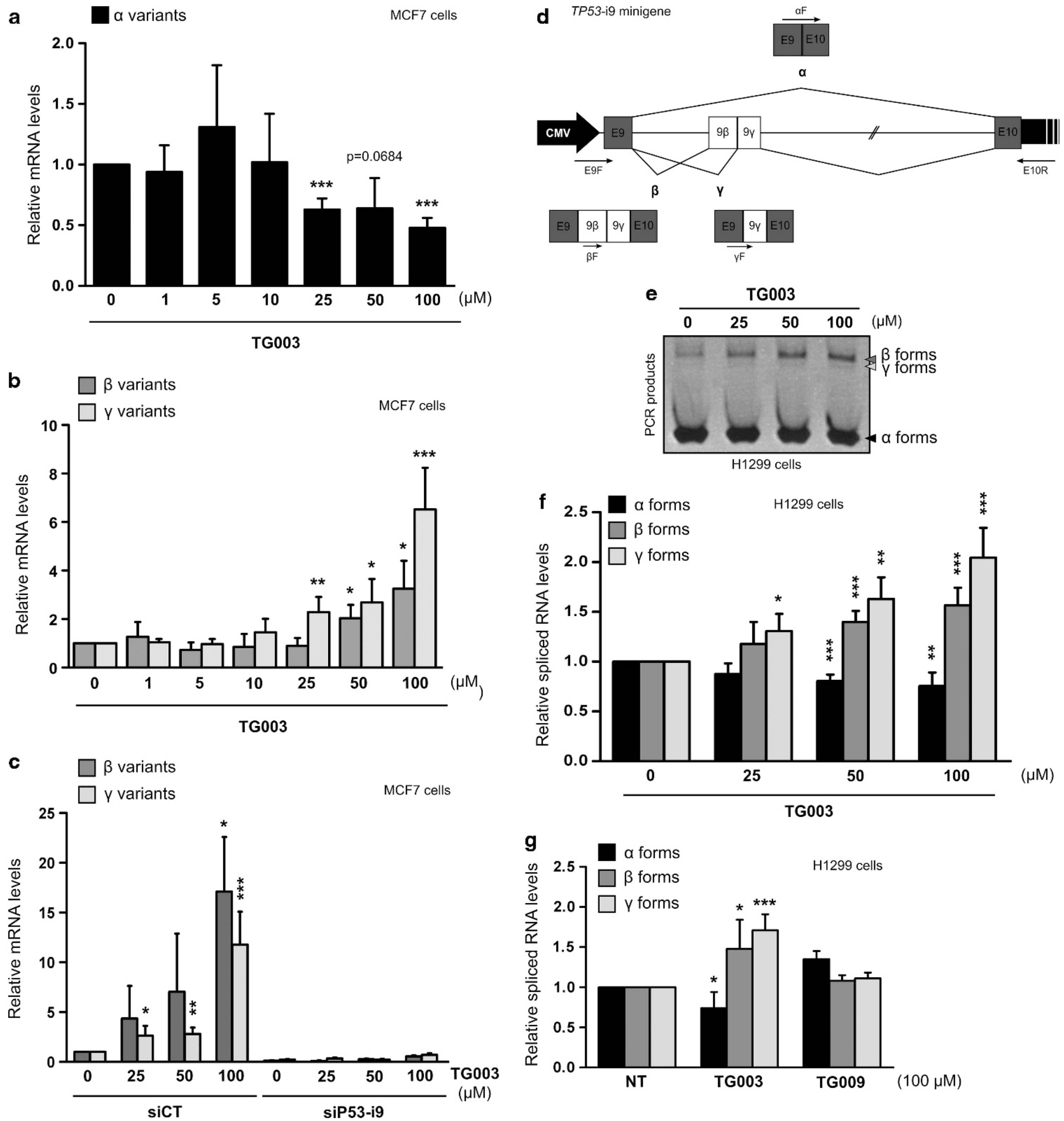

Figure 2 The small drug TG003 modulates expression of spliced p53 mRNA isoforms. (**a** and **b**) Impact of TG003 on expression of endogenous p53 mRNA isoforms analyzed by qPCR. In MCF7 cells, TG003 treatment significantly reduced expression levels of endogenous α mRNA variants (**a**), while treatment promoted a strong increase in endogenous β and γ mRNA variants in a dose-dependent manner (**b**). (**c**) Introduction of siP53-i9 targeting exons 9β and 9γ abolished the induction of endogenous β and γ mRNA variants in response to TG003 treatment, compared with cells transfected with a nonspecific siRNA (siCT). (**d**) Schematic representation of *TP53*-i9 minigene used to study alternative splicing of intron 9. CMV, CMV promoter; 3' black boxes, SV40 polyadenylation site; gray boxes, constitutive *TP53* exons; white boxes, alternative exons located in *TP53* intron 9; bold lines, non-coding *TP53* introns; arrows, location of primers; E9F, forward primer used for RT-PCR; αF, βF, γF, forward primers used for qPCR. (**e** and **f**) In p53-null H1299 cells transfected with *TP53*-i9 minigene, TG003 significantly reduced the production of α spliced forms and increased the one of β and γ spliced forms, as observed on polyacrylamide gels by RT-PCR (**e**) and by qPCR, normalized to *Neomycin* expression to account for variation in transfection efficiency (**f**). (**g**) In contrast to TG003, TG009 treatment did not alter alternative splicing of intron 9. NT, cells treated with the corresponding amount of DMSO. All graphs show means and s.d. of at least three independent experiments and stars represented *P*-value of the Student's *t*-test. *$P<0.05$; **$P<0.01$; ***$P<0.001$

an increase in endogenous p53β and p53γ protein isoforms, without altering expression of p53α protein (Figure 3b). Moreover, transfection of siSFRS1 significantly reduced by 60% the levels of α mRNA variants, and concomitantly induced a significant 1.5-fold induction of β and γ mRNA levels (Figures 3c–e). This suggests that SFRS1 splicing factor regulates expression of *TP53* at mRNA levels.

To determine whether SFRS1 regulates the splicing of *TP53* intron 9, we performed sequential transfections of siSFRS1 and *TP53*-i9 minigene in p53-null cells (Supplementary Figure 4d). Introduction of siSFRS1 promoted generation of both β and γ RNA products, although it decreased α RNA product (Figures 3f and g). Thus, SFRS1 regulates alternative splicing of *TP53* intron 9 and favors

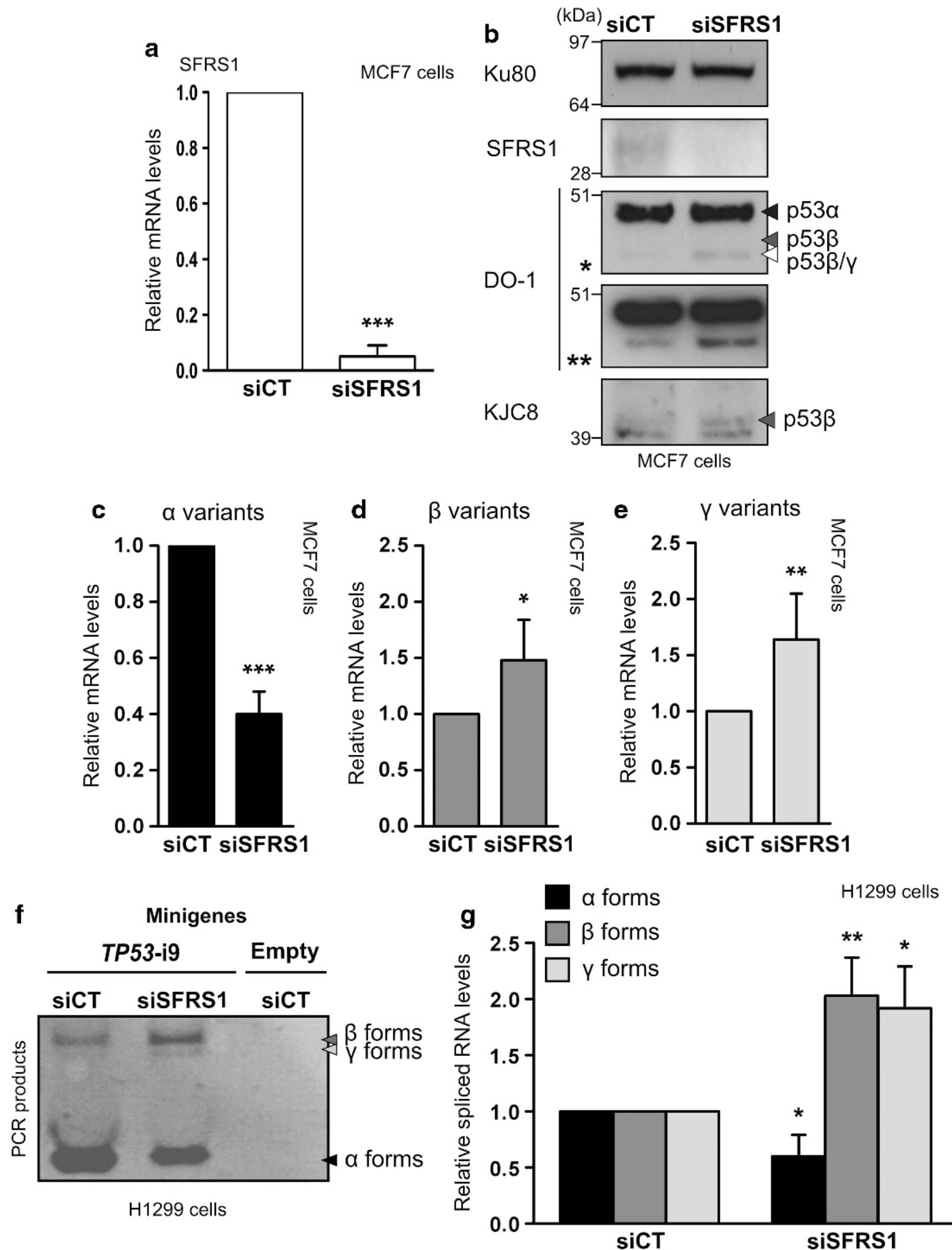

Figure 3 The splicing factor SFRS1 regulates the expression of p53 isoforms. (**a** and **b**) Knockdown of SFRS1 modulates expression of endogenous p53 protein isoforms. In MCF7 cells, siRNA targeting specifically SFRS1 reduced SFRS1 mRNA (**a**) and protein levels (**b**). Compared with cells transfected with a negative siRNA (siCT), knockdown of SFRS1 increased the expression of endogenous p53β and p53γ proteins (**b**).DO-1, antibody specific for p53α, p53β and p53γ; KJC8, antibody specific for p53β; Ku80, loading control; *, short exposure; **, long exposure. (**c**–**e**) Knockdown of SFRS1 modulates expression of endogenous p53 mRNA isoforms. Compared with siCT, introduction of siSFRS1 significantly decreased the expression of endogenous α mRNA variants (**c**), while it significantly promoted β (**d**) and γ (**e**) mRNA variants. (**f** and **g**) Knockdown of SFRS1 modulates alternative splicing of *TP53* intron 9. In p53-null H1299 cells transfected with *TP53*-i9 minigene, knockdown of SFRS1 reduced the production of α spliced forms and increased the ones of β and γ spliced forms, as shown by PCR (**f**) and qPCR (**g**). All graphs showed means and S.D. of at least three independent experiments and stars represented *P*-values calculated by Student's *t*-test. *$P<0.05$; **$P<0.01$; ***$P<0.001$

exclusion of the entire intron 9. Moreover, knockdown of SFRS1 abolished the TG003-mediated modulation of α and β production (Supplementary Figure 4e), suggesting that SFRS1 splicing factor has, at least in part, a role in the modulation of *TP53* alternative splicing of intron 9 in response to TG003 treatment.

Additional analyses were performed to establish association between expression of SFRS1 and exclusion of intron 9. mRNA levels of SFRS1 and α variants were quantified by qPCR in a cohort of 85 primary breast tumors previously described[7] (Supplementary Table 2). A significant and positive correlation was observed between expression of SFRS1 and α variants, showing that high SFRS1 levels were correlated with high expression levels of α mRNA variants (Table 1). The positive correlation was observed in breast tumors expressing wild-type but not mutant *TP53* (Supplementary Table 4). A similar correlation was observed in a panel of 18 cancer cell lines (Supplementary Tables 3 and 4). Moreover, high levels of SFRS1 in primary breast tumors were significantly associated with lack of detection of p53β (Table 1 and Supplementary Table 5). Altogether, correlation analyses in cancer cell lines and breast tumors showed a positive correlation of mRNA expression between SFRS1 and α variants.

p53β and p53γ isoforms regulate G1 cell cycle arrest and apoptosis in MCF7 cells. We investigated whether combined knockdown of p53β and p53γ expression affects cell growth of MCF7 cells. Cell proliferation was determined by MTT assays after introduction of siP53-i9 specifically targeting p53β and p53γ (Figures 1e–h). Knockdown of p53β and p53γ expression was sufficient to significantly increase cell population by 10% compared with siCT-transfected cells (Figure 4a). In contrast, cells with combined knockdown of p53α, p53β and p53γ using siP53-tot presented a percentage of cell growth not significantly different from siCT-transfected cells (Supplementary Figure 5a). This suggests that p53β and p53γ isoforms inhibit cell growth in MCF7 cells in a p53α-dependent manner.

To determine whether cell growth inhibition involves cell cycle progression and/or apoptosis, we performed BrdU and annexin V flow cytometer analyses. In MCF7 cells, a significant decrease in the G1/S ratio was observed in siP53-i9 compared with siCT-transfected cells (Figure 4b). This was due to a 10% decrease in the percentage of cells in G0/G1 phase accompanied with a 10% increase in the percentage of cells in S phase, with no variation in G2/M phase (Supplementary Figure 5b). This suggests that p53β and p53γ inhibit cell cycle progression in MCF7 cells. Moreover, the number of apoptotic cells decreased by 35% on knockdown of p53β and p53γ (Figure 4c). We monitored the expression of two p53-inducible genes, the cyclin-dependent kinase inhibitor *p21* and the pro-apoptotic *Bax* genes that were used as markers of cell cycle arrest and apoptosis. Knocking-down expression of p53β and p53γ, either alone or together, was associated with reduced levels of Bax protein and increased levels of p21 protein, known to inhibit apoptosis[19] (Figure 6a and Supplementary Figure 3c). Altogether, it suggests that p53β and p53γ inhibit cell cycle progression and promote apoptosis of MCF7 cells.

Table 1 Correlation and association of mRNA expression levels between SFRS1 and α, β and γ variants of *TP53* gene in a cohort of breast cancer tumors

Isoforms	Number (*n*)	*P*-value		
α	85	0.0017**[a]	Pearson's *r* 0.335	95% CI (0.131–0.512)
			Mean levels of SFRS1 mRNA	S.D.
β	$\beta+$ 30	0.0123*[b]	2.667	1.313
	$\beta-$ 52		8.278	18.90
γ	$\gamma+$ 35	0.1528[c]	6.028	16.13
	$\gamma-$ 47		6.372	14.74

*$P<0.05$; **$P<0.01$
[a]Correlation between SFRS1 and α variant levels using Pearson's test (two-tailed *P*-value)
[b]Association between SFRS1 expression level and lack of β detection using Mann–Whitney test (two-tailed *P*-value)
[c]No association between SFRS1 expression level and detection of γ using - Mann–Whitney test (two-tailed *P*-value)

Luciferase assays revealed that in contrast to p53α, p53β and p53γ do not directly regulate activities of *p21* and *Bax* promoters (Supplementary Figure 6). To investigate the effect of p53β and p53γ on p53α transcriptional activity, we transfected an increasing amount of p53β or p53γ with a constant amount of p53α that gives 50% of maximum p53α transcriptional activity on *p21* or *Bax* promoter[16] (Figures 5a and b). Increasing amount of p53β, but not of p53γ, significantly increases in a dose-dependent manner the p53α transcriptional activity on the *p21* promoter. In contrast, both p53β or p53γ enhance p53α transcriptional activity on the *Bax* promoter in a dose-dependent manner. Compared with p53β, a lower amount of p53γ expression vector is sufficient to enhance p53α transcriptional activity on the *Bax* promoter. Thus, p53β and p53γ enhance differentially and in a promoter-dependent manner the p53α transcriptional activity.

To assess whether p53β and/or p53γ modulate p53α transcriptional activity on *p21* and *Bax* promoters through direct interaction with p53α, we performed co-immunoprecipitation assays after co-transfection of p53α with p53β or p53γ in p53-null cells. As no or weak interaction was observed between p53α and p53β or p53γ after transfection, we tested whether the presence of DNA containing p53-responsive promoter influences p53α/p53 isoform interaction. p53α and p53β or p53γ were co-transfected with either a DNA plasmid free of p53 response element (pBlueScript) or a plasmid containing p53 responsive elements (p21-Luc and Bax-Luc). In the presence of pBlueScript, no or weak interaction between p53α and p53β or p53γ was observed (Figures 5c and d). In contrast, p53β or p53γ can be co-immunoprecipitated with p53α in the presence of *p21* or *Bax* promoters. These data indicate that p53β and p53γ modulate p53α transcriptional activity through, at least in part, the formation of DNA-mediated p53α/p53β and p53α/p53γ protein complex.

In response to alteration of alternative splicing pathway, p53β and p53γ maintain cell proliferation. We recently reported that alteration of alternative splicing pathway activates p53, regulates p53-target gene expression and induces a p53-dependent G1 cell cycle arrest in HCT116 cells.[14] Here we determined whether combined knockdown of p53β and p53γ by siP53-i9 affects MCF7 cell response to

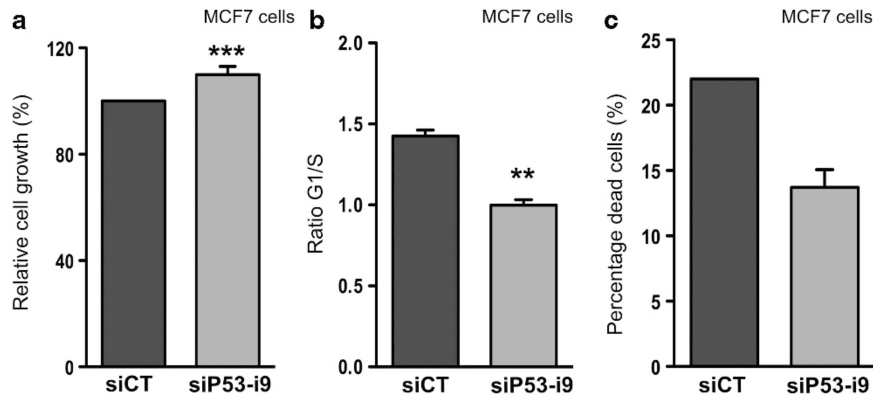

Figure 4 p53 isoforms promote apoptosis in MCF7 cells. (**a**) MTT assays performed 48 h after knockdown of p53β and p53γ expression showed that siP53-i9 transfection significantly increased cell growth by 10 % compared with the introduction of siCT in MCF7 cells. Error bars:S.E. (**b**) Analysis of BrdU/PI staining by flow cytometer showed that knockdown of both p53β and p53γ in standard condition of cell culture significantly decreases the ratio G1/S. (**c**) Analysis of annexin V/PI staining by flow cytometer showed that knockdown of both p53β and p53γ in MCF7 cells reduces percentage of apoptotic cells. All graphs show means and S.D. of at least three independent experiments and stars represented *P*-value of the Student's *t*-test. *$P<0.05$; **$P<0.01$; ***$P<0.001$

TG003 treatment. MCF7 cells were treated with increasing concentration of TG003 and cell proliferation was determined by MTT assay (Figure 6). To exclude difference between siCT and siP53-i9-transfected cells due to variation in basal apoptosis, MTT assays were normalized at the day of TG003 addition (Figure 6b). Although cell growth was significantly inhibited in a dose-dependent manner from 50 μM TG003 in siCT-transfected cells, cell growth was significantly reduced from 25 μM TG003 in siP53i9-transfected cells. Thus, cell growth inhibition in response to TG003 was significantly stronger in siP53-i9 than in siCT-transfected cells, suggesting that knockdown of p53β and p53γ sensitizes cells to TG003 treatment. These data suggest that in response to TG003 treatment, p53β and p53γ enable cell proliferation.

To determine whether p53β and p53γ alter apoptosis in response to TG003, annexin V staining and western blottings were performed. No significant variation in apoptosis or in Bax levels was observed in response to TG003 treatment in cells transfected with either siCT or siP53-i9 (Figure 6a and Supplementary Figure 7a), suggesting that TG003 has no toxic effect as previously described.[12]

We then investigated the impact on cell cycle progression by BrdU assay. Increased concentrations of TG003 were accompanied with a dose-dependent increase in the percentage of cells in G2/M phase in siCT-transfected cells, but also in siP53-i9- and siP53-tot-transfected cells, indicating that TG003 promotes G2 arrest in a p53-independent manner. (Figure 6c and Supplementary Figure 7b). In siCT-transfected cells, TG003 treatment significantly reduces G1/S ratio from 25 μM, whereas an increase in G1/S ratio was observed in response to both TG003 and knockdown of p53β and p53γ expression. These data indicate that p53β and p53γ promote cell cycle progression in response to TG003 treatment. Moreover, the latter condition was accompanied with increased levels of p21 protein (Figure 6a). Interestingly, knockdown of SFRS1 that results in an increased expression of p53β and p53γ also reduced the percentage of cell in G0/G1 phase (Supplementary Figure 7c). To decipher the role of p53α in the cellular response to TG003, we reproduced the same experiment in cells depleted from all isoforms, including p53α, using siP53-tot (Figure 6d). Knocking-down expression of all p53 isoforms abolished the increased number of cells in G0/G1 observed in siP53-i9 cells in response to TG003. Our data suggest that p53β and p53γ promote cell cycle progression in response to TG003 in a p53α-dependent manner. Moreover, we observed that TG003 treatment reduced the enhanced effects of p53β and p53γ on p53α transcriptional activity on the *Bax* promoter and increased the phosphorylation of serine 15 of p53α, and also of p53β and p53γ (Supplementary Figures 7d and e). Overall, our data indicate that TG003 not only modulates the expression of p53 protein isoforms but also their intrinsic activities.

Discussion

Alternative splicing of *TP53* intron 9 results in the generation of three C-terminal p53 isoforms:[3] the canonical p53α, p53β and p53γ isoforms (Figure 1a). Here we observed that the Clk inhibitor TG003 and knockdown of SFRS1 promoted endogenous expression of p53β and p53γ at mRNA and protein levels, at least through modulation of p53 pre-RNA splicing. We cannot rule out a role of TG003 and SFRS1 in regulating *TP53* expression using additional and complementary transcriptional processes. Indeed, SFRS1 has been shown to regulate mRNA stability and protein translation.[20,21] Using a panel of 18 cancer cell lines and a series of 85 breast tumors, we observed that SFRS1 expression is positively correlated with α mRNA variants but negatively associated with p53β transcripts. These clinical data support our experimental conclusion, indicating that SFRS1 can regulate alternative splicing of *TP53* intron 9. Moreover, our data support the notion that TG003 regulates *TP53* intron 9 splicing through inactivation of SFRS1, as already shown for the alternative splicing of β-globin.[12] The Clk kinases regulate the activity of a subset of splice factors and their inhibition affects only a subset of targets without altering the global alternative splicing. Indeed, our data showed that TG003 treatment leads to inclusion of *TP53* exons 9β and 9γ, while, for example, splicing of *Ku80* pre-mRNA is not altered and occur normally. Moreover, TG003 treatment results in increased expression of p53β and p53γ protein isoforms in different wild-type p53 cell lines, suggesting a conserved effect of

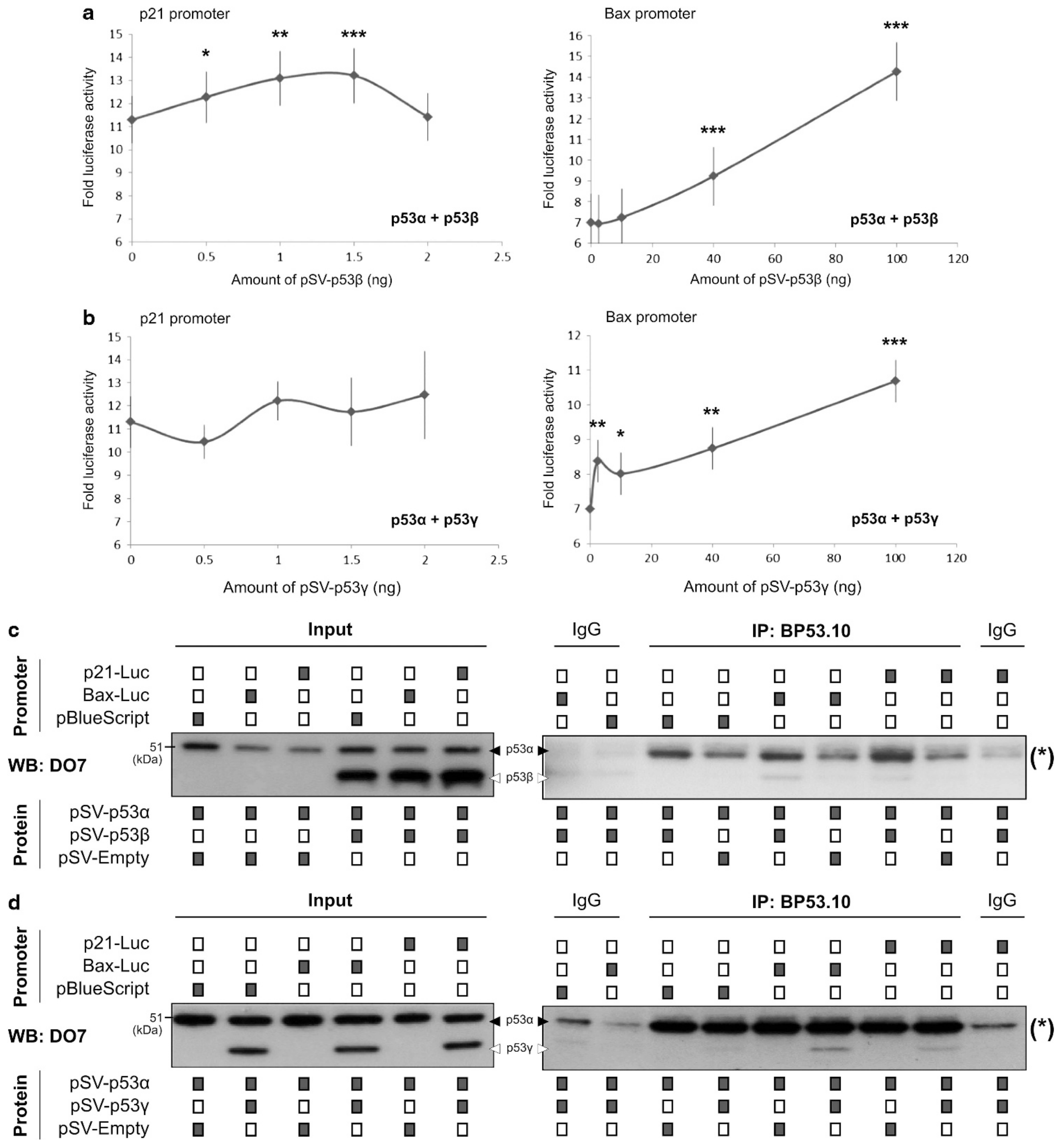

Figure 5 p53 isoforms modulate p53α transactivation activity through direct interaction in the presence of p53-responsive promoters. (**a** and **b**) Luciferase assays performed in p53-null H1299 cells showed that p53β, but not p53γ, enhances p53α transcriptional activity on the *p21* promoter, and that both p53β and p53γ enhances p53α transcriptional activity on the *p21* and *Bax* promoters. (**c** and **d**) Co-immunoprecipitation of p53α and p53β/p53γ carried out in the presence of the *p21* or *Bax* promoters reveals that p53α interacts with both p53β and p53γ in the presence of a p53-responsive promoter. Black box, plasmid present in the transfection mix; white box, plasmid absent in the transfection mix; IP, anti-α p53 mouse monoclonal antibody BP53.10 (epitope 374–378 only present in p53α but absent in p53β and p53γ); WB, anti-p53 mouse monoclonal antibody DO7 (epitope 21–25 present in p53α, p53β and p53γ) Input, control of transfection; IgG, negative control of immunoprecipitation; star, p53α detection corresponds to positive control of immunoprecipitation. All graphs show means and S.D. of at least three independent experiments and stars represented *P*-value of the Student's *t*-test. *$P<0.05$; **$P<0.01$; ***$P<0.001$

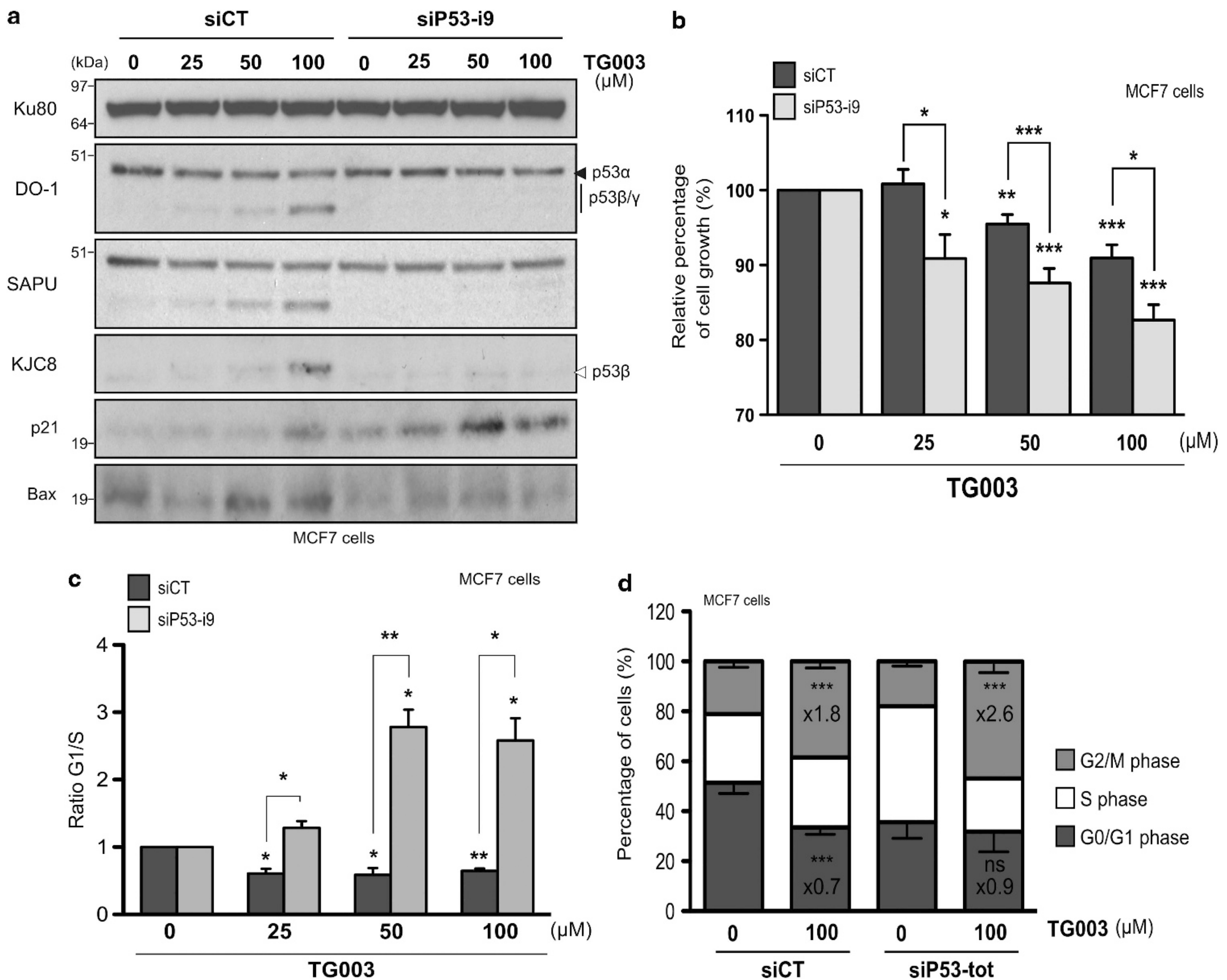

Figure 6 TG003 modulates cell cycle progression in a p53 isoform-dependent manner. (**a**) Analysis of protein expression in response to siP53-i9 and TG003 treatments. Transfection with siP53-i9 abolishes the TG003-induced p53β and p53γ expression. Ku80, loading control. (**b**) Impact of knocking down p53β and p53γ expression on cell growth in response to TG003 treatment. A significant decrease in cell growth was observed by MTT assays in response to TG003 in a dose-dependent manner in siCT-transfected cells. This effect was stronger in siP53-i9-transfected cells. Error bars:S.E. (**c**) Impact of knocking down p53β and p53γ expression on the ratio G1/S in response to TG003 treatment. TG003 treatment decreased ratio G1/S in siCT cells, while TG003 treatment increases ratio G1/S in siP53-i9 cells. (**d**) Impact of knocking down all p53 isoforms expression on the cell cycle progression in response to TG003 treatment. TG003 treatment for 24 h increased percentage of cells in G2/M phase, in both siCT and siP53-tot conditions. In contrast to what was observed in response to knocking down p53β and p53γ expression, no variation in the percentage of G0/G1 cells was observed in response to TG003 treatment in siP53-tot cells compared with untreated cells. All graphs show means and S.D. of at least two independent experiments and stars represented *P*-value of the Student's *t*-test. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

TG003 on *TP53* splicing. However, the impact of TG003 on p53α protein is cell line-dependent, p53α expression being either increased or decreased, supporting a cell type-dependent effect of TG003.[14]

Early studies reported that p53β has some tumor suppressor-like activity in non-treated normal fibroblasts and cancer cells, p53β promoting apoptosis and senescence by differentially regulating gene expression.[3,6] Consistently, endogenous p53β and p53γ expression promotes G1 cell cycle arrest and basal apoptosis in MCF7 cells. This observation is consistent with recent study, reporting that p53β and p53γ overexpression reduced cell growth.[22] Importantly, knockdown of p53β and p53γ expression using siP53-i9 did not alter p53α expression, suggesting that the regulation of cell cycle and basal apoptosis is dependent on p53β and p53γ. Indeed, we show that endogenous p53β and p53γ differentially regulate protein expression such as p21 and Bax without altering p53α expression level. The molecular mechanism by which p53β and p53γ modulate gene expression in basal condition was unclear. Here we show that p53β enhances p53α transcriptional activity on both the *p21* and *Bax* promoters, whereas p53γ enhances p53α transcriptional activity on the *Bax* promoter only. Moreover, we reported that these two isoforms modulate p53α transcriptional activity in a dose-dependent manner. The regulation of p53α transcriptional activity by p53β and p53γ can be explained at least in part through the formation of DNA-mediated p53 protein complex composed of p53α and p53β or p53γ. We show that

the detection by co-immunoprecipitation assay of p53α/p53β and p53α/p53γ protein complex requires the presence of a p53-responsive promoter, suggesting that the p53α/p53β and p53α/p53γ protein complex formation and/or stabilization is promoter dependent.

In response to TG003 treatment, we observed variation in expression of p53-target genes, as recently reported.[14] Using siRNA specific for p53β and p53γ, we demonstrated that TG003 treatment and knockdown of SFRS1 induced endogenous p53β and p53γ protein expression in a dose-dependent manner without altering p53α expression in MCF7 cells. Moreover, we observed no significant induction of apoptosis and no alteration of cell proliferation in response to 25 μM TG003 in control transfected cell. However, higher concentrations of TG003 and knockdown of SFRS1 reduce cell proliferation. Interestingly, knockdown of endogenous p53β and p53γ induces G1 cell cycle arrest in response to 25 μM TG003 in a p53α-dependent manner, with concomitant induction of p21 protein. It suggests that p53β and p53γ confer resistance to TG003 treatment, thus maintaining cell proliferation in response to alteration of alternative splicing by TG003. In addition to modifying *TP53* expression, TG003 also affects the posttranslational modification status of p53 protein isoforms and alters the activity of p53β and p53γ towards p53α transcriptional activity. The different layers of *TP53* regulation induced by TG003 may explain the dual role of p53β and p53γ isoforms on cell growth observed in untreated and TG003-treated cells.

Altogether, this is the first proof-of-principle that alternative splicing can be used to modulate p53 isoform expression and thus cellular response, suggesting that regulation of alternative splicing of *TP53* can be a new therapeutic target to control cell growth in human cancers.

Materials and Methods

Cells and treatments. Two human cancer cell lines were used: the luminal MCF7 breast cancer cells expressing wild-type *TP53* gene and all p53 isoforms, as well as the p53-null H1299 lung cancer cells expressing no p53 isoform.[15] Cells were maintained in DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum and 0.5 % gentamycin under 5% CO_2 atmosphere at 37 °C. Small drugs were diluted in DMSO at a stock concentration of 50 mM and stored at −80 °C. Cells at 60 % of confluence were treated for 24 h with increasing concentrations of TG003, a Clk inhibitor[12] (Sigma, Dorset, UK, reference T5575). Amount of TG003 was balanced with the corresponding quantity of DMSO to introduce the same amount of DMSO in each condition. DMSO and/or TG009, kindly provided by M. Hagiwara (Tokyo, Japan),[12] were used as negative controls. In knockdown experiments, cells at 30 % of confluence were transfected 48 h prior TG003 treatment using Lipofectamine RNAiMAX Reagent (Invitrogen) with siRNAs targeting p53β and p53γ isoforms (siP53-i9), all p53 isoforms (siP53-tot) or a nonspecific siRNA used as a negative control (siCT) (Figure 1a, Supplementary Figure 1a and Supplementary Table 1). Cells were maintained for 48 h in contact of siRNAs before removing the medium and addition of new medium supplemented with TG003 treatment for the next 24 h.

Western blotting. Proteins were extracted in NP40 extraction buffer by scrapping and purified by centrifugation (Igepal extraction buffer stored at 4 °C: 50 mM Tris pH 7.5, 10 % glycerol, 0.1 % Igepal (Calbiochem, Darmstadt, Germany), 100 mM NaCl, 0.2 mM EDTA; the buffer was supplemented extemporaneously with 1 × CompleteTM Protease Inhibitor Cocktail (Roche, Mannheim, Germany)). About 20–30 μg of proteins were loaded on NuPAGE Novex 10% Bis-Tris Mini Gels (Invitrogen) and analyzed by immunoblotting using the following antibodies: anti-p53 isoforms, sheep polyclonal antibody Sapu, rabbit polyclonal antibody CM1, mouse monoclonal antibody DO1 and DO12, rabbit polyclonal antibody KJC8 (Dundee, UK) (Figure 1b and Supplementary Figure 1b),[4,16] anti-Clk1 (Santa Cruz, Dallas, TX, USA), anti-p21 (Calbiochem), anti-Bax (Cell Signaling, Danvers, MA, USA). Ku80 (Abcam, Cambridge, UK) and actin (Cell Signaling) were used as loading controls. The secondary antibodies from Jackson Immunodiagnostics (West Grove, PA, USA) were used.

RNA analysis. Total RNAs were extracted and purified using RNeasy Mini Kit (Qiagen, Crawley, UK) with an additional step of DNase treatment (Qiagen). Reverse transcription was performed on 1 μg of total RNAs using SuperScript II Reverse Transcriptase (Invitrogen). Quantification of mRNA levels was carried out by real-time PCR using TaqMan 2X Universal PCR Master Mix No AmpErase UNG (Applied Biosystem, Carlsbad, CA, USA) supplemented with specific sets of primers and probes (Supplementary Table 1).[17] Relative mRNA levels were determined by the $2-\Delta\Delta Ct$ method using *Actin* mRNA levels to normalize measures.[23] mRNA levels were quantified in triplicate on at least three independent experiments and compiled to perform statistical analyses.

***TP53*-i9 minigene.** A fragment of 3.0 kb was amplified from Human Genomic DNA (Promega, Madison, WI, USA) as template using primers in exon 9 and 10 as described. The PCR fragment was cloned into *Eco*RI restriction sites of pcDNA3-Empty vector. This construct was named *TP53*-i9 minigene and pcDNA3-Empty vector was used as a negative control. *TP53*-i9 minigene expresses also the neomycin-resistant gene, which reflects transfection efficiency. H1299 cells at 40% of confluence were transfected with 400 ng of *TP53*-i9 minigene using Fugene 6 Transfection Reagent (Roche). Two different analyses were developed. The qualitative analysis corresponds to a RT-PCR using GoTaq DNA Polymerase (Promega) in the presence of E9F and E10R primers to amplify all spliced RNAs produced by *TP53*-i9 minigene (Figure 2d and Supplementary Table 1). PCR products were separated on Novex 8% TBE Gel (Invitrogen) and identity of the individual band was verified by sequencing. The quantitative analysis corresponds to a quantitative PCR using Brillant III UltraFast SYBR Green QPCR Master Mix (Agilent, Santa Clara, CA, USA) in the presence of forward primers specific of each spliced RNAs (Figure 2d and Supplementary Table 1). Quantification of *TP53*-i9 minigene mRNA products was normalized to the quantity of *Neomycin* mRNA co-expressed in *TP53*-i9 plasmid, to take into account variation in transfection efficiency.

Breast cancer tumors. A cohort of breast cancer tissues collected from untreated Caucasian women and available at the Tayside Tissue Bank (www.tissuebank.dundee.ac.uk) has been previously described and analyzed for p53 mutation status.[9] Among this cohort, 85 breast cancers samples with different characteristics were used (Supplementary Table 2). Relative mRNA levels of SFRS1 and α forms were determined by qPCR using total RNAs extracted from U2OS cells as a reference control measured in each run.

Cell growth assays. Cell growth was determined by MTT assays using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega) as described by the manufacturer. Briefly, after 2 h of incubation with CellTiter, absorbance of MCF7 cell medium was recording at 490 nm with a 96-well plate reader. Absorbance were measured in triplicate on at least four independent experiments and compiled to perform statistical analyses. Cell growth is shown as a relative cell growth, using non-treated cells as reference.

Flow cytometry. Analyses by flow cytometry were performed in MCF7 cells incubated for 24 h with increasing concentration of TG003. Cell cycle status was determined by BrdU/PI staining. Briefly, MCF7 cells were incubated 30 min with 30 μM BrdU (Calbiochem) and then collected by trypsination. Fixed cells were successively treated with 1 mg/ml pepsin diluted in 30 mM HCl, anti-BrdU antibody (1 : 50, Becton Dickinson, Franklin Lakes, NJ, USA), secondary-FITC antibody (1 : 64, Sigma) and 25 μg/ml PI (Invitrogen). Apoptotic status was determined by Annexin V/PI staining. MCF7 cells were washed and suspended in 1 × PBS after trypsination. Annexin V-FITC reagent diluted in 1X Buffer (BioVision, Milpitas, CA, USA) were added as well as 25 μg/ml PI (Invitrogen).

Luciferase assays and co-immunoprecipitation. Luciferase assays were performed as already described.[16] Briefly, p53-null H1299 cells were transfected with vectors expressing p53 isoforms (pSV-p53α, pSV-p53β, pSV-p53γ) or an empty vector, together with a firefly luciferase reporter gene driven by two different human promoters, *Bax* and *p21*, both containing p53 response elements. In addition, to normalize for any variation due to transfection efficiency,

a *renilla* luciferase reporter gene was used in all transfections. Dual-Luciferase Reporter Assay System (Promega) was used according to manufacturer's recommendation to measure luciferase activity.

Co-immunoprecipitations were carried out in p53-null H1299 cells co-transfected with the expression vectors pSV-p53α (1 μg) and pSV-p53β or pSV-p53γ or pSV-Empty (7 μg) in combinations with the Bax-Luc or p21-Luc or control empty pBluescript (2 μg). In addition, 0.2 μg of *renilla* luciferase reporter gene was included in all transfections mix to allow for constant immunoprecipitation of transfected proteins from total protein extract (i.e., to take into account variation in transfection efficiency). Cells were collected 24 h after transfection in cold NP40 buffer (see 'Western blotting' section) supplemented with Complete TM Protease Inhibitor and DTT to final concentration of 1 mM. Once normalization was achieved, 10 μg of mouse monoclonal p53 antibody BP53.10 (specific of the α peptide recognizing only p53α) or mouse IgG was added and samples incubated overnight with rotation, at 4 °C. This was followed by the addition of Dynabeads Protein G and a further 2 h incubation. Immunoprecipitation was completed by washers (2 × NP40 and 2 × PBS) and elution in SDS Laemmli buffer. Immunoprecipitated samples were then analyzed by western blotting using the DO7 antibody specific of TA forms (p53α, p53β and p53γ).

Statistical analysis. Statistical analyses were performed using GraphPad Instat3 software (GraphPad Software Inc., La Jolla, CA, USA). Results are shown as a mean of at least three independent experiments and error bars correspond to S.D. For experimental studies, statistical analyses were performed using Student's *t*-test (ns: not significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$). For panels of cancer cell lines and breast cancer tumors, correlation of expression was determined using two-tailed Pearson's test. Additional methods and related references are available in the 'Supplementary Methods' section.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

VM, KF and OT designed the experiments; VM and J-CB supervised the project; VM, KF and OT conducted the experiments; VM and J-CB performed the data analysis; VM wrote the first draft of the manuscript; VM, OT, DPL and J-CB wrote the manuscript.

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