Citation: Cell Death and Disease (2012) 3, e400; doi:10.1038/cddis.2012.138
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p53 is a major component of the transcriptional and apoptotic program regulated by PI 3-kinase/Akt/GSK3 signaling

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The phosphatidylinositol (PI) 3-kinase/Akt signaling pathway has a prominent role in cell survival and proliferation, in part, by regulating gene expression at the transcriptional level. Previous work using global expression profiling identified FOXOs and the E-box-binding transcription factors MITF and USF1 as key targets of PI 3-kinase signaling that lead to the induction of proapoptotic and cell cycle arrest genes in response to inhibition of PI 3-kinase. In this study, we investigated the role of p53 downstream of PI 3-kinase signaling by analyzing the effects of inhibition of PI 3-kinase in Rat-1 cells, which have wild-type p53, compared with Rat-1 cells expressing a dominant-negative p53 mutant. Expression of dominant-negative p53 conferred partial resistance to apoptosis induced by inhibition of PI 3-kinase. Global gene expression profiling combined with computational and experimental analysis of transcription factor binding sites demonstrated that p53, along with FOXO, MITF and USF1, contributed to gene induction in response to PI 3-kinase inhibition. Activation of p53 was mediated by phosphorylation of the histone acetyltransferase Tip60 by glycogen synthase kinase (GSK) 3, leading to activation of p53 by acetylation. Many of the genes targeted by p53 were also targeted by FOXO and E-box-binding transcription factors, indicating that p53 functions coordinately with these factors to regulate gene expression downstream of PI 3-kinase/Akt/GSK3 signaling.
Cell Death and Disease (2012) **3,** e400; doi:10.1038/cddis.2012.138; published online 11 October 2012
Subject Category: Cancer

The phosphatidylinositol (PI) 3-kinase/Akt signaling pathway has a central role in the growth and survival of both normal and malignant cells.[1,2] Signaling by PI 3-kinase downstream of growth factor receptors is required to suppress apoptosis of most mammalian cell types, as well as acting to stimulate cell proliferation. Oncogenic mutations affecting the PI 3-kinase/Akt pathway are among the most common abnormalities found in human tumors, and inhibitors of PI 3-kinase are currently being evaluated as cancer chemotherapeutic agents in clinical trials.[1,2]

Downstream targets of PI 3-kinase/Akt signaling include the protein kinases mTOR, which is a key regulator of translation and autophagy,[3,4] and glycogen synthase kinase (GSK) 3, which is a proapoptotic protein kinase inhibited by Akt phosphorylation.[5,6] Akt and GSK3 act to phosphorylate several direct regulators of cell survival and proliferation, including Mcl1, Bad and cyclin D.[7–10] GSK3 also regulates translation via phosphorylation of eIF2B,[11,12] and both Akt and GSK3 regulate gene expression by phosphorylating a variety of transcription factors.[13–15]

In order to elucidate the program of gene expression regulated by PI 3-kinase, we have used global gene expression profiling to identify changes in gene expression that result from inhibition of PI 3-kinase in proliferating cells.[16] Early changes in gene expression, coinciding with the onset of apoptosis, included the induction of genes encoding proteins that mediate apoptosis and cell cycle arrest as well as the downregulation of genes that mediate cell survival and proliferation. Bioinformatic analysis, chromatin immunoprecipitation (ChIP) and RNA interference further allowed us to identify key transcription factors that were regulated by PI 3-kinase signaling and contributed to these changes in gene expression. Downregulation of genes in response to inhibition of PI 3-kinase resulted in part from a loss of NFκB activity,[16] as well as from increased mRNA degradation due to activation of the mRNA destabilizing proteins BRF1 and KSRP.[17] Induction of upregulated genes in response to PI 3-kinase inhibition was mediated in part by FOXO transcription factors,[18] which are well-established inhibitory targets of Akt.[19,20] In addition, many of the genes induced following inhibition of PI 3-kinase were targeted by the E-box-binding transcription factors MITF and USF1, which were activated as a result of phosphorylation by GSK3.[18]

The gene expression analyses discussed above were performed principally in T98G glioblastoma cells, which are widely used as a model for growth factor-dependent human cells.[21,22] However, *TP53* is inactivated by mutation in T98G cells,[23] so any role for p53 in the transcriptional response to PI 3-kinase inhibition would not have been detected in this cell line. The p53 tumor suppressor is a major regulator of cell proliferation and apoptosis, which is activated in response to DNA damage and is mutated in $\sim$50% of human cancers.[24–27] Although PI 3-kinase signaling can affect p53 activity,[28–30] the role of p53 in the cellular response to

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Keywords: apoptosis; phosphatidylinositol 3-kinase; p53; Tip60; glycogen synthase kinase 3
Abbreviations: PI 3-kinase, phosphatidylinositol 3-kinase; GSK3, glycogen synthase kinase 3
Received 26.7.12; revised 23.8.12; accepted 31.8.12; Edited by G Raschellà

inhibition of PI 3-kinase in cells without DNA damage has not been determined.

In this study, we investigated the role of p53 in apoptosis and changes in the program of gene expression resulting from inhibition of PI 3-kinase in otherwise normally proliferating cells. We addressed this question by characterizing the transcriptional response to inhibition of PI 3-kinase in Rat-1 cells, which have a normal *Tp53* gene,[31,32] compared with Rat-1 cells expressing a dominant-negative p53 mutant. Analysis of apoptosis and gene regulation in these cells indicates that p53 is a major component of the network that contributes to cell survival and alterations in gene expression downstream of PI 3-kinase signaling, along with FOXO, MITF and USF1. The principal mechanism leading to activation of p53 in response to inhibition of PI 3-kinase is phosphorylation of the histone acetyltransferase Tip60 by GSK3, leading to acetylation and activation of p53. Major changes in gene expression and cell survival following inhibition of PI 3-kinase thus result from the activation of p53, MITF and USF1 via GSK3, in addition to the activation of FOXOs resulting directly from inhibition of Akt.

Results

Characterization of Rat-1 cells expressing dominant-negative p53. In order to investigate the role of p53 in apoptosis and transcriptional regulation downstream of PI 3-kinase, we characterized the effects of inhibition of PI 3-kinase in Rat-1 cells, which have wild-type p53,[31,32] compared to Rat-1 cells expressing a dominant-negative p53 mutant. Rat-1 cells were transfected with a plasmid conferring resistance to G418 and driving expression of the dominant-negative p53 mutant V143A.[6,33] Two stably transformed clones (designated DN1 and DN2) were selected for further study, both of which expressed the transfected dominant-negative p53 at levels ~fourfold greater than endogenous p53 in Rat-1 cells (Figure 1a). Induction of the p53 target genes *Bax* and *Cdkn1a* in response to activation of p53 by treatment with a low dose of actinomycin D[34,35] was blocked in both clones expressing the dominant-negative mutant (Figure 1b), indicating that p53 was effectively inhibited.

We investigated the effect of dominant-negative p53 expression on apoptosis in response to inhibition of PI 3-kinase by treating cells with the small-molecule inhibitor, PI-103.[36] Inhibition of PI-3 kinase rapidly induced apoptosis in both wild-type Rat-1 cells and cells expressing dominant-negative p53, as indicated by DNA fragmentation as early as 1 h after treating with PI-103 (Figure 1c). However, quantification by TUNEL assays indicated that apoptosis was significantly inhibited in both clones expressing the dominant-negative p53 mutant (Figure 1d). These results indicate that p53 contributes to but is not essential for apoptosis in response to inhibition of PI 3-kinase. Similar results were

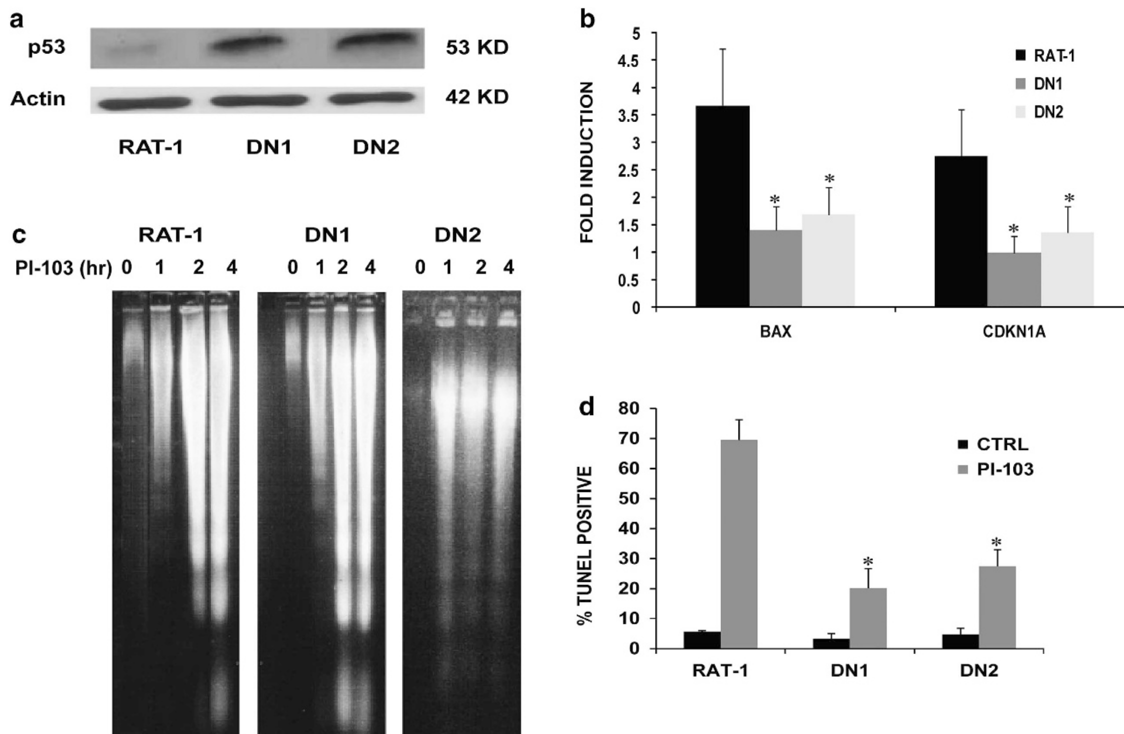


Figure 1 Effect of dominant-negative p53 on apoptosis induced by inhibition of PI 3-kinase. Characterization of two stably-transformed clones (DN1 and DN2) of Rat-1 cells expressing p53 V143A, as compared with wild-type Rat-1 cells. (**a**) Whole cell extracts were immunoblotted with anti-p53 and anti-β-actin antibodies. (**b**) Cells were treated with 10 nM actinomycin D for 24 h. Expression of *Bax* and *Cdkn1a* mRNAs were determined by real-time RT-PCR. Data are presented as fold induction compared with untreated cells and represent the means of three independent experiments ± S.E. *Indicates a significant reduction ($P<0.05$) in DN1 or DN2 compared with wild-type cells as determined by Student's *t*-test. (**c**) Cells were treated with 5 μM PI-103 for 0–4 h. Cytosolic nucleic acids were isolated and DNA fragmentation was assessed by gel electrophoresis. (**d**) Cells were treated with DMSO (control) or 5 μM PI-103 for 24 h. Apoptosis was analyzed after TUNEL staining by flow cytometry. Data represent mean ± S.E of 2–3 independent experiments. Cell death was reduced significantly in DN1 and DN2 cells compared with wild type ($P<0.01$, indicated by *)

obtained following treatment with GDC-0941, which is a more specific PI 3-kinase inhibitor that does not affect mTOR or related protein kinases[37] (see Figure 8).

Identification of genes induced by PI-3 kinase inhibition in proliferating Rat-1 cells. We used global expression profiling to investigate the role of p53 in the transcriptional changes resulting from inhibition of PI 3-kinase. Wild-type Rat-1 cells and Rat-1 cells expressing dominant-negative p53 were treated with PI-103 for 1 h to identify changes in gene expression coinciding with the onset of apoptosis. Microarray analyses were performed in three independent experiments and results for genes that were induced in response to inhibition of PI 3-kinase were verified by reverse transcription (RT)-PCR.

Treatment of wild-type Rat-1 cells with PI-103 for 1 h resulted in the upregulation of 26 genes, which were similarly induced by treatment with GDC-0941 (induction >1.7-fold, $P \leqslant 0.01$) (Figure 2). The upregulation of 14 of these genes was significantly inhibited in the Rat-1 clones-expressing dominant-negative p53, indicating that p53 contributed to their induction following inhibition of PI 3-kinase (Figure 2a). We also observed p53-dependent induction of the well-characterized p53 target gene *Cdkn1a*, which encodes p21, in response to inhibition of PI 3-kinase with either GDC-0941 or PI-103. Similar results were obtained for several representative genes in both the DN1 and DN2 cells expressing dominant-negative p53 (Figure 2b). Four of the p53-dependent genes induced by inhibition of PI 3-kinase (*Ccng2, Gadd45A, Tp53inp1* and *Nupr1*) have previously been identified as p53 targets.[38–40]

The genes induced in response to inhibition of PI 3-kinase in Rat-1 cells included genes encoding proteins with well-characterized roles in cell cycle arrest and/or apoptosis (ATROGIN-1, CCNG2 and TXNIP) that were also induced by inhibition of PI 3-kinase in T98G cells.[16,18] The role of representative p53 target genes (*Atrogin-1, Gadd45A, Nupr1, Tp53inp1* and *Txnip)* in apoptosis induced by PI 3-kinase inhibition was tested by siRNA knockdowns (Figure 2c). Inhibition of PI 3-kinase for 24 h resulted in 50–55% cell death (Figure 2c). Knockdown of four of the five p53 target genes tested (all except *Nupr1*) significantly reduced cell death, indicating the biological relevance of these genes in the cellular response to PI 3-kinase inhibition.

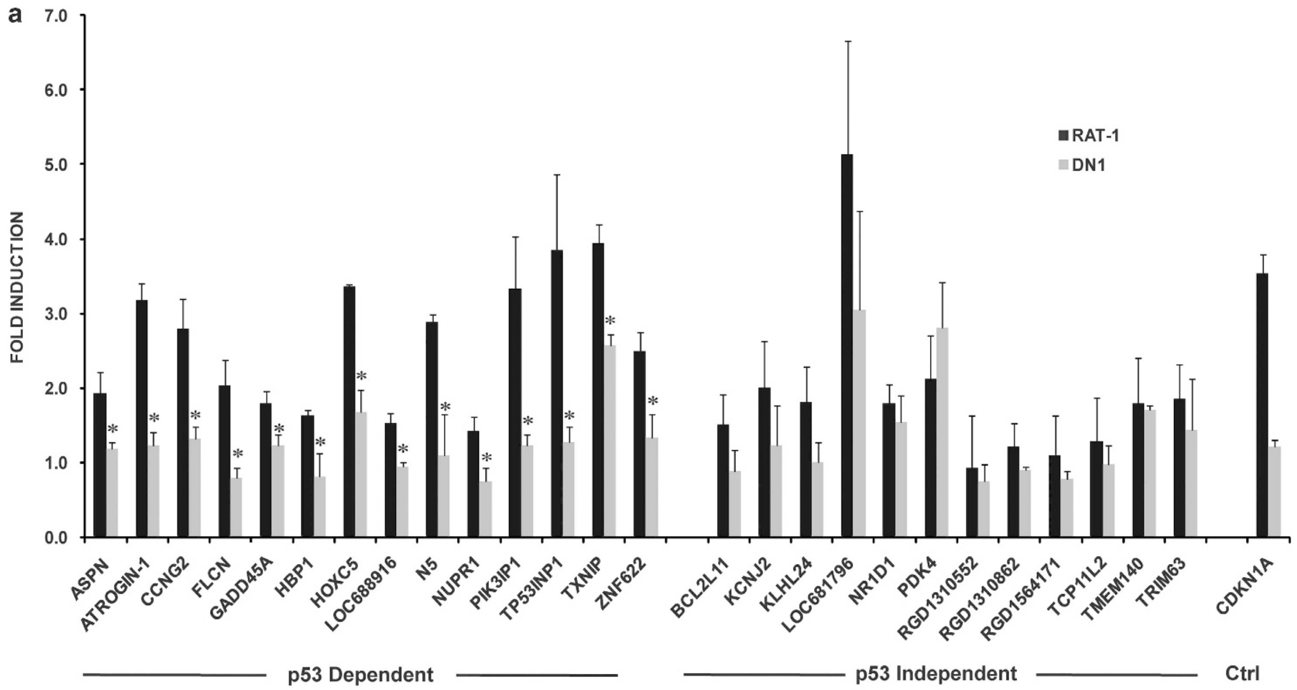
Identification of genes targeted by FOXO, MITF, USF1 and p53. The inhibition of induction of a subset of genes by expression of dominant-negative p53 indicates that inhibition of PI 3-kinase leads to activation of p53, which has a role in the transcriptional response. However, as the induction of most genes was unaffected or only partially inhibited by dominant-negative p53, it appears that p53 is only one component of the transcriptional response to PI 3-kinase inhibition. We previously reported that FOXO and the E-box-binding transcription factors MITF and USF1 had a major role in the induction of cell cycle arrest and proapoptotic genes in response to inhibition of PI 3-kinase in the p53-deficient T98G cell line.[18] FOXO, MITF and USF1 were also activated in Rat-1 cells in response to PI 3-kinase inhibition,[18] suggesting that these factors as well as p53 contribute to gene induction.

To further characterize the roles of FOXO, MITF, USF1 and p53 in the transcriptional program activated by PI-3 kinase inhibition, we identified transcription factor-binding sites that were overrepresented in the upstream regions of the genes induced by PI 3-kinase inhibition in Rat-1 cells. Computational analysis, as previously described,[16,41] was used to identify predicted binding sites that were conserved in human and rat sequences and were statistically overrepresented in the genes induced by inhibition of PI 3-kinase. Eighteen of the 26 induced genes were used for this analysis, as transcription start sites have not been identified for the other eight genes. FOXO and E-box-binding sites were the most significantly overrepresented regulatory sequences upstream of the genes induced by PI 3-kinase inhibition in Rat-1 cells, consistent with previous results in T98G cells.[16] FOXO-binding sites were predicted upstream of about 70% of the upregulated genes ($P<0.0001$, FDR corrected) and E-box sites upstream of about 50% of the upregulated genes ($P \leqslant 0.01$, FDR corrected).

Computational predictions of FOXO and E-box binding sites were verified by ChIP assays. Binding of FOXO3a, and to a lesser extent FOXO1, was detected to predicted sites upstream of 10 genes that were induced in response to inhibition of PI 3-kinase, including *Atrogin-1, Ccng2* and *Txnip*[18] (Figure 3a). These FOXO target genes included one gene whose induction was independent of p53 (*Pdk4*), as well as nine genes whose induction was inhibited by expression of dominant-negative p53 (*Aspn, Atrogin-1,Ccng2, Gadd45A, Flcn, Hbp1, Hoxc5, Tp53inp1* and *Txnip*). Similarly, binding of MITF was detected to predicted E-box sites upstream of six of the genes induced by inhibition of PI 3-kinase (Figure 3b). USF1 bound to the predicted E-box sequences upstream of the same six genes targeted by MITF, as well as to sequences upstream of *Nr1d1* (Figure 3c). As in the case of FOXO, the genes targeted by MITF and USF1 included both genes whose induction was inhibited (*Atrogin-1, Ccng2, Gadd45A, Hbp1, Hoxc5* and *Txnip*) and whose induction was unaffected by dominant-negative p53 (*Nr1d1*).

ChIP assays also confirmed the binding of p53 to predicted sites upstream of all of the genes tested whose induction was inhibited by expression of dominant-negative p53 (*Loc68916* and *N5* were not included, as their upstream sequences could not be obtained for computational analysis) (Figure 4a). The extent of binding of p53 to the predicted sequences upstream of these PI 3-kinase regulated genes was comparable to the binding of p53 upstream of *Cdkn1a*, which was included as a positive control.

There was substantial overlap in the genes targeted by FOXO, MITF, USF1 and p53 (Figure 4b). In total, 14 of the 18 genes analyzed were targeted by either FOXO, MITF, USF1 or p53 transcription factors. All but five of these genes were targeted by members of at least two families of these transcription factors and six were targeted by all three. A total of 12 genes were targeted by p53, with nine of these genes also targeted by FOXO and six targeted by both FOXO and the E-box factors. It thus appears that p53 contributes to gene regulation downstream of PI 3-kinase signaling as part of an overlapping network of transcription factors that includes FOXO, MITF and USF1.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TXNIP
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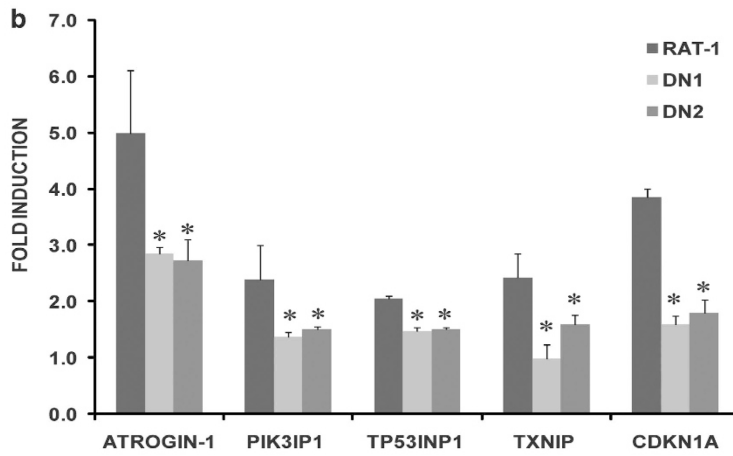
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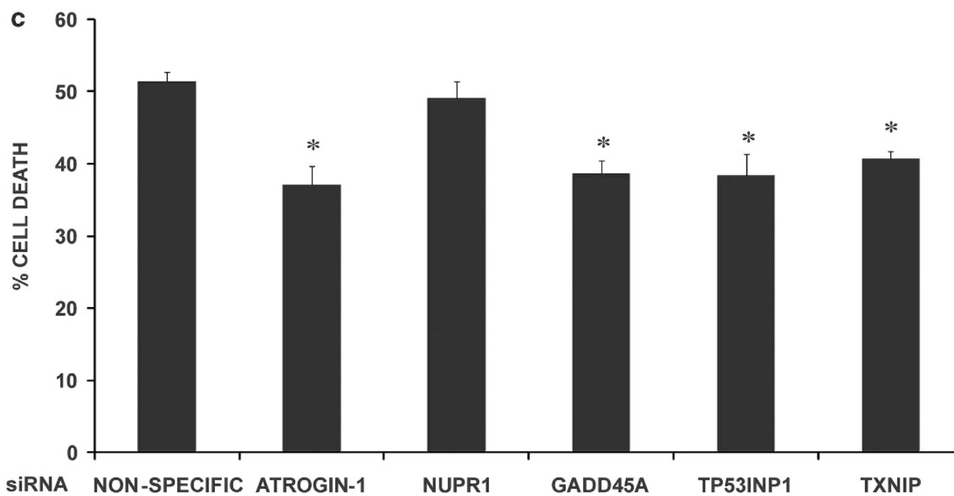
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GSK3 mediates activation of p53 in response to PI-3 kinase inhibition. We further explored the molecular mechanism underlying p53 regulation in response to PI-3 kinase inhibition. Intracellular levels of p53 are generally controlled by degradation mediated by the ubiquitin ligase MDM2.[24,26] Previous studies have shown that MDM2 is stimulated by Akt phosphorylation, providing a potential link between p53 and PI 3-kinase signaling.[28,29] However, we did not observe any detectable increase in p53 levels after inhibition of PI-3 kinase, whereas the expected increase in p53 was readily apparent after treatment with low doses of actinomycin D (Figure 5). It thus appears that regulation of MDM2 and subsequent stabilization of p53 is not the major mechanism underlying p53 activation following inhibition of PI 3-kinase.

It has been recently shown that PI 3-kinase signaling can also regulate the activity of p53 as a result of phosphorylation of the acetyltransferase Tip60 by GSK3.[30] GSK3 phosphorylation on serine 86 activates Tip60, which then activates p53 via acetylation of lysine 120.[30] As inhibition of PI 3-kinase leads to activation of GSK3, we sought to determine whether GSK3 was responsible for activation of p53 by this mechanism.

As expected, inhibition of PI 3-kinase led to rapid dephosphorylation of both Akt and GSK3 (Figure 6a), corresponding to activation of GSK3. Phosphorylation of Tip60 on serine 86 increased ~fourfold following inhibition of PI 3-kinase with either PI-103 or GDC-0941 (Figure 6b). Likewise, inhibition of PI 3-kinase increased the acetylation of p53 lysine 120 by three-to-four-fold (Figure 6c). Importantly, both Tip60 phosphorylation and p53 acetylation were blocked by treatment with the GSK3 inhibitor SB-21676, demonstrating the functional role of GSK3 in p53 activation. Inhibition of PI 3-kinase similarly resulted in GSK3-dependent phosphorylation of Tip60 (Figure 6d) and acetylation of p53 (Figure 6e) in PC12 rat pheochromocytoma cells.

These results indicated that activation of GSK3 following inhibition of PI 3-kinase led to activation of p53 via acetylation. We therefore sought to determine whether inhibition of GSK3 blocked transcriptional activation of p53 target genes following PI 3-kinase inhibition (Figure 7). Treatment with the GSK3 inhibitor SB-216763 interfered with the induction of most of the p53-target genes, including *Cdkn1a*, confirming the role of GSK3 in p53 activation. However, the induction of some genes that were dependent on p53 (*Flcn, Hoxc5*, *Loc688916* and *Txnip*) (Figure 2a) was not significantly reduced by inhibition of GSK3, consistent with the induction of these genes by other transcription factors or with the possibility that inhibition of PI-3 kinase can also activate p53 independently of GSK3 activity.

As GSK3 has a major role in activation of p53, as well as in activation of MITF and USF1,[18,30] in response to inhibition of PI 3-kinase, we further investigated the role of GSK3 in apoptosis both in wild-type Rat-1 cells and in cells expressing dominant-negative p53. Inhibition of GSK3 significantly inhibited apoptosis induced by inhibition of PI 3-kinase in both wild-type Rat-1 cells and in the DN1 and DN2 cells expressing the dominant-negative p53 mutant (Figure 8). These results indicate a role for GSK3 in both p53-dependent and p53-independent pathways, leading to apoptosis downstream of PI 3-kinase signaling, consistent with the role for GSK3 in gene regulation by p53, MITF and USF1.

Discussion

PI3-kinase/Akt signaling is required for the survival and growth of mammalian cells by coordinating the activities of a variety of downstream targets, including direct effectors of cell cycle progression and apoptosis, regulators of protein synthesis and autophagy, and proteins that control transcription and mRNA degradation. We have used global gene expression profiling to characterize the transcriptional program that is maintained by PI 3-kinase signaling in proliferating cells by investigating the changes in gene expression that result from inhibition of PI 3-kinase.[16,18] These studies have indicated that inhibition of PI 3-kinase results in rapid induction of genes that inhibit cell cycle progression and/or induce apoptosis. The FOXO and E-box-binding transcription factors MITF and USF1 have been shown to have key roles in this transcriptional response, with FOXO being regulated by Akt and MITF and USF1 being activated via phosphorylation by GSK3.[18]

The p53 transcription factor, which is a major regulator of cell proliferation and survival, can also be affected by PI 3-kinase signaling. p53 is typically activated in response to DNA damage, which results in stabilization of p53 and induction of target genes that induce cell cycle arrest and apoptosis.[24–27] PI 3-kinase/Akt signaling can impinge on the regulation of p53 via phosphorylation of MDM2, the ubiquitin ligase that targets p53 for proteasomal degradation.[28,29] In this study, we therefore sought to determine whether p53 had a role in the cellular response to inhibition of PI 3-kinase signaling by comparing the effects of PI 3-kinase inhibition in Rat-1 cells (which express wild-type p53) to transfected cell clones stably-expressing a dominant-negative p53 mutant.

Expression of dominant-negative p53 inhibited but did not prevent apoptosis resulting from inhibition of PI 3-kinase. This is consistent with previous reports that p53 contributes to apoptosis induced by growth factor deprivation[42–44] and indicates that p53 is one of the effectors contributing to cell

Figure 2 p53-dependent and -independent changes in gene expression and functional role of representative p53-dependent genes. (**a**) Rat-1 cells and cells expressing dominant-negative p53 (DN1) were treated with DMSO (control) or 1 μM GDC-0941 for 1 h. Levels of mRNA for induced genes identified by microarrays and for *Cdkn1a* were determined by real-time RT-PCR. Results represent means of three independent experiments $\pm$ S.E. The induced genes were classified into p53-dependent and -independent groups based upon 50% inhibition of gene induction in DN1 compared with wild-type cells ($P<0.05$, indicated by *). (**b**) Rat-1 cells and cells expressing dominant-negative p53 (DN1 and DN2) were treated with DMSO (control) or 5 μM PI-103 for 1 h. Levels of mRNA were determined by RT-PCR. Results represent means of three independent experiments $\pm$ S.E. *Indicates a significant reduction in DN1 or DN2 cells compared with wild type ($P<0.05$). (**c**) Rat-1 cells were transfected with the indicated siRNAs for 48 h and then treated with 1 μM GDC-0941 for 24 h. Cell viability was assessed by MTT assay both before and after GDC-0941 treatment. Results are presented as the percentage of cell death following GDC-0941 treatment. Data represent the means of six independent transfections $\pm$ S.E. Knockdown of ATROGIN-1, GADD45A, TP53INP1 and TXNIP significantly reduced cell death ($P<0.05$, indicated by *). Transfection with all siRNAs resulted in 80–90% reduction in target mRNA levels

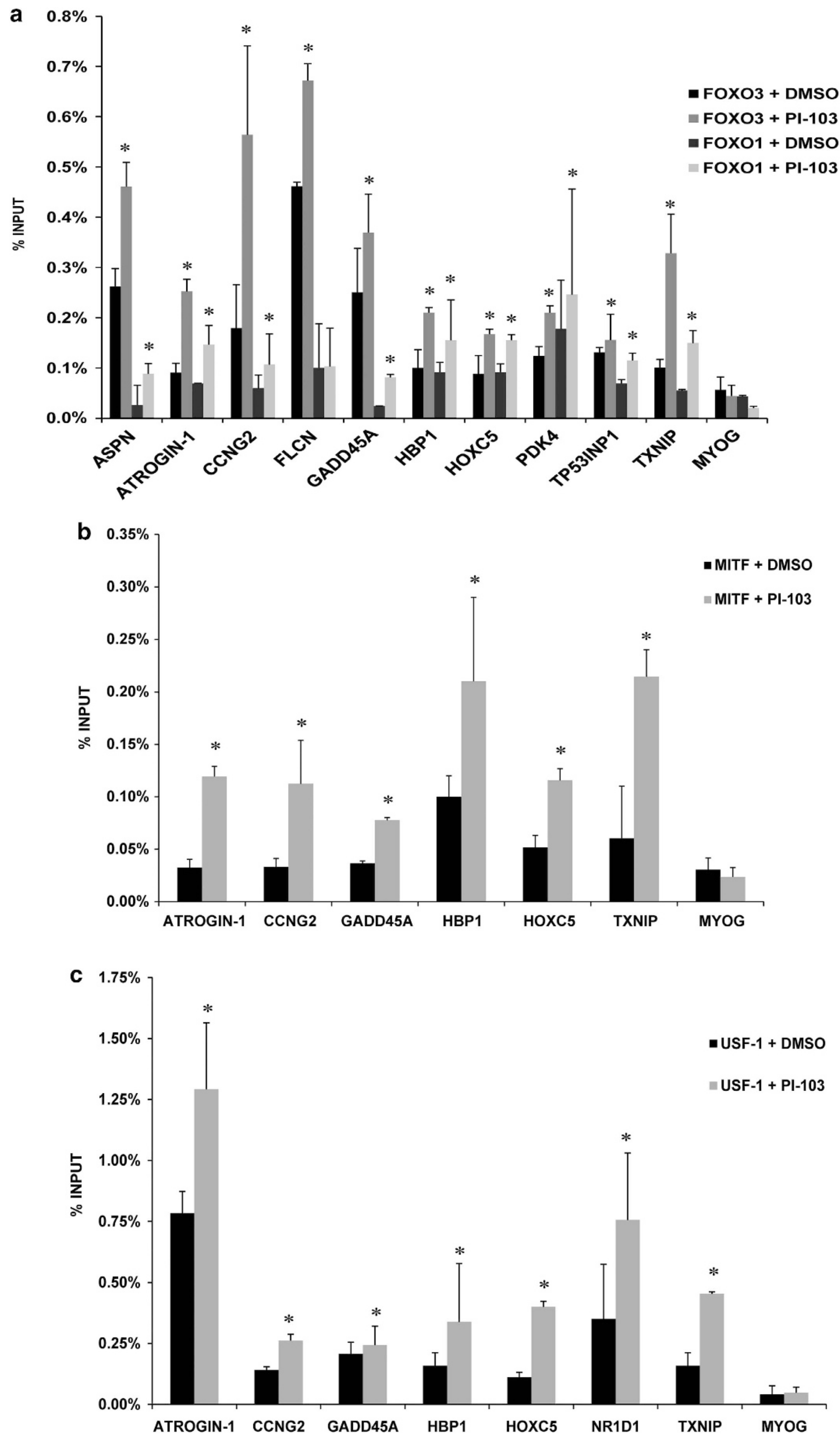

Figure 3 FOXO, MITF and USF1 binding upstream of genes induced by PI 3-kinase inhibition. Rat-1 cells were treated with DMSO or 5 μM PI-103 for 1 h. Chromatin fragments were immunoprecipitated with antibodies to (**a**) FOXO1 and FOXO3a, (**b**) MITF and (**c**) USF1 and quantified by real-time PCR. Positions of the FOXO and E-box sites tested are presented in Supplementary Table 1. Data are presented as the percentage of input DNA bound and are the means of at least five independent experiments ± S.E. *Myog* was used as the negative control. *Indicates significant binding ($P < 0.05$) following PI-103 treatment

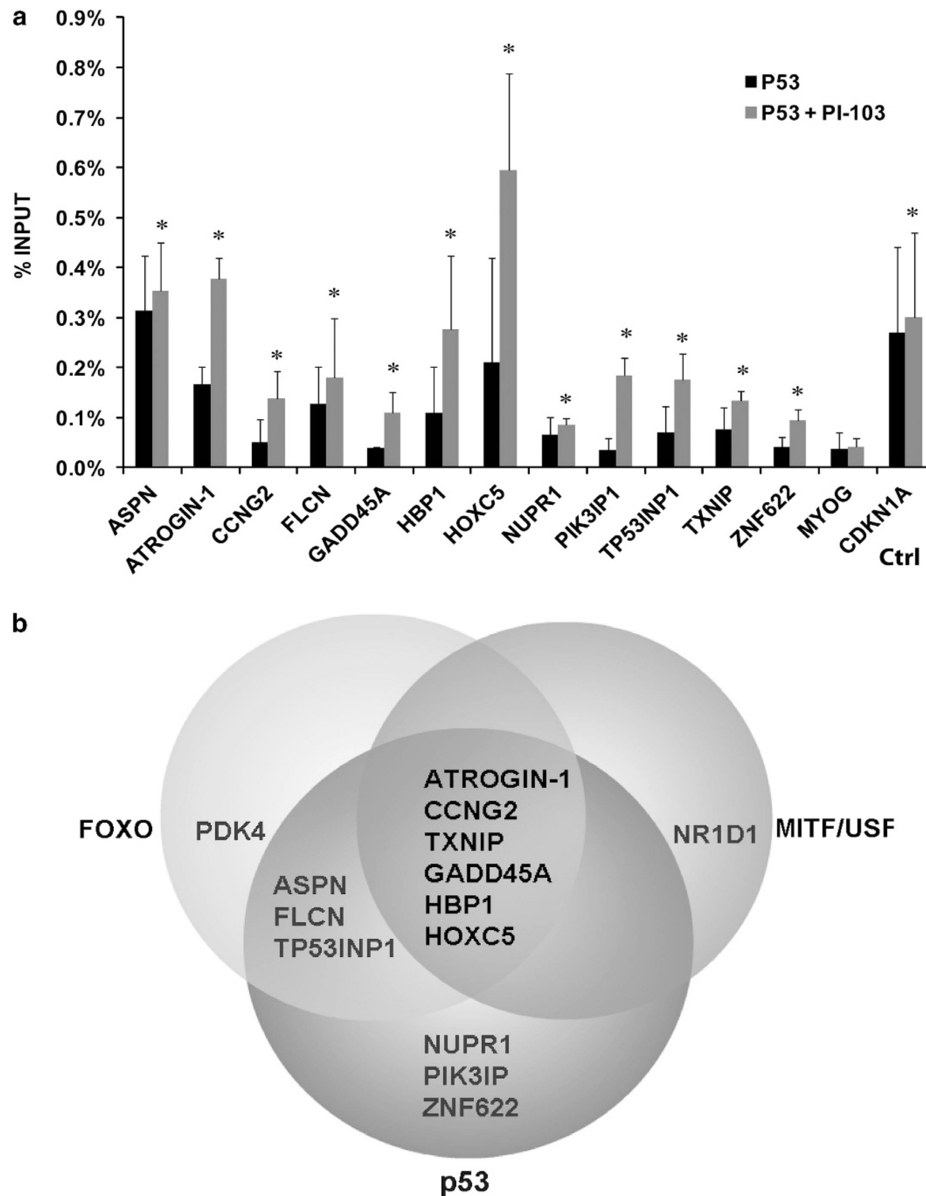

Figure 4 Analysis of p53 binding upstream of genes induced by PI 3-kinase inhibition. (**a**) Rat-1 cells were treated with DMSO or 5 μM PI-103 for 1 h. Chromatin fragments were immunoprecipitated with antibody to p53 and quantified by real-time PCR. Positions of the p53 sites tested are presented in Supplementary Table 1. Data are presented as the percentage of input DNA bound and are the means of three independent experiments $\pm$ S.E. *Myog* was used as the negative control. *Indicates significant binding ($P<0.05$) following PI-103 treatment. (**b**) Venn diagram showing the overlap between the genes targeted by FOXO, E-box binding (MITF and USF1) and p53 transcription factors

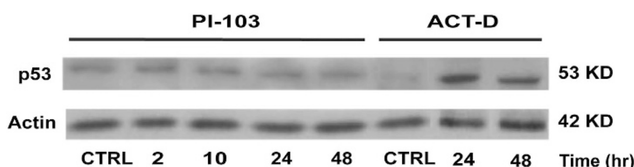

Figure 5 p53 levels remain constant following PI 3-kinase inhibition. Rat-1 cells were treated with DMSO (CTRL), 5 μM PI-103 or 10 nM actinomycin D (ACT-D) for the indicated times. Whole cell extracts were analyzed by immunoblotting with anti-p53 and anti-β-actin antibodies

death when PI 3-kinase signaling is blocked. Our current results on the effects of p53 on transcriptional changes in response to PI 3-kinase inhibition were consistent with this partial effect on apoptosis. The expression of about half of the genes induced in response to inhibition of PI 3-kinase was reduced in cells expressing dominant-negative p53, although the effect of dominant-negative p53 on most of these genes was a partial rather than complete block to their induction. Consistent with the partial effect of p53 on gene induction, the genes induced by inhibition of PI 3-kinase in Rat-1 cells were

also targeted by FOXO, MITF and USF1 transcription factors, as previously reported in human T98G cells.[16,18]

Notably, many of the genes targeted by p53 were co-regulated by FOXO and E-box transcription factors. Approximately 70% of the genes targeted by p53 were also targeted by FOXO, and nearly 50% of the genes targeted by p53 were also targeted by both FOXO and E-box-binding factors, indicating synergistic gene regulation by the transcription factors acting downstream of PI 3-kinase signaling. In agreement with this, FOXO transcription factors are known to cooperate with p53 and previous studies have identified cell cycle arrest and proapoptotic genes that are coregulated by p53 and FOXO transcription factors.[45]

Activation of p53 in response to DNA damage and other forms of cell stress is generally mediated by inhibition of p53 degradation, leading to increased intracellular p53 levels. Although Akt can regulate p53 degradation via phosphorylation of MDM2, this did not appear to be a major mechanism of p53 regulation in response to PI 3-kinase inhibition, as intracellular levels of p53 did not significantly increase.

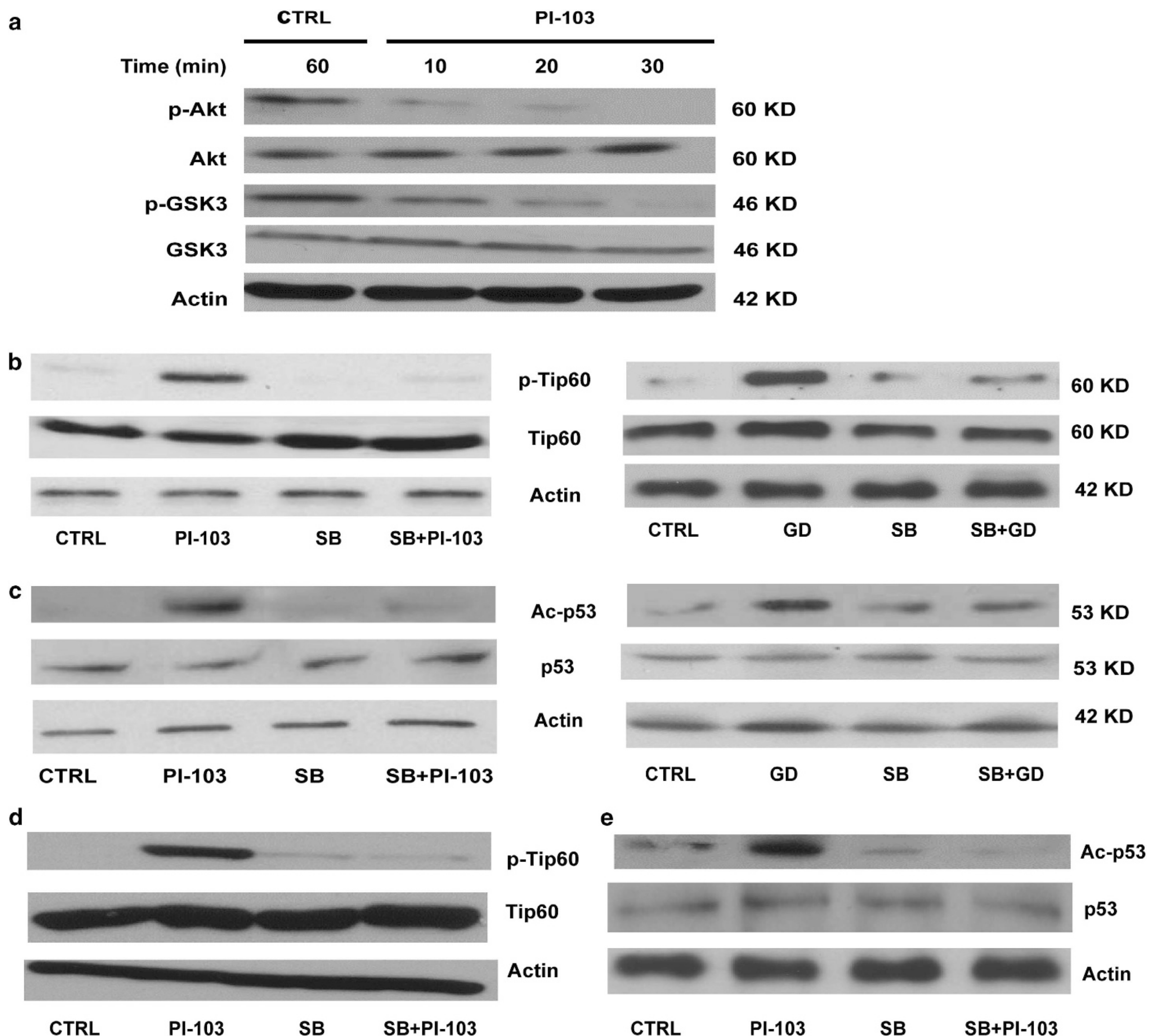

Figure 6 Activation of GSK3, phosphorylation of Tip60 and acetylation of p53 following inhibition of PI 3-kinase. (**a**) Rat-1 cells were treated with DMSO or 5 μM PI-103 for the indicated times. Whole cell extracts were analyzed by immunoblotting with antibodies to phospho-S473-Akt, pan Akt, phospho-S9-GSK3β, pan GSK3β and β-actin. (**b**) Rat-1 cells were treated for 1 h with DMSO (CTRL), 1 μM GDC-0941(GD), 5 μM PI-103, 5 μM SB-216763 (SB), or pretreated with SB-216763 for 1 h followed by 1 h with PI-103 (SB + PI-103) or GDC-0941 (SB + GD). Whole cell extracts were immunoblotted with antibodies against phospho-S86-Tip60, pan Tip60 and β-actin. (**c**) Rat-1 cells were treated as described for panel **b** and immunoblotted with antibodies against acetyl-K120-p53, pan p53 and β-actin. (**d**) PC-12 cells were treated for 1 h with DMSO (CTRL), 5 μM PI-103, 5 μM SB-216763 (SB) or pretreated with SB-216763 for 1 h followed by 1 h with PI-103 (SB + PI-103). Whole cell extracts were immunoblotted with antibodies against phospho-S86-Tip60, pan Tip60 and β-actin. (**e**) PC-12 cells were treated as described for panel **d** and immunoblotted with antibodies against acetyl-K120-p53, pan p53 and β-actin

Instead, activation of p53 was mediated primarily by a recently described pathway[30] in which GSK3 phosphorylates and activates the lysine acetyltransferase Tip60, which then activates p53 by acetylation. In this study, we confirmed this mechanism by demonstrating phosphorylation of Tip60 and acetylation of p53 in response to PI 3-kinase inhibition, both of which were blocked by inhibition of GSK3. Inhibition of GSK3 also blocked the induction of most p53-dependent genes in response to inhibition of PI 3-kinase, confirming the role of GSK3 in p53 activation. However, some p53-dependent genes were unaffected by inhibition of GSK3, suggesting that inhibition of PI 3-kinase may also lead to activation of p53 by other mechanisms, possibly as a result of phosphorylation of p53 by other stress-activated protein kinases, such as the c-Jun N-terminal kinase.[46]

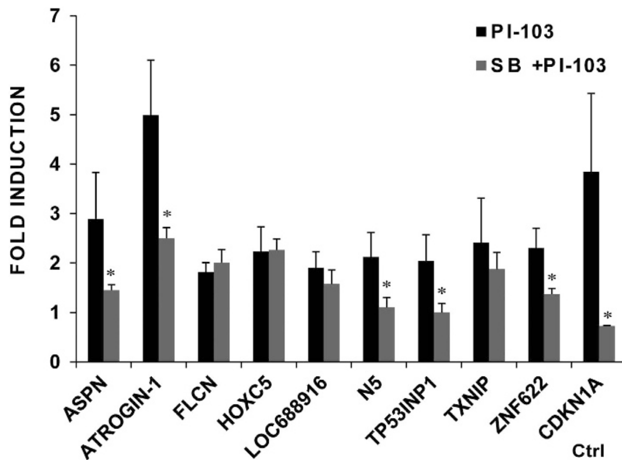

Figure 7 GSK3 regulates induction of p53-dependent genes. Rat-1 cells were treated with 5 μM PI-103 for 1 h with or without 1 h pretreatment with 5 μM SB-216763. Gene induction was determined by real-time RT-PCR. Data represent the mean of three independent experiments ± S.E. *Indicates a significant reduction in gene expression ($P<0.05$) in response to pretreatment with SB-216763

Inhibition of GSK3 also reduced, but did not completely block, apoptosis resulting from inhibition of PI 3-kinase in both wild-type Rat-1 cells and cells expressing dominant-negative p53. The reduction in apoptosis due to inhibition of GSK3 in both cell types suggests that p53 is one of several proapoptotic targets of GSK3, consistent with the identification of other GSK3 targets including MITF and USF1,[18,47–49] Mcl-1[7] and eIF2B.[12] The finding that GSK3 reduced but did not eliminate apoptosis is also consistent with the direct effects of Akt on other proapoptotic effectors, including FOXO[19,20] and Bad.[8,9] p53 is thus one component of the network of transcription factors that contributes to changes in

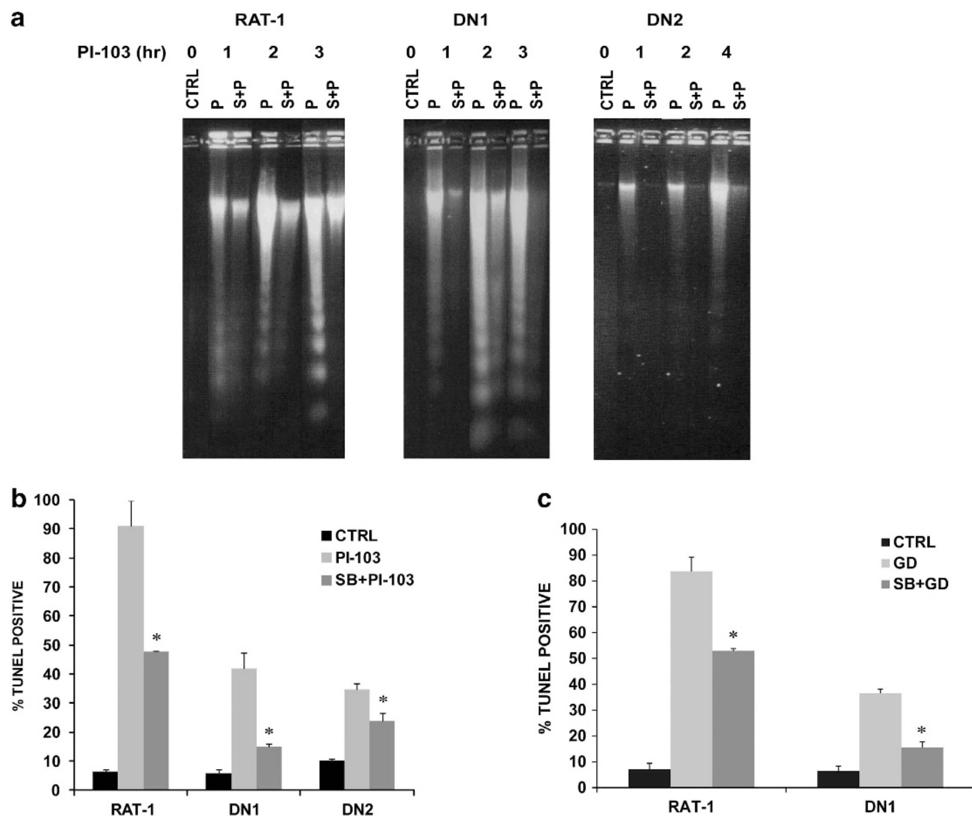

Figure 8 Role for GSK3 in apoptosis resulting from PI 3-kinase inhibition. (**a**) Rat-1 cells and cells expressing dominant-negative p53 (DN1 and DN2) were either treated with 5 μM PI-103 (P) or pretreated with 5 μM SB-216763 for 1 h followed by PI-103 addition (S + P). Cytosolic nucleic acids were isolated after 0–4 h treatment with PI-103 and DNA fragmentation was assessed by gel electrophoresis. (**b**) Cells were either treated with 5 μM PI-103 or pretreated with 5 μM SB-216763 for 1 h followed by PI-103 addition (SB + PI-103). Apoptosis was analyzed after 24 h of PI-103 treatment by TUNEL staining and flow cytometry. Data represent the means of three independent experiments ± S.E. *Indicates a significant reduction in TUNEL positive cells ($P<0.05$) in response to pretreatment with SB-216763. (**c**) Cells were either treated with 1 μM GDC-0941 or pretreated with 5 μM SB-216763 for 1 h followed by GDC-0941 addition (SB + GD). Apoptosis was analyzed after 24 h of GDC-0941 treatment. Data represent the means of three independent experiments ± S.E. *Indicates a significant reduction in TUNEL-positive cells ($P<0.05$) in response to pretreatment with SB-216763

gene expression and cell death following inhibition of PI 3-kinase and Akt, with resulting activation of GSK3.

Finally, we note that the function of p53 downstream of PI 3-kinase signaling may be relevant to tumor therapy with drugs targeting PI 3-kinase. Mutations that activate PI 3-kinase signaling are frequently found in human cancers, and several inhibitors of PI 3-kinase are currently being evaluated in clinical trials.[1,2] Mutations of p53 are also extremely common in human cancer,[24,26] and the role of p53 in inducing apoptosis downstream of PI 3-kinase signaling suggests that p53 mutations in tumors may confer partial resistance to PI 3-kinase inhibitors.

Materials and Methods

Cell culture and inhibitor treatments. Rat-1 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. PC12 rat pheochromocytoma cells were grown in DMEM supplemented with 10% fetal bovine serum and 5% horse serum. For inhibitor treatments, cells were plated at 10^6 cells per 100 mm dish and cultured for 48 h. Actively proliferating cells were treated with GDC-0941 (1 μM, Selleck Chemicals, Houston, TX, USA), PI-103 (5 μM, EMD Biosciences, San Diego, CA, USA), SB-216763 (5 μM, Biomol, Hamburg, Germany) or actinomycin D (10 nM, Sigma-Aldrich, St. Louis, MO, USA).

Isolation of Rat-1 cell clones expressing dominant-negative p53. Rat-1 cells were transfected with 1 μg of a plasmid expressing the dominant-negative V143A p53 cDNA under the control of a cytomegalovirus promoter and containing a geneticin (G418) resistance gene for selection.[33] 3×10^5 cells were plated in 35 mm plates 24 h before transfection. Transfections were performed with Lipofectamine (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. The following day, the standard medium was replaced with medium containing G418 (600 μg/ml). Individual G418-resistant colonies were isolated and expression of the exogenous mutant p53 was tested by immunoblot analysis. Cells were maintained in medium supplemented with 400 μg/ml G418.

Microarray analysis. Gene expression microarrays were performed with three independent RNA samples. Total RNA was extracted with TRIzol reagent (Invitrogen) and RNAs were further purified using a Qiagen (Valencia, CA, USA) RNeasy kit according to manufacturer's instructions. The quality of the RNA was determined using an Agilent (Santa Clara, CA, USA) bioanalyzer before analysis on Affymetrix (Santa Clara, CA, USA) Rat Gene 1.0ST microarrays. Microarray sample preparation/labeling, hybridization, scanning and subsequent data analysis were conducted by the Boston University Microarray Facility.

Real-time RT-PCR. Total RNA used for real-time RT-PCR was extracted using TRIzol extraction as per the manufacturer's protocol. Real-time RT-PCR was carried out as previously described.[41] Primer sequences can be found in Supplementary Table 2.

Immunoblotting. Cells were lysed in 2 $\times$ Laemmli buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis through a 10% gel, transferred to polyvinylidene difluoride membrane, and immunoblotted overnight at 4 °C with antibodies to p53 (Cell Signaling Technology, Danvers, MA, USA, 9282), β-actin (Sigma-Aldrich), KAT5/Tip60 (Abcam, Cambridge, MA, USA, 23886), KAT5/Tip60 phospho S86 (Abcam, 73207), p53 acetyl K120 (Abcam, 8316), GSK3 (BD Biosciences, San Jose, CA, USA, g22320), GSK3 phospho S9 (Cell Signaling Technology, 9336), Akt (Cell Signaling Technology, 9272) and Akt phospho S473 (Cell Signaling Technology, 9271) as recommended by the manufacturers. Membranes were washed thrice in 1 $\times$ Tris-buffered saline with 0.05% Tween 20, incubated in horseradish peroxidase linked to secondary antibody for 1 h at room temperature, washed thrice in Tris-buffered saline with 0.05% Tween 20 and then visualized using chemiluminescence. Densitometry was performed using Image J software (NIH).

Prediction of transcription factor binding sites. The prediction of transcription factor binding sites was carried out as described,[16,41] utilizing the TRANSFAC 12.1 database and the MinSUM threshold in regions spanning 3-kb upstream of the transcription start sites. Overrepresented binding sites were determined by comparing the frequency of predicted sites in the set of upregulated genes to the frequencies in a background set of genes that were expressed in proliferating Rat-1 cells but not affected by inhibition of PI 3-kinase.

Chromatin immunoprecipitation. ChIPs were performed as previously described,[16,18] using 5 μg of the following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA): anti-MITF (SC-25386), anti-USF1 (SC-229), anti-FOXO1 (SC-11350), anti-FOXO3a (SC-11351) and anti-p53 from Cell Signaling (9282). Immunoprecipitated DNA was quantified by real-time PCR using primers within 100 bp of predicted transcription factor binding sites. Primer sequences can be found in Supplementary Table 2.

Apoptosis assay by DNA fragmentation. DNA fragmentation was assayed as previously described.[50] Briefly cells (5×10^6 cells per 100 mm dish) were plated 24 h before treatment with PI-103. The cells were harvested, washed once with phosphate-buffered saline, and cytosolic nucleic acids were electrophoresed through a 1.5% agarose gel containing ethidium bromide. The gels were treated with 20 μg/ml RNase A for 4 h at 37 °C before visualization by UV transillumination.

TUNEL assay. Cells (1×10^6 cells per 60 mm dish) were plated 24 h before treatment with PI-103, GDC-0941 or SB216763 and apoptotic cells were detected using a fluorescent-based FragEL DNA fragmentation kit (Calbiochem, San Diego, CA, USA, QIA39) according to the manufacturer's protocol. Following staining and washing, samples were passed into 5-ml polystyrene tubes containing cell strainer (352235, BD Biosciences) and analyzed by flow cytometry.

RNA interference and cell viability. Cells were plated at $\sim$2000 cells per well in 96-well plates and transfected with predesigned siRNAs against ATROGIN-1 (Ambion, Carlsbad, CA, USA, s139579), GADD45A (Ambion, s129010), NUPR1 (Ambion, s137294), TP53INP1 (Ambion, s128542), TXNIP (Ambion, s138748) or a nonspecific negative control (Ambion, 4390843) using Lipofectamine 2000 per the manufacturer's protocol. Cells were transfected with 10 nM siRNAs against ATROGIN-1 and GADD45A and 20 nM siRNAs against NUPR1, TP53INP1 and TXNIP, optimized for the individual gene targets. Cells were transfected for 48 h followed by PI 3-kinase inhibition for 24 h with 1 μM GDC-0941. The effect of target gene knockdown on cell viability was assayed using a standard MTT assay (Promega, Madison, WI, USA) per the manufacturer's protocol.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We are grateful to Steve Mullenbrock, John Tullai and Ulla Hansen for helpful comments on the paper. This work was supported by National Institutes of Health Grant RO1 CA018689.

1. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* 2009; **9**: 550–562.
2. Vanhaesebroeck B, Stephens L, Hawkins P. PI3K signalling: the path to discovery and understanding. *Nat Rev Mol Cell Biol* 2012; **13**: 195–203.
3. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011; **12**: 21–35.
4. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 2009; **10**: 307–318.
5. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995; **378**: 785–789.
6. Pap M, Cooper GM. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J Biol Chem* 1998; **273**: 19929–19932.
7. Maurer U, Charvet C, Wagman AS, Dejardin E, Green DR. Glycogen synthase kinase-3 regulates mitochondrial outer membrane permeabilization and apoptosis by destabilization of MCL-1. *Mol Cell* 2006; **21**: 749–760.
8. Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y *et al.* Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997; **91**: 231–241.
9. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 1997; **278**: 687–689.
10. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998; **12**: 3499–3511.

11. Welsh GI, Miller CM, Loughlin AJ, Price NT, Proud CG. Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Lett* 1998; **421**: 125–130.
12. Pap M, Cooper GM. Role of translation initiation factor 2B in control of cell survival by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3β signaling pathway. *Mol Cell Biol* 2002; **22**: 578–586.
13. Brazil DP, Yang ZZ, Hemmings BA. Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci* 2004; **29**: 233–242.
14. Jope RS, Johnson GV. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 2004; **29**: 95–102.
15. Duronio V. The life of a cell: apoptosis regulation by the PI3K/PKB pathway. *Biochem J* 2008; **415**: 333–344.
16. Terragni J, Graham JR, Adams KW, Schaffer ME, Tullai JW, Cooper GM. Phosphatidylinositol 3-kinase signaling in proliferating cells maintains an anti-apoptotic transcriptional program mediated by inhibition of FOXO and non-canonical activation of NF-κB transcription factors. *BMC Cell Biol* 2008; **9**: 6.
17. Graham JR, Hendershott MC, Terragni J, Cooper GM. mRNA degradation plays a significant role in the program of gene expression regulated by phosphatidylinositol 3-kinase signaling. *Mol Cell Biol* 2010; **30**: 5295–5305.
18. Terragni J, Nayak G, Banerjee S, Medrano JL, Graham JR, Brennan JF *et al.* The E-box binding factors Max/Mnt, MITF, and USF1 act coordinately with FoxO to regulate expression of proapoptotic and cell cycle control genes by phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3 signaling. *J Biol Chem* 2011; **286**: 36215–36227.
19. Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BM. Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* 1999; **398**: 630–634.
20. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS *et al.* Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* 1999; **96**: 857–868.
21. Stein GH. T98G: an anchorage-independent human tumor cell line that exhibits stationary phase G1 arrest *in vitro*. *J Cell Physiol* 1979; **99**: 43–54.
22. Takahashi Y, Rayman JB, Dynlacht BD. Analysis of promoter binding by the E2F and pRB families *in vivo*: distinct E2F proteins mediate activation and repression. *Genes Dev* 2000; **14**: 804–816.
23. Van Meir EG, Kikuchi T, Tada M, Li H, Diserens AC, Wojcik BE *et al.* Analysis of the p53 gene and its expression in human glioblastoma cells. *Cancer Res* 1994; **54**: 649–652.
24. Levine AJ, Oren M. The first 30 years of p53: growing ever more complex. *Nat Rev Cancer* 2009; **9**: 749–758.
25. Kruse JP, Gu W. Modes of p53 regulation. *Cell* 2009; **137**: 609–622.
26. Vousden KH, Prives C. Blinded by the light: the growing complexity of p53. *Cell* 2009; **137**: 413–431.
27. Vousden KH, Lu X. Live or let die: the cell's response to p53. *Nat Rev Cancer* 2002; **2**: 594–604.
28. Mayo LD, Donner DB. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci USA* 2001; **98**: 11598–11603.
29. Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat Cell Biol* 2001; **3**: 973–982.
30. Charvet C, Wissler M, Brauns-Schubert P, Wang SJ, Tang Y, Sigloch FC *et al.* Phosphorylation of Tip60 by GSK-3 determines the induction of PUMA and apoptosis by p53. *Mol Cell* 2011; **42**: 584–596.
31. Ling CC, Guo M, Chen CH, Deloherey T. Radiation-induced apoptosis: effects of cell age and dose fractionation. *Cancer Res* 1995; **55**: 5207–5212.
32. Allan LA, Duhig T, Read M, Fried M. The $p21^{WAF1/CIP1}$ promoter is methylated in Rat-1 cells: stable restoration of p53-dependent $p21^{WAF1/CIP1}$ expression after transfection of a genomic clone containing the $p21^{WAF1/CIP1}$ gene. *Mol Cell Biol* 2000; **20**: 1291–1298.
33. Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW, Vogelstein B. Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science* 1992; **256**: 827–830.
34. Dai MS, Lu H. Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. *J Biol Chem* 2004; **279**: 44475–44482.
35. Choong ML, Yang H, Lee MA, Lane DP. Specific activation of the p53 pathway by low dose actinomycin D: a new route to p53 based cyclotherapy. *Cell Cycle* 2009; **8**: 2810–2818.
36. Fan QW, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D *et al.* A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* 2006; **9**: 341–349.
37. Folkes AJ, Ahmadi K, Alderton WK, Alix S, Baker SJ, Box G *et al.* The identification of 2-(1H-indazol-4-yl)-6-(4-methanesulfonyl-piperazin-1-ylmethyl)-4-morpholin-4-yl-t hieno [3,2-d]pyrimidine (GDC-0941) as a potent, selective, orally bioavailable inhibitor of class I PI3 kinase for the treatment of cancer. *J Med Chem* 2008; **51**: 5522–5532.
38. Wei CL, Wu Q, Vega VB, Chiu KP, Ng P, Zhang T *et al.* A global map of p53 transcription-factor binding sites in the human genome. *Cell* 2006; **124**: 207–219.
39. Riley T, Sontag E, Chen P, Levine A. Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol* 2008; **9**: 402–412.
40. Huang B, Vassilev LT. Reduced transcriptional activity in the p53 pathway of senescent cells revealed by the MDM2 antagonist nutlin-3. *Aging (Albany NY)* 2009; **1**: 845–854.
41. Tullai JW, Schaffer ME, Mullenbrock S, Kasif S, Cooper GM. Identification of transcription factor binding sites upstream of human genes regulated by the phosphatidylinositol 3-kinase and MEK/ERK signaling pathways. *J Biol Chem* 2004; **279**: 20167–20177.
42. Vaghefi H, Hughes AL, Neet KE. Nerve growth factor withdrawal-mediated apoptosis in naive and differentiated PC12 cells through p53/caspase-3-dependent and -independent pathways. *J Biol Chem* 2004; **279**: 15604–15614.
43. Aloyz RS, Bamji SX, Pozniak CD, Toma JG, Atwal J, Kaplan DR *et al.* p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. *J Cell Biol* 1998; **143**: 1691–1703.
44. Lotem J, Sachs L. Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. *Blood* 1993; **82**: 1092–1096.
45. Zhang X, Tang N, Hadden TJ, Rishi AK. Akt, FoxO and regulation of apoptosis. *Biochim Biophys Acta* 2011; **1813**: 1978–1986.
46. Hu MC, Qiu WR, JNK1 Wang YP. JNK2 and JNK3 are p53 N-terminal serine 34 kinases. *Oncogene* 1997; **15**: 2277–2287.
47. Takeda K, Takemoto C, Kobayashi I, Watanabe A, Nobukuni Y, Fisher DE *et al.* Ser298 of MITF, a mutation site in Waardenburg syndrome type 2, is a phosphorylation site with functional significance. *Hum Mol Genet* 2000; **9**: 125–132.
48. Khaled M, Larribere L, Bille K, Aberdam E, Ortonne JP, Ballotti R *et al.* Glycogen synthase kinase 3β is activated by cAMP and plays an active role in the regulation of melanogenesis. *J Biol Chem* 2002; **277**: 33690–33697.
49. Khaled M, Larribere L, Bille K, Ortonne JP, Ballotti R, Bertolotto C. Microphthalmia associated transcription factor is a target of the phosphatidylinositol-3-kinase pathway. *J Invest Dermatol* 2003; **121**: 831–836.
50. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990; **348**: 334–336.

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