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TIGAR, a p53-Inducible Regulator of Glycolysis and Apoptosis

Karim Bensaad,[1] Atsushi Tsuruta,[1,3] Mary A. Selak,[1] M. Nieves Calvo Vidal,[2] Katsunori Nakano,[1,4] Ramon Bartrons,[2] Eyal Gottlieb,[1] and Karen H. Vousden[1,*]
[1] The Beatson Institute for Cancer Research, Switchback Road, Glasgow G61 1BD, UK
[2] Unitat de Bioquimica i Biologia Molecular, Departament de Ciencies Fisiologiques II, Campus de Ciencies de la Salut, IDIBELL–Universitat de Barcelona, Feixa Llarga s/n, E-08907 L'Hospitalet, Spain
[3] Present address: Department of Surgery, Kurashiki Central Hospital, Miwa 1-1-1, Kurashiki, Okayama, Japan 710-8602.
[4] Present address: Surgical Department, Omihachiman Municipal Hospital, 395 Demachi, Omihachiman, Shiga, Japan 523-0892.
*Contact: k.vousden@beatson.gla.ac.uk
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SUMMARY

The p53 tumor-suppressor protein prevents cancer development through various mechanisms, including the induction of cell-cycle arrest, apoptosis, and the maintenance of genome stability. We have identified a p53-inducible gene named *TIGAR* (*TP53-i*nduced *g*lycolysis and *a*poptosis *r*egulator). TIGAR expression lowered fructose-2,6-bisphosphate levels in cells, resulting in an inhibition of glycolysis and an overall decrease in intracellular reactive oxygen species (ROS) levels. These functions of TIGAR correlated with an ability to protect cells from ROS-associated apoptosis, and consequently, knockdown of endogenous TIGAR expression sensitized cells to p53-induced death. Expression of TIGAR may therefore modulate the apoptotic response to p53, allowing survival in the face of mild or transient stress signals that may be reversed or repaired. The decrease of intracellular ROS levels in response to TIGAR may also play a role in the ability of p53 to protect from the accumulation of genomic damage.

INTRODUCTION

The p53 tumor-suppressor protein plays a critical role in responding to cellular stress and inhibiting malignant development (Vousden and Lu, 2002). Loss of p53 function contributes to the development of most cancers (Hollstein et al., 1999), making the potential reactivation of p53 an attractive therapeutic goal (Lain and Lane, 2003). However, success in these endeavors depends largely on a clear understanding of the consequences of p53 activation. p53 has been shown to be involved in the induction of apoptosis, cell-cycle arrest, senescence, and differentiation—responses that prevent further proliferation of stressed or damaged cells and so protect from the outgrowth of cells harboring malignant alterations (Vogelstein et al., 2000). A role for p53 in the repair of DNA damage has also been described (Sengupta and Harris, 2005), and the ability of p53 to induce reversible cell-cycle arrest may contribute to the ability of cells to repair and recover from damage before reentering a normal proliferative state.

One of the key functions of p53 is as a transcription factor. Stress-induced activation of p53 leads to the induction of expression of a large number of p53 target genes, several of which have been shown to play an important role in mediating the various responses to p53 (Vogelstein et al., 2000). For example, activation of expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ by p53 plays an important role in the induction of G1 cell-cycle arrest (Polyak et al., 1996; Waldman et al., 1995), and the expression of the BH3-domain protein PUMA is an essential component of p53-activated apoptotic pathways in several cell types (Jeffers et al., 2003; Villunger et al., 2003). Several lines of evidence suggest that one mechanism by which the response to p53 can be regulated is through differential activation of different groups of p53 target genes. In particular, the ability to induce expression of cell-cycle arrest targets without induction of the apoptotic target genes may allow for the resolution of damage in a stressed cell without recourse to permanent cell-cycle arrest or cell death (Vousden, 2000).

Recent studies have suggested that another important role for p53 in determining the death or survival of cells is the regulation of intracellular reactive oxygen species (ROS) levels. Organisms living in aerobic conditions are continuously subjected to ROS, and the cellular response to different ROS levels ranges from proliferation to growth arrest or senescence and cell death (Martindale and Holbrook, 2002). A number of studies have suggested a role for p53 in the regulation of ROS levels and therefore in the determination of the response to stress (Macip et al., 2003). p53 can activate numerous genes that result in increased generation of ROS, which contributes to apoptosis (Johnson et al., 1996; Li et al., 1999; Polyak et al., 1997) and also functions in a feedback loop in which ROS can signal to the further activation of p53 (Chen et al., 2003;

Martindale and Holbrook, 2002). However, more recent evidence suggests that p53 can also induce the expression of proteins that function to lower ROS levels and that this antioxidant function of p53 is important in preventing DNA damage and tumor development under low-stress conditions (Sablina et al., 2005). The sestrins have been identified as a family of p53-inducible proteins that provide an antioxidant defense to protect cells from hydrogen peroxide (H_2O_2) induced damage (Budanov et al., 2004). p53-dependent activation of ALHD4 has also been shown to decrease intracellular ROS (Yoon et al., 2004). It seems likely, therefore, that the regulation of ROS levels by different targets of p53 may play a key role in determining the choice of response to p53.

In this study we describe TIGAR (*T*P53-*i*nduced *g*lycolysis and *a*poptosis *r*egulator), a novel p53-inducible protein that functions to regulate glycolysis and protect against oxidative stress. TIGAR can lower ROS levels and decrease sensitivity to p53 and other ROS-associated apoptotic signals and is likely to be an important component in mediating the tumor-suppressive effects of p53.

RESULTS

Identification of *TIGAR* as a p53-Inducible Gene

Microarray analysis of gene expression following p53 induction that previously identified *p53R2* and *PUMA* (Nakano et al., 2000) yielded one further p53-responsive gene (accession number NM_020375; Jen and Cheung, 2005), which was named *TIGAR*. A time course following p53 induction indicated that *TIGAR* mRNA expression was elevated after 6 hr, with kinetics similar to those seen for established direct targets of p53 such as $p21^{WAF1/CIP1}$ and somewhat faster than the induction of the apoptotic target gene *BAX* (Figure 1A). Induction of *TIGAR* mRNA by endogenous p53 was assessed by treatment of wild-type p53-expressing U2OS or RKO cells with actinomycin D (Figure 1B). A robust activation of *TIGAR* mRNA expression was detected, although this was not seen in RKO cells in which p53 was inactivated by expression of HPV16 E6 (Figure 1B).

The *TIGAR* gene is located on chromosome 12p13-3 and contains six potential coding exons and two possible p53 binding sites, one upstream of the first exon (BS1) and one within the first intron (BS2) (Figure 1C). Binding of either inducible p53 in SAOS-2 cells or endogenous p53 in adriamycin-treated U2OS cells could be detected to BS1 and BS2 (Figure 1D). While the binding of p53 to BS2 appeared to be similar in efficiency to that seen for the p53 binding site in the $p21^{WAF1/CIP1}$ promoter, the binding to BS1 was less efficient. This lower binding efficiency was also seen in vitro, where only BS2 oligonucleotides could compete for binding of p53 to the high-affinity consensus binding sequence (Figure 1E). Binding of p53 to BS2 has also recently been detected using ChIP-on-chip analysis for novel p53 target genes (Jen and Cheung, 2005). Examination of the mouse genome revealed a similar organization of the mouse and human *TIGAR* genes, although only one potential p53 binding site was found upstream of the first exon of mouse *Tigar*. Preliminary analysis indicated that mouse cells show a weaker p53-dependent induction of *Tigar* expression than human cells (data not shown).

Comparison of the predicted amino acid sequence of the TIGAR protein revealed a high degree of conservation through vertebrate species from human to fish (Figure 2A). We raised mouse monoclonal and rabbit polyclonal antibodies against two peptides derived from sequences within the central and the COOH-terminal parts of the putative TIGAR protein (Figure 2A). Both antibodies immunoprecipitated Flag-tagged TIGAR (see Figure S1 in the Supplemental Data available with this article online), and Western blotting of cell lysates using these antibodies detected a single endogenous protein band of 30 kDa, consistent with the predicted size of TIGAR. Expression of this protein decreased following knockdown of TIGAR expression using two independent small interfering RNAs (siRNAs) (Figure 2B), which paralleled a reduction in mRNA levels (data not shown). Treatment of a variety of human cell lines expressing endogenous wild-type p53, including tumor cell lines (U2OS, RKO, and MCF-7) and untransformed epithelial (RPE) and fibroblast (MRC5) cells, with adriamycin resulted in the expected stabilization of p53 and elevation of $p21^{WAF1/CIP1}$ expression (Figure 2C). As expected from the mRNA expression analysis, induction of p53 also led to an increased expression of TIGAR protein. The p53 dependence of this induction was illustrated by the lack of TIGAR induction following adriamycin treatment of the p53 null human cancer cell line H1299 (Figure 2C). Interestingly, the tumor cell lines H1299, U2OS, and RKO each showed a significant basal level of TIGAR protein expression, demonstrating the existence of p53-independent mechanisms to regulate TIGAR expression.

p53-inducible genes can be broadly placed into two groups: those induced rapidly by low levels of stress (generally cell-cycle arrest or antioxidant targets) and those that are induced by higher levels of p53/stress (generally apoptotic targets) (Sablina et al., 2005). The results shown in Figure 1A suggested that *TIGAR* expression is rapidly induced in response to p53, like $p21^{WAF1/CIP1}$. Consistent with this observation, activation of endogenous p53 by low levels of adriamycin treatment in RKO cells resulted in the induction of TIGAR and $p21^{WAF1/CIP1}$ protein expression, while higher levels of stress were required to elevate expression of the apoptotic protein PIG3 (Figure 2D). Interestingly, extended exposure to stress and the induction of the p53-mediated apoptotic response correlated with a reduction in the expression of TIGAR and $p21^{WAF1/CIP1}$, suggesting that TIGAR may play a role in controlling the switch in p53 response (Figure 2E and Figure S2A). Another mechanism that can distinguish classes of p53 targets is their transcriptional sensitivity to the p53R175P mutant, which selectively fails to activate the expression of some apoptotic target genes (Ludwig et al., 1996).

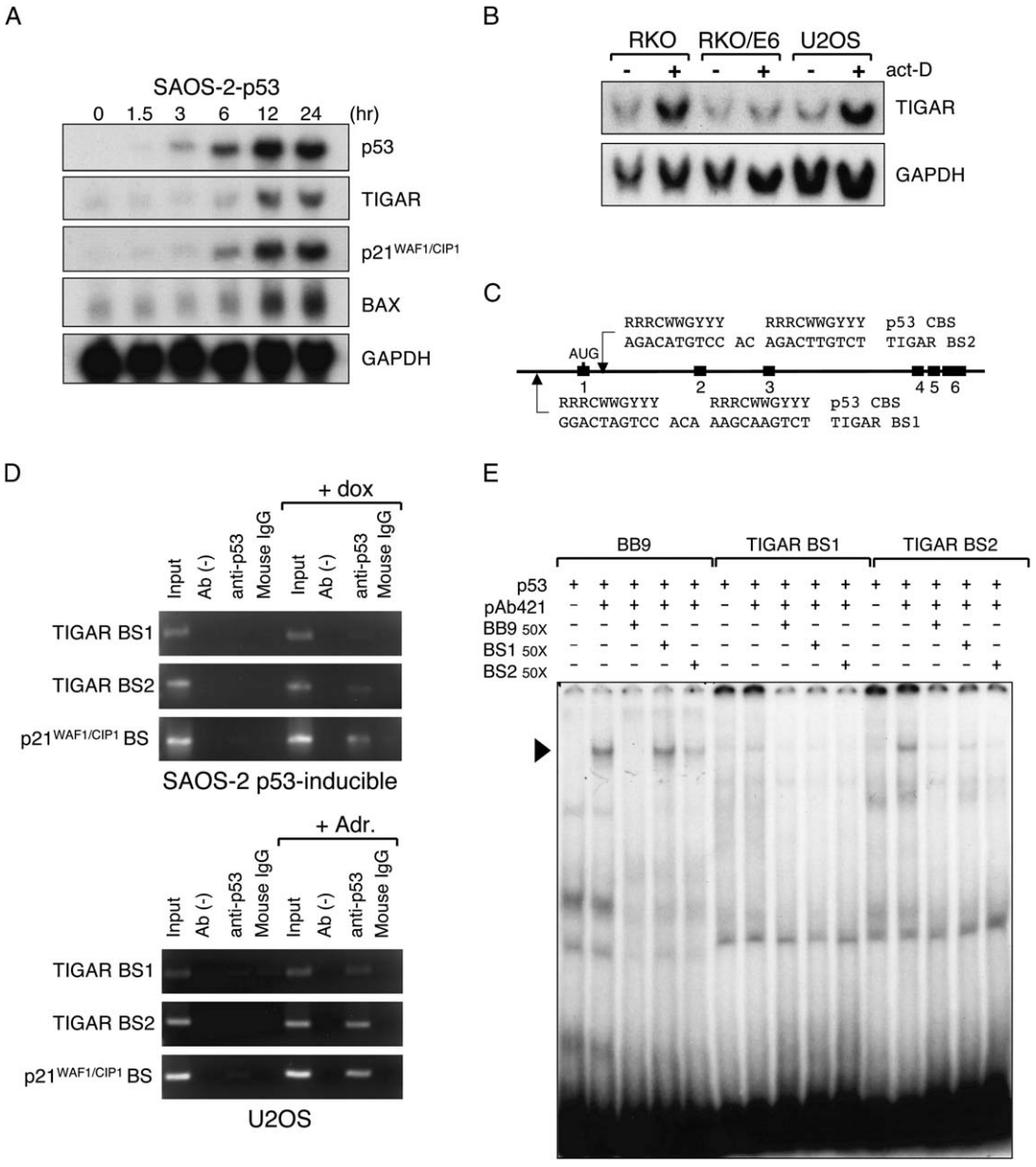

Figure 1. Identification of *TIGAR* as a p53-Inducible Gene

(A) Northern blot using specific probes showing expression of p53, *TIGAR*, *p21*$^{WAF1/CIP1}$, and *BAX* in p53-inducible SAOS-2 cells without (0 hr) and with (1.5 to 24 hr) doxycycline treatment (2 μg/ml). GAPDH expression was examined as a loading control.

(B) Northern blot using *TIGAR*-specific probes, showing expression of *TIGAR* in RKO, RKO/E6, and U2OS cells without (−) and with (+) actinomycin D treatment (5 nM) for 24 hr.

(C) The genomic structure of human *TIGAR*, showing the exon/intron organization and the two potential p53 binding sites upstream of the first exon (*TIGAR* BS1) and within the first intron (*TIGAR* BS2) compared to the consensus p53 binding site (p53 CBS). R, purine; Y, pyrimidine; W, adenine or thymine. The predicted initiation codon (AUG) is also indicated.

(D) ChIP assay for *TIGAR* and *p21*$^{WAF1/CIP1}$ in p53-inducible SAOS-2 cells without and with doxycycline treatment (2 μg/ml) for 24 hr and in U2OS cells without and with adriamycin treatment (0.2 μg/ml) for 24 hr. Crosslinked p53 protein/DNA complexes were immunoprecipitated with an anti-p53 antibody (DO-1). Control immunoprecipitations were carried out without antibody (Ab (−)) or with normal mouse IgG (Mouse IgG).

(E) EMSA showing the DNA binding activity of p53 to oligonucleotides containing a consensus p53 binding site (BB9), *TIGAR* BS1, and *TIGAR* BS2. The DNA binding activity of in vitro-translated p53 protein was activated using the COOH-terminal anti-p53 antibody pAb421 as indicated. Specificity of the binding was checked using an excess (50×) of nonradiolabeled competitive *TIGAR* BS1, *TIGAR* BS2, and BB9 oligonucleotides.

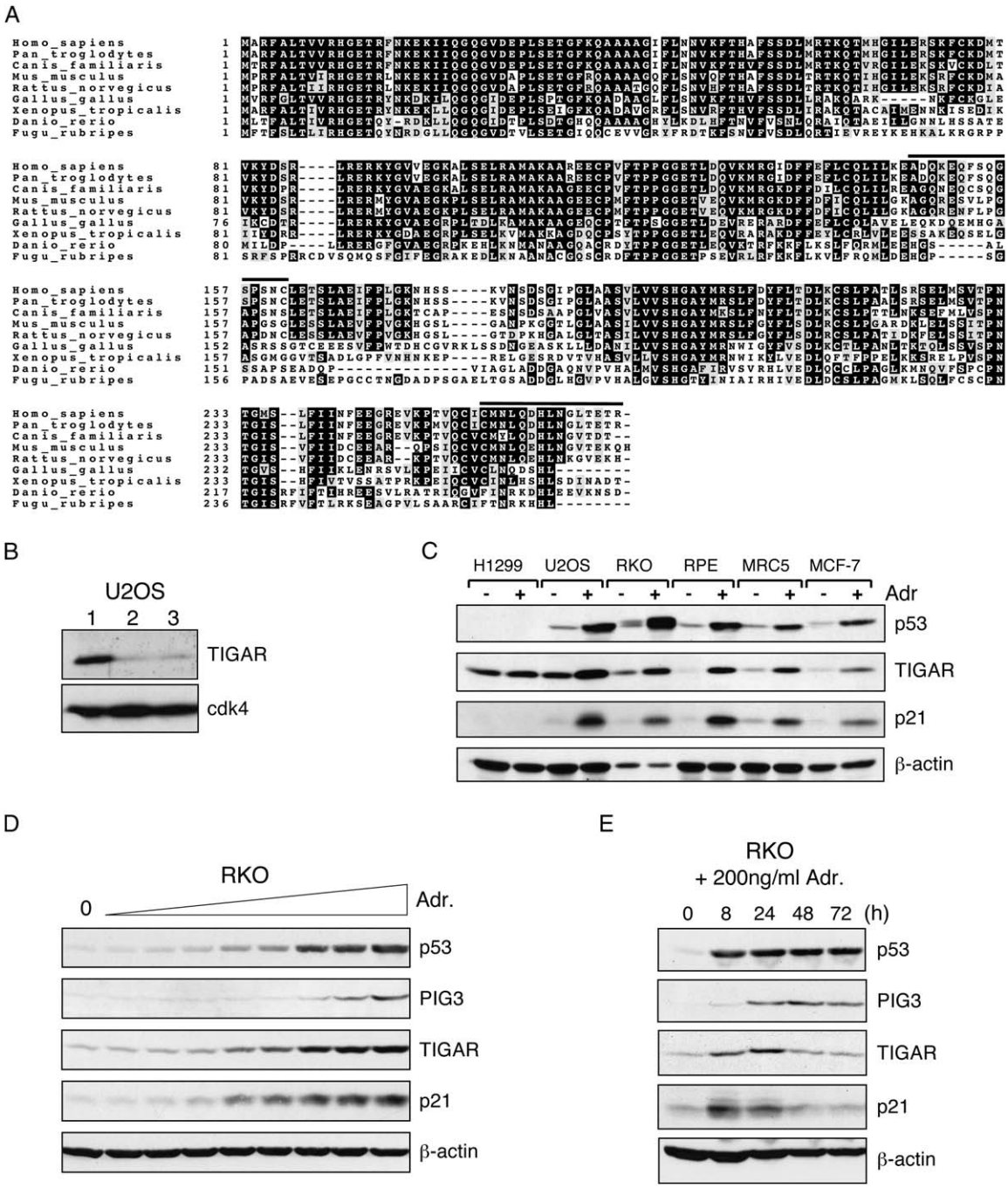

Figure 2. TIGAR Protein Is Induced by p53

(A) Multiple alignment of human TIGAR protein with homologous proteins from different species. The alignment was generated using the ClustalW program. Black shading shows amino acid residues conserved in aligned sequences. The positions of the aligned regions in the respective protein sequences are indicated, as are the peptides used to raise antibodies.

(B) Expression of endogenous TIGAR in U2OS cells transfected with scrambled siRNA (1), TIGAR siRNA1 (2), or TIGAR siRNA2 (3). TIGAR was detected by Western blotting using the anti-TIGAR monoclonal antibody. Cdk4 expression was examined as a loading control.

(C) Expression of endogenous p53, TIGAR, and p21$^{WAF1/CIP1}$ in various cells without (−) and with (+) adriamycin treatment (0.2 μg/ml) for 24 hr. p53, TIGAR, and p21$^{WAF1/CIP1}$ were detected by Western blotting. β-actin expression was examined as a loading control.

(D) Western blot showing expression of endogenous p53, PIG3, TIGAR, and p21$^{WAF1/CIP1}$ in RKO cells without (0) and with treatment with an increasing amount (2.5–200 ng/ml) of adriamycin for 24 hr.

(E) Western blot showing expression of endogenous p53, PIG3, TIGAR and p21$^{WAF1/CIP1}$ in RKO cells without (0) and with treatment with 200 ng/ml adriamycin for the indicated times. β-actin expression was examined as a loading control.

Like $p21^{WAF1/CIP1}$, TIGAR was activated by p53R175P, but not the transcriptionally inactive p53R175H mutant (Figure S2B).

TIGAR Shows Similarities to the Bisphosphatase Domain of PFK-2/FBPase-2

Having established TIGAR as a bone fide p53 target, we examined the TIGAR protein sequence for any information about possible function. Using predicted amino acid sequence on the entire genomic database, TIGAR was found to share similarity with proteins in the phosphoglycerate mutase (PGM) family. This similarity was mainly limited to the catalytic regions, and within these, the highest degree of similarity was observed with the bisphosphatase domain of the different isoform products of the four genes (*pfkfb1–4*) encoding the enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) (Figure 3A) (Okar et al., 2001). PFK-2/FBPase-2 is a bifunctional enzyme with both kinase and bisphosphatase activities that are catalyzed at different sites on each subunit of this protein. The kinase domain, PFK-2, is localized within the NH_2-terminal part of the enzyme, and the bisphosphatase domain, FBPase-2, is in the COOH-terminal region. Interestingly, the similarity between TIGAR and PFK-2/FBPase-2 is limited to the bisphosphatase domain. Although overall the sequence similarity between TIGAR and FBPase-2 was relatively weak, the regions in the FBPase-2 domain that have previously been identified as essential for catalytic activity are well conserved in TIGAR (Figure 3A). These include the three amino acids (H, E, and H; Figure 3A) that constitute the catalytic triad of the active site (Lin et al., 1992) and the RHG signature (Figure 3A, region 1) that is characteristic of the PGM family (Bazan et al., 1989). While TIGAR shows similarity with these regions of all members of the PGM family, sequences surrounding the third residue (H) of the catalytic triad are more similar between TIGAR and the corresponding region in the bisphosphatase domain of PFK-2/FBPase-2 than the other PGM family members (Figure 3A, region 2). These regions are also conserved in TIGAR of different species (Figure 2A). Taken together, these observations suggest that TIGAR may function as a fructose bisphosphatase but would not be predicted to show any kinase activity associated with the full-length PFK-2/FBPase.

PFK-2/FBPase-2 regulates both the synthesis (through PFK-2) and the degradation (through FBPase-2) of intracellular fructose-2,6-bisphosphate (Fru-2,6-P_2), a potent positive allosteric effector of 6-phosphofructo-1-kinase (PFK-1), which stimulates glycolysis. Fru-2,6-P_2 is also an inhibitor of fructose-1,6-bisphosphatase (FBPase-1), a regulatory enzyme of gluconeogenesis (Okar et al., 2001). The function of FBPase-2 is therefore to lower Fru-2,6-P_2 levels, thereby decreasing the activity of PFK-1 and enhancing the activity of FBPase-1, and so inhibiting glycolysis.

In order to determine whether TIGAR can function in a manner similar to FBPase-2, we examined the levels of Fru-2,6-P_2 in cells either transiently or constitutively overexpressing TIGAR following transfection (Figure 3B). These experiments showed a reproducible decrease in Fru-2,6-P_2 levels following TIGAR expression. The extent of this decrease was comparable to that seen following expression of a truncated form of PFK-2/FBPase-2 expressing only the bisphosphatase domain (FBPase-2) (Figure 3B). In order to assess whether the three amino acids in TIGAR that are analogous to the key residues of the catalytic triad in the FBPase-2 domain (H, E, and H; Figure 3A) are active sites for a putative bisphosphatase activity, we generated the TIGAR-TM mutant (triple mutant H11A/E102A/H198A). Interestingly, mutation in these amino acids, shown to be necessary for enzyme activity in FBPase-2, abrogated the ability of TIGAR expression to lower Fru-2,6-P_2 levels (Figure 3B). To establish an activity for endogenous TIGAR, we examined the effect of siRNA knockdown (Figure 3C). As predicted, lowering TIGAR expression resulted in an increase in Fru-2,6-P_2 levels. Further analysis showed that the decrease of Fru-2,6-P_2 levels in cells overexpressing TIGAR correlated with a decrease in glycolytic rates in the murine IL-3-dependent pro-B FL5.12 cell line (Figure 3D) and human U2OS cells (Figure 3E). Although this effect was modest, it was similar to the extent of reduction in glycolysis seen following expression of the FBPase-2 domain (Figure 3E). Furthermore, knockdown of endogenous TIGAR expression using siRNAs resulted in an increase in glycolytic rates (Figure S3).

TIGAR Expression Can Modulate Apoptosis in a Cell-Type-Dependent Manner

Several recent studies have shown that modulation of glycolytic rates can have a profound effect on the apoptotic sensitivity of cells (Vander Heiden et al., 2001). After growth-factor withdrawal, the FL5.12 cell line has been shown to be extremely sensitive to changes in glycolysis, with lower glycolytic rates leading to enhanced cell death by apoptosis (Vander Heiden et al., 2001). IL-3-deprived cells overexpressing TIGAR showed enhanced apoptosis (Figure 4A), which correlated with the level of TIGAR expression and the decrease in glycolytic rate (Figure 3D). While these results are consistent with previous published observations linking inhibition of glycolysis with enhanced death in this cell system, we were also interested in a series of studies showing that decrease in flux through the major glycolytic pathway by inhibition of PFK-1 activity might be linked to increased resistance to cell death (Boada et al., 2000; Perez et al., 2000). More specifically, overexpression of the FBPase-2 domain showed that the inhibition of PFK-1 resulted in the accumulation of fructose-6-phosphate (F6P). F6P is then isomerized to glucose-6-phosphate (G6P) in cells, and this accumulated G6P is diverted into the pentose phosphate pathway (PPP), an alternative metabolic pathway that can provide substrates for the later steps in glycolysis. The increased flow through the PPP lowered apoptosis due to an increased generation of reduced glutathione and removal

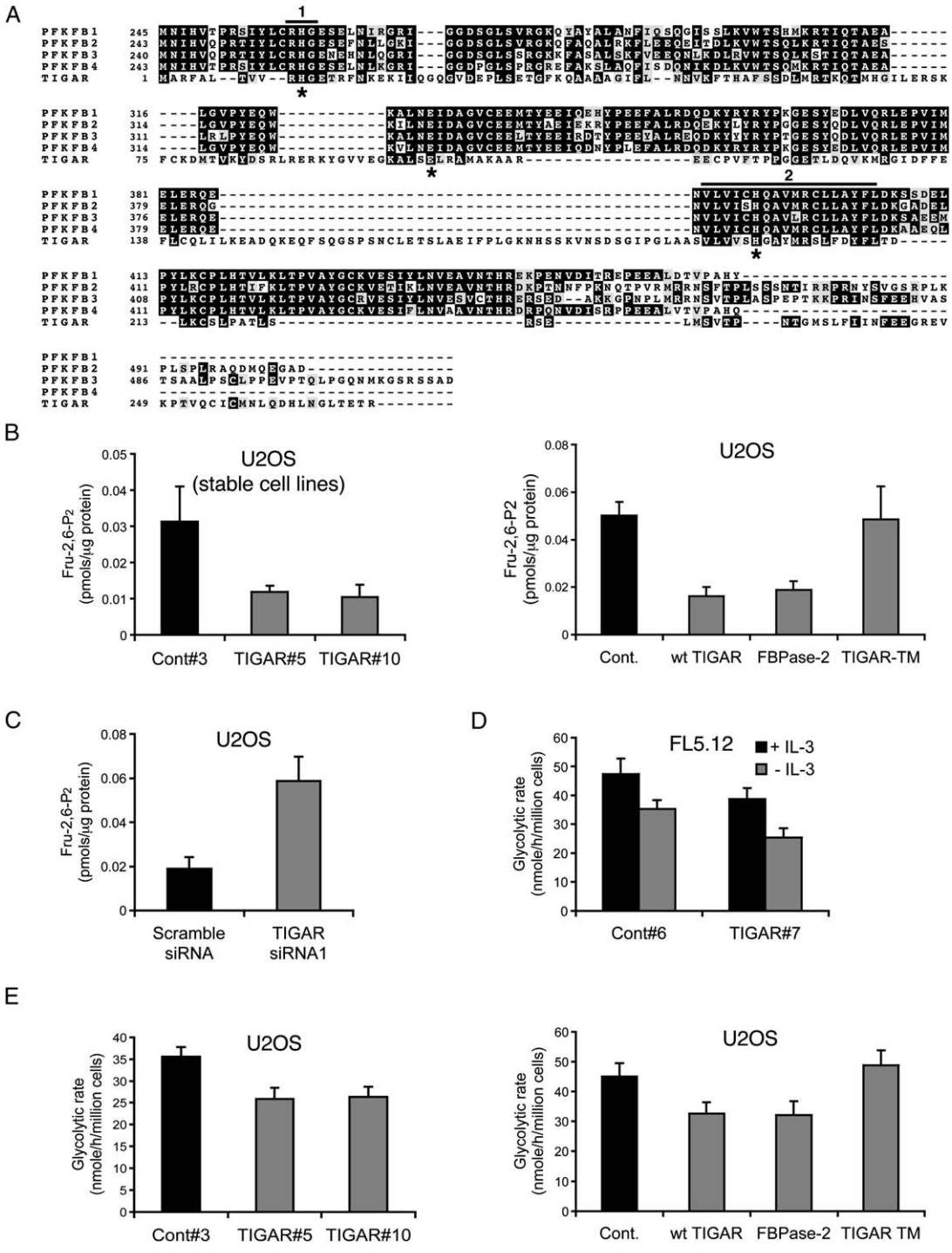

Figure 3. TIGAR Is a Protein with Similarities to PFK-2/FBPase-2 that Can Inhibit Glycolysis

(A) Multiple alignment of human TIGAR protein with the sequences of the products of the four PFK-2/FBPase-2 genes, *pfkfb1*, *pfkfb2*, *pfkfb3*, and *pfkfb4*. The alignment was generated using the MACAW program. Black shading shows amino acid residues conserved in aligned sequences. The two essential domains for activity are indicated (1 and 2), and the three amino acids that are part of the catalytic triad of the bisphosphatase are denoted by asterisks. The positions of the aligned regions in the respective protein sequences are indicated.

(B) Intracellular Fru-2,6-P_2 concentration determination in U2OS control cells (clone Cont#3) and cells stably overexpressing Flag-tagged TIGAR (clones TIGAR#5 and TIGAR#10) (left panel) and U2OS cells transiently transfected with empty vector pcDNA3.1 or expression plasmids for Flag-tagged TIGAR, HA-tagged FBPase-2, or Flag-tagged TIGAR-TM (right panel). In this and all other figures, unless stated otherwise, the mean and standard deviation of the mean for at least three independent experiments are shown.

of ROS in cells. The similarity in function between TIGAR and the FPBase-2 domain prompted us to examine the effect of TIGAR overexpression on p53-induced apoptosis, which has been associated with increased ROS (Polyak et al., 1997). This system is different from the IL-3-withdrawal-induced death seen in the FL5.12 cells, which is neither clearly p53 dependent nor ROS generating. In U2OS cells, the apoptosis induced by p53 expression was lowered by coexpression of either TIGAR or the isolated FBPase-2 domain (Figure 4B). TIGAR overexpression lowered even background levels of apoptosis, and, when coexpressed with p53, TIGAR decreased apoptotic rates slightly more efficiently than expression of FBPase-2 (Figure 4C). To determine whether this survival effect reflects levels of TIGAR expression, we analyzed different populations of transfected cells with no, low, or high TIGAR levels and showed that increased levels of TIGAR correlated with decreased apoptosis (Figure 4D). Although TIGAR clearly modulated the apoptotic response seen after extended stress, the cell-cycle arrest induced by p53 following a shorter exposure to stress was unaffected by TIGAR (Figure S4). This is consistent with retention of the ability to induce $p21^{WAF1/CIP1}$ (Figure 2E). Interestingly, coexpression of full-length PFKFB1, which, like other PFK-2/FBPase-2 isoforms, has a kinase/bisphosphatase ratio that results in a net increase of phosphofructokinase activity and an accumulation of Fru-2,6-P_2 in the cells (Perez et al., 2000), can overcome the effect of TIGAR on apoptosis (Figure 4E). These results indicated that an FBPase-2 activity of TIGAR may be responsible for its ability to reduce apoptosis, a suggestion that was further supported by the analysis of TIGAR mutants that were altered in the key residues essential for bisphosphatase activity (Figure 5A). While each of these mutants was efficiently expressed (Figure 5B), only wild-type TIGAR efficiently inhibited p53-induced apoptosis (Figure 5C). The loss of activity of the TIGAR-TM mutant correlated with the failure of this mutant to lower intracellular Fru-2,6-P_2 levels (Figure 3B) and decrease the glycolytic rate (Figure 3E).

To determine the role of endogenous TIGAR in modulating p53-dependent apoptosis, we used siRNAs to knock down TIGAR expression in a number of cell lines. In U2OS cells, siRNAs that reduced TIGAR expression (Figure 2B) led to an enhanced sensitivity of cells to apoptosis following activation of endogenous p53 by adriamycin (Figure 6A) or overexpression of ectopic p53 by transfection (Figure 6B). To confirm that the enhanced apoptosis is a specific result of a decrease in TIGAR levels rather than an off-target effect of the siRNAs, we constructed a siRNA-resistant *TIGAR* mutant that expressed wild-type TIGAR protein. Expression of this construct reversed the effect of the TIGAR siRNA, allowing expression of TIGAR protein (Figure S7) and a decrease in apoptosis (Figure 6B). Knockdown of endogenous TIGAR expression also resulted in enhanced apoptosis following p53 activation in a number of other cell lines, including MCF-7 cells (Figure S5A), RKO cells (Figure S5B), and HCT116 cells (Figure 6C). To further assess the ability of TIGAR to modulate p53-induced apoptosis, we examined the effect of treatment of the cells with Nutlin-3, a direct and specific activator of p53 (Vassilev et al., 2004). As previously reported, treatment with Nutlin-3 enhanced death in HCT116 cells in a manner dependent on the presence of p53 (Figure 6C). Although inhibition of TIGAR expression enhanced the p53-dependent death as expected, no increase in apoptosis in p53 null cells treated with Nutlin-3 was observed (Figure 6C), demonstrating a direct role of TIGAR in modulating the p53 response. Interestingly, $p21^{WAF1/CIP1}$ was not required for TIGAR function (Figure S5C).

The isolated bisphosphatase domain of PFK-2/FBPase-2 (Boada et al., 2000) functions by decreasing ROS levels and therefore lowers apoptosis induced directly by H_2O_2 treatment of cells. Similarly, protection against apoptosis induced by H_2O_2 was also seen following overexpression of TIGAR in both p53-expressing cells (U2OS) and in a p53 null background (H1299) (Figure 6D). We also found that TIGAR lowered the sensitivity of cells to sodium n-butyrate treatment, a p53-independent apoptotic signal that can induce ROS (Hsiao et al., 2006) (Figure 6E). Furthermore, knockdown of TIGAR enhanced the p53-independent apoptosis induced by adriamycin (Figure S5C), a response that reflects increased ROS levels (Tsang et al., 2003). These results are consistent with an antiapoptotic function of TIGAR in modulating ROS-sensitive apoptosis rather than a direct or specific inhibition of p53 activation or function. Importantly, apoptosis induced by the anti-Fas antibody CH11, which does not require ROS (Huang et al., 2003), was not affected by TIGAR expression either in U2OS cells (Figure 6F) or in HeLa cells, where the anti-Fas antibody directly induces cell death without additional treatment with cycloheximide (data not shown). These results, in conjunction with the effect of TIGAR in FL5.12 cells (Figure 4A), demonstrate that TIGAR selectively inhibits ROS-sensitive apoptosis.

TIGAR Functions through the Pentose Phosphate Pathway to Decrease Intracellular ROS Levels

The suggestion that the antiapoptotic activities of TIGAR are a consequence of the regulation of glycolysis predicts that TIGAR should not be able to function under circumstances where glycolysis has been blocked. We therefore

(C) Intracellular Fru-2,6-P_2 concentration determination in U2OS cells transiently transfected with scrambled siRNA or TIGAR siRNA1.
(D) Glycolytic rate in FL5.12 control cells (clone Cont#6) and cells stably overexpressing Flag-tagged TIGAR (clone TIGAR#7), determined by measuring the conversion of 5-^{3}H-glucose to ^3H-H_2O. FL5.12 cells were grown with (+IL-3) or without (−IL-3) interleukin-3 for 4 hr.
(E) Glycolytic rates in U2OS control cells (clone Cont#3) and cells stably overexpressing Flag-tagged TIGAR (clones TIGAR#5 and TIGAR#10) (left panel) and in U2OS cells transiently transfected with empty vector pcDNA3.1 or expression plasmids for Flag-tagged TIGAR, HA-tagged FBPase-2, or Flag-tagged TIGAR-TM (right panel). Glycolytic rates were determined by measuring the conversion of 5-^{3}H-glucose to ^3H-H_2O.

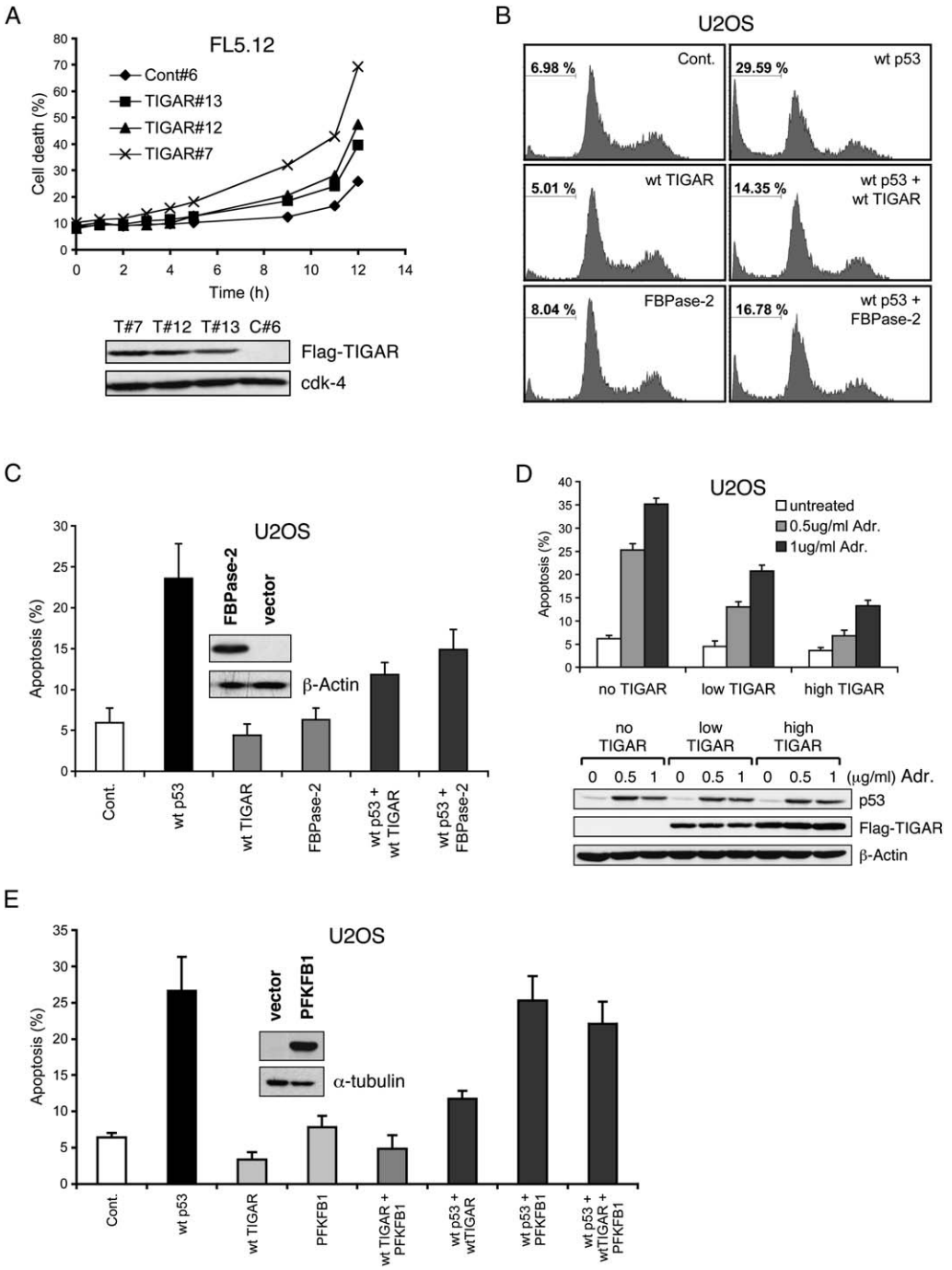

Figure 4. TIGAR Expression Can Modulate Apoptosis in a Cell-Type-Dependent Manner

(A) FL5.12 cells stably expressing Flag-tagged TIGAR (TIGAR#7, #12, and #13) and control cells (Cont#6) were cultured following IL-3 withdrawal for the indicated times, and cell viability was measured using annexin V staining. The percentage of cell death following growth-factor withdrawal over time is shown for one representative experiment. Levels of TIGAR expression in each line were determined by Western blotting with an anti-Flag antibody; levels of cdk-4 expression were determined as a loading control.

(B) Apoptosis in U2OS cells, as measured by cells with a sub-G1 DNA content, 48 hr following transfection of expression plasmids for various combinations of wild-type p53, Flag-tagged TIGAR, and/or HA-tagged FBPase-2 or empty vector pcDNA3.1 as a control.

(C) Apoptosis in U2OS cells 48 hr after transfection of expression plasmids for various combinations of wild-type p53, Flag-tagged TIGAR, and/or HA-tagged FBPase-2 or empty vector pcDNA3.1 as a control. Expression of exogenous HA-tagged FBPase-2 was detected by Western blotting.

(D) Apoptosis in U2OS cells. Twenty-four hours following transfection of expression plasmids for Flag-tagged TIGAR or empty vector pcDNA3.1 as a control, cells were left untreated or treated with 0.5 or 1 μg/ml adriamycin. The apoptotic rate, as measured by cells with a sub-G1 DNA content, was

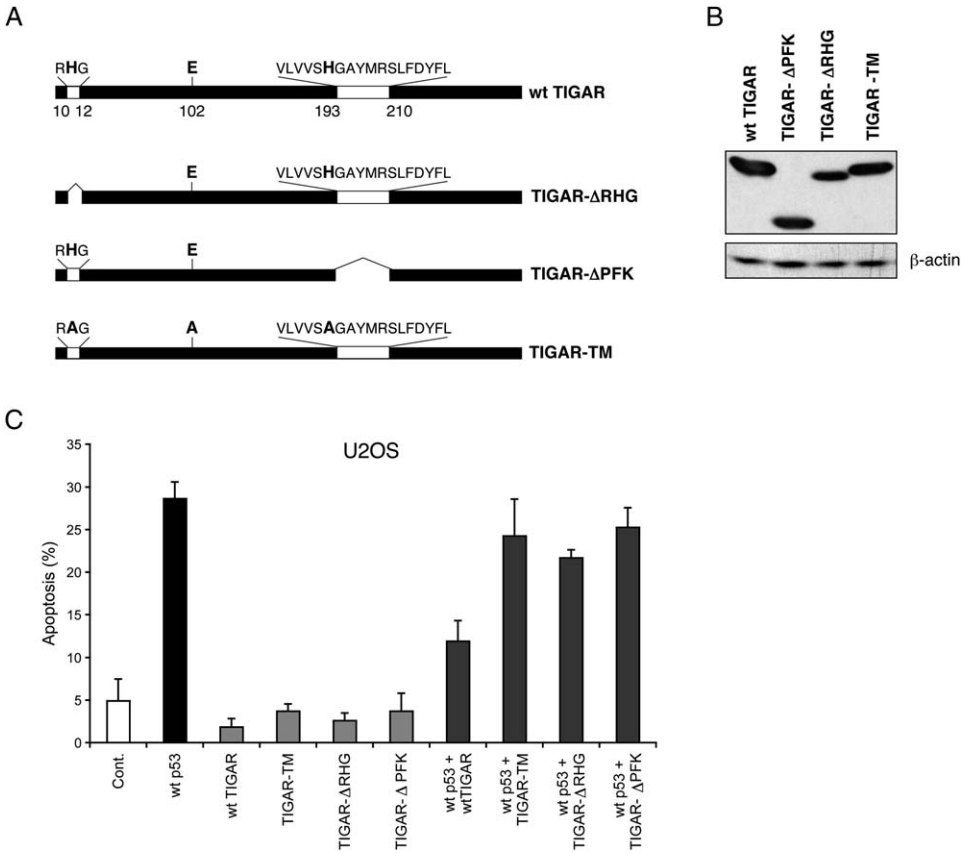

Figure 5. TIGAR Mutants Show an Impaired Antiapoptotic Activity

(A) Schematic representation of the predicted domain structure of the human TIGAR protein, indicating the two regions (amino acids 10–12 and 193-210) and the three amino acids (H11, E102, and H198) essential for activity. TIGAR-ΔRHG, TIGAR-ΔPFK, and TIGAR-TM (H11A/E102A/H198A) mutants are shown.

(B) Expression of Flag-tagged TIGAR, TIGAR-ΔPFK, TIGAR-ΔRHG, and TIGAR-TM following transient transfection of U2OS cells. TIGAR proteins were detected by Western blotting using an anti-Flag antibody. β-actin expression was examined as a loading control.

(C) Apoptosis in U2OS cells, as measured by cells with a sub-G1 DNA content, 48 hr after transfection of expression plasmids for various combinations of wild-type p53, Flag-tagged TIGAR, TIGAR-TM, TIGAR-ΔRHG, and/or TIGAR-ΔPFK or empty vector pcDNA3.1 as a control.

examined the effects of TIGAR expression on p53-induced apoptosis in U2OS cells treated with 2-deoxyglucose (2-DG), a glucose analog that specifically inhibits glycolysis. 2-DG can be phosphorylated to 2-deoxyglucose-6-phosphate (2-DG6P), which cannot be metabolized further through the glycolytic pathway, although it retains some ability to be metabolized into PPP intermediates (Le Goffe et al., 2002; Ozer et al., 2001). As expected, expression of TIGAR under these conditions was unable to reduce p53-induced cell death (Figure 7A).

Our model for TIGAR function also predicts a requirement for nicotinamide adenine dinucleotide (NADPH) produced by the PPP to generate reduced glutathione (GSH) and thereby decrease levels of ROS. GSH, a tripeptide with a free sulfhydryl group, is required to combat oxidative stress and maintain the normal reduced state in the cell (Kletzien et al., 1994). Oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase using NADPH, which is generated by glucose-6-phosphate-dehydrogenase (G6PDH), the rate-limiting enzyme of the PPP, and 6-phosphogluconate dehydrogenase. Glutathione peroxidase reduces H_2O_2 to H_2O by oxidizing GSH. Consequently, the PPP plays an essential role in protection from oxidative-stress-induced apoptosis (Fico et al., 2004; Tian et al., 1999), and an ability of TIGAR to increase the flux through the PPP should lead to the removal of intracellular ROS. To directly test the involvement of this pathway in the function of TIGAR, we examined the effects

measured 48 hr later. For Western blotting, cells were sorted simultaneously into different populations (no, low, or high expression) based on Flag-tagged TIGAR protein expression using the BD FACSVantage SE Flow Cytometer. Endogenous p53 and Flag-tagged TIGAR were detected by Western blotting using an anti-p53 antibody (DO-1) and an anti-Flag monoclonal antibody. β-actin expression was examined as a loading control.

(E) Apoptosis in U2OS cells 48 hr after transfection of expression plasmids for various combinations of wild-type p53, Flag-tagged TIGAR, and/or wild-type PFKFB1 or empty vector pcDNA3.1 as a control. Expression of exogenous PFKFB1 was detected by Western blotting.

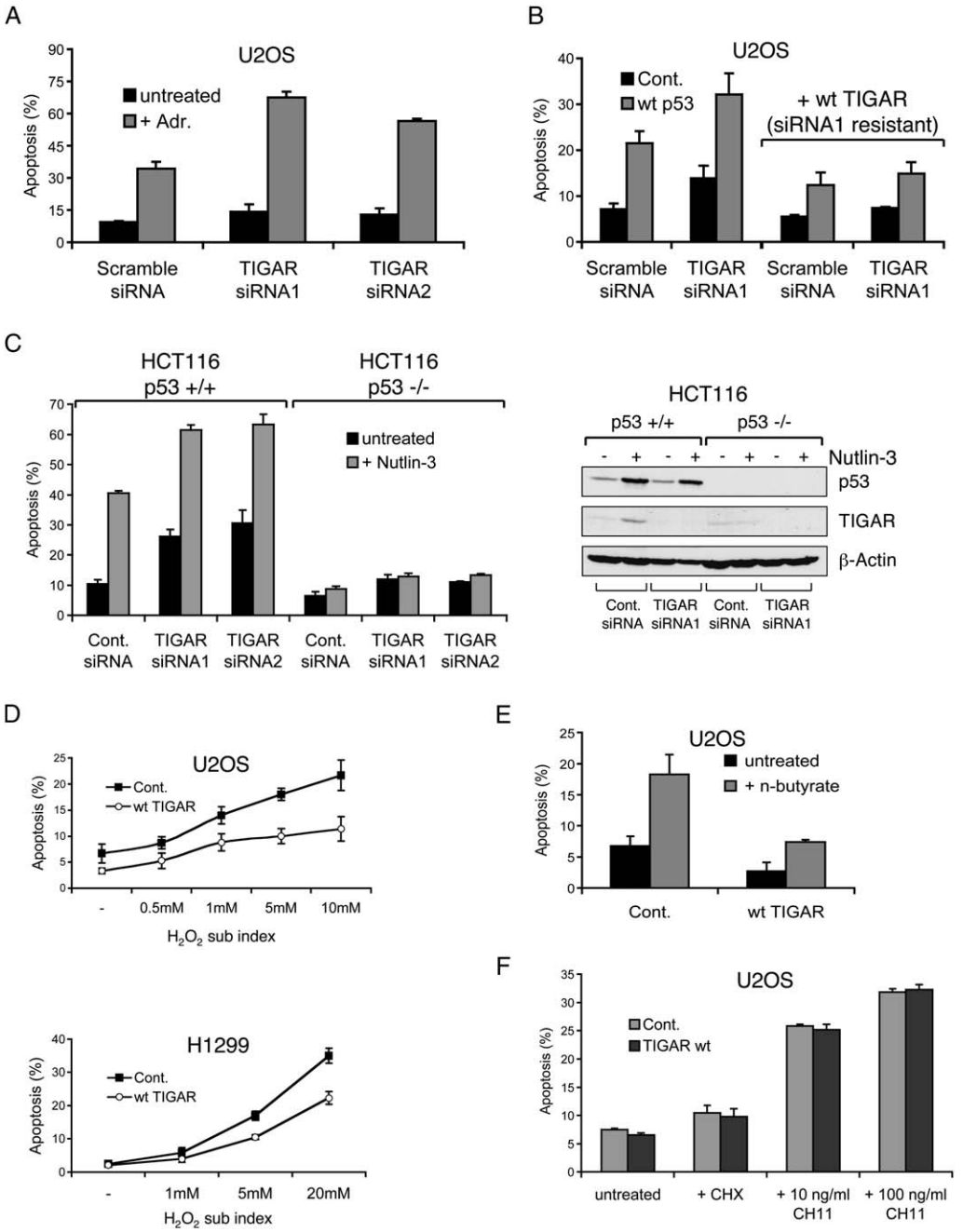

Figure 6. Modulation of TIGAR Expression Influences ROS-Sensitive Apoptosis

(A) Apoptosis in U2OS cells left untreated or treated (+Adr.) with 1 μg/ml adriamycin to induce apoptosis in the presence of either scrambled or TIGAR siRNAs as indicated. The apoptotic rate, as measured by cells with a sub-G1 DNA content, was measured 48 hr later.

(B) Flow cytometric analysis of U2OS cells transfected with scrambled siRNA or TIGAR siRNA1 and then, 24 hr later, transiently transfected with expression plasmids for wild-type p53, Flag-tagged TIGAR (siRNA1 resistant), or empty vector pcDNA3.1 as a control. The apoptotic rate, as measured by cells with a sub-G1 DNA content, was determined after 48 hr.

(C) Apoptosis in HCT116 cells (wild-type and *p53*$^{-/-}$) left untreated or treated (+Nutlin-3) with 10 μM of Nutlin-3 (Cayman Chemicals) to induce p53-dependent apoptosis in the presence of either scrambled or TIGAR siRNAs as indicated. After 48 hr, the apoptotic rate was measured by percentage of cells with a sub-G1 DNA content. p53 and TIGAR were detected by Western blotting. β-actin expression was examined as a loading control.

(D) Apoptosis, as measured by cells with a sub-G1 DNA content, in U2OS (top panel) and H1299 (bottom panel) cells following transient transfection of expression plasmids for Flag-tagged TIGAR or empty vector pcDNA3.1 as a control. After 48 hr, transfected cells were incubated for 10 hr (U2OS) and 20 hr (H1299) in the presence of the indicated concentrations of H_2O_2.

(E) Apoptosis, as measured by cells with a sub-G1 DNA content, in U2OS cells following transfection of expression plasmids for Flag-tagged TIGAR or empty vector pcDNA3.1 as a control. After 48 hr, transfected cells were incubated for 24 hr in the presence of 5 mM sodium n-butyrate.

of TIGAR expression on apoptosis in cells treated with *trans*-androsterone (TA), a specific inhibitor of G6PDH. As predicted, inhibition of the PPP prevented the antiapoptotic activity of TIGAR (Figure 7B).

Since TIGAR inhibits ROS-associated apoptosis and requires the PPP to function, we determined directly the effect of TIGAR expression on the GSH/GSSG ratio. As expected, expression of TIGAR or the isolated FBPase-2 domain increased the GSH/GSSG ratio, while decrease of endogenous TIGAR expression lowered it (Figure 7C). Finally, we measured intracellular ROS in cells overexpressing wild-type TIGAR, the TIGAR-TM mutant, and the isolated FBPase-2 domain. Expression of TIGAR prevented a p53-induced increase in ROS levels in U2OS cells (Figure 7D). TIGAR expression also lowered the increase in ROS levels resulting from treatment of cells with adriamycin to an extent similar to that seen with the FBPase-2 domain (Figure 7E). The mutant TIGAR protein had no effect on ROS levels. Knockdown of endogenous TIGAR expression using siRNA slightly enhanced intracellular ROS levels in U2OS cells, an effect that was more evident following induction of ROS by adriamycin (Figure 7F; Figure S6). Taken together, these results suggest that TIGAR expression can modulate the levels of intracellular ROS.

Our results have demonstrated that TIGAR functions to reduce cellular sensitivity to ROS-associated apoptosis rather than directly affecting p53 function. However, in light of previous studies showing that increased ROS levels can signal to stabilize and activate p53 (Chen et al., 2003; Martindale and Holbrook, 2002), we examined the relationship between TIGAR and p53 more closely. In cotransfection, modulation of TIGAR expression only slightly affected exogenous p53 levels (Figure S7A), an effect that was not detectable when examining the levels of endogenous p53 (Figure 4D; Figures S7A and S7B). Induction of p53 target genes was also only slightly affected by TIGAR expression. These results indicate that, while the regulation of intracellular ROS levels has the potential to feed back to the p53 response under some conditions, TIGAR does not directly modulate p53.

DISCUSSION

In this paper, we report the identification of a new p53-inducible gene named *TIGAR*. Expression of TIGAR reduced ROS levels and protected from ROS-sensitive apoptotic responses, such as those induced by p53. However, ROS-insensitive apoptosis was not decreased by TIGAR, and, as illustrated by the effects of TIGAR expression in FL5.12 cells, modulation of glycolysis can also increase sensitivity to apoptosis under some circumstances. The effects of TIGAR expression on cell survival are therefore likely to be cell and context dependent. Although we have not demonstrated bisphosphatase activity directly, TIGAR shows functional similarities to the bisphosphatase domain of PFK-2/FBPase-2 in modulating glycolysis, ROS levels, and apoptosis. However, the sequence similarity between TIGAR and the FBPase-2 domain is limited, and we have reproducibly noted a more efficient protection from apoptosis following overexpression of TIGAR compared to the FBPase-2 domain. It is possible that TIGAR also has other activities that contribute to the protection from apoptotic stimuli.

While we found TIGAR to be conserved in vertebrates, there is no clear homolog of TIGAR in *Drosophila* or *Caenorhabditis elegans*. There is also some evidence for the expression of an alternative form of TIGAR with an altered, truncated NH_2-terminal region, although this is detected at only very low levels by RT-PCR, with no evidence for protein expression by Western blotting. We have therefore not yet pursued the functional consequences of expression of this putative protein.

Our identification of *TIGAR* as a p53-inducible gene extends the complex balance of ROS-inducing and ROS-decreasing signals propagated by p53. The ability of p53 to protect the genome from oxidative damage by decreasing ROS levels has recently been shown to play a critical role in tumor suppression (Sablina et al., 2005), and it seems most likely that TIGAR will contribute to this activity. Indeed, our data suggest that *TIGAR* falls into the group of genes that are activated by low levels of stress and may play a role in the nonrestrictive tumor-suppressor function of p53. Interestingly, the switch from p53-induced cell-cycle arrest to apoptosis following prolonged stress is associated with a decrease in expression of both TIGAR and $p21^{WAF1/CIP1}$, suggesting that the induction of the apoptotic response may reflect the loss of protection by these p53-inducible survival signals. p53 also plays a role in allowing DNA repair through several mechanisms (Sengupta and Harris, 2005), and the activation of p53-dependent survival signals such as TIGAR may be essential to allow the opportunity for such repair. Indeed, TIGAR may contribute directly to DNA repair, in addition to decreasing ROS levels and allowing cell survival, since the intermediate metabolites of the PPP, including NADPH and ribose-5-phosphate, are important precursors of DNA biosynthesis and repair (Zhang et al., 2003). Our preliminary analysis of tumor cell lines clearly shows that TIGAR expression is not completely p53 dependent, with relatively high levels of TIGAR expressed in some p53 null cells. It will therefore be of interest to identify factors that influence TIGAR expression or function independently of p53. Finally, one of the hallmark of cancer cells is an increased glycolytic rate (Warburg, 1956), which is frequently accompanied by elevated levels of Fru-2,6-P_2 (Chesney et al., 1999; Nissler et al.,

(F) Flow cytometric analysis of U2OS cells transfected with expression plasmids for Flag-tagged TIGAR or empty vector pcDNA3.1 as a control and then, 24 hr later, left untreated or treated with CH11 (anti-Fas antibody, Upstate) in the presence of cycloheximide (1 μg/ml). The apoptotic rate, as measured by cells with a sub-G1 DNA content, was measured 24 hr later.

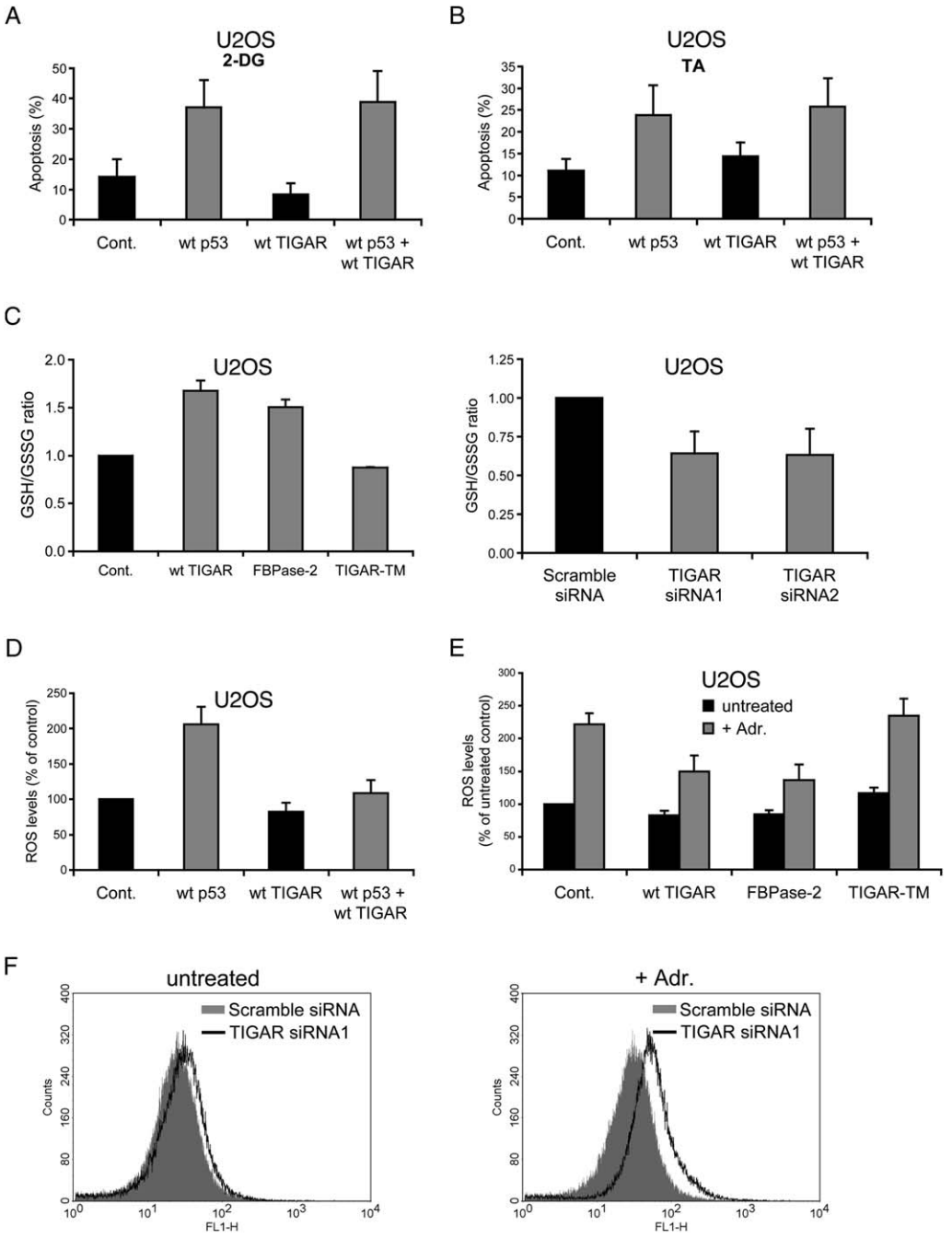

Figure 7. TIGAR Functions through the Pentose Phosphate Pathway to Decrease Intracellular ROS Levels

(A) Effect of inhibition of the glycolytic pathway by 6 mM 2-deoxyglucose (2-DG) on TIGAR antiapoptotic activity in U2OS cells. Cells were analyzed by flow cytometry 48 hr after transfection with expression plasmids for wild-type p53 and/or Flag-tagged TIGAR or empty vector pcDNA3.1 as a control.

(B) Effect of inhibition of the pentose phosphate pathway by 50 μM *trans*-androsterone (TA) on TIGAR antiapoptotic activity in U2OS cells. Cells were analyzed by flow cytometry 48 hr after transfection with expression plasmids for wild-type p53 and/or Flag-tagged TIGAR or empty vector pcDNA3.1 as a control.

(C) GSH/GSSG ratio (normalized to the controls) in U2OS cells transiently transfected with empty vector pcDNA3.1 or expression plasmids for Flag-tagged TIGAR, HA-tagged FBPase-2, or Flag-tagged TIGAR-TM (left panel) or with scrambled or TIGAR siRNAs (right panel).

(D) ROS levels in U2OS cells transiently cotransfected for 12 hr with expression plasmids for GFP and wild-type p53 and/or Flag-tagged TIGAR or empty vector pcDNA3.1. Cells were stained with DHE and measured by counting ROS-positive versus GFP-positive cells using a fluorescence microscope. The results are expressed as the mean and standard deviation of the mean of DHE fluorescence from three experiments.

(E) Basal (untreated) and adriamycin-induced (+Adr., 0.15 μg/ml, 10 hr) ROS levels in U2OS cells transiently cotransfected with expression plasmids

1995). Whether changes in TIGAR expression can contribute or respond to these cancer-specific effects also remains to be determined.

EXPERIMENTAL PROCEDURES

Array Screening and Cloning of *TIGAR*

Total RNA from p53-inducible SAOS-2 cells with or without treatment with doxycycline was screened by Incyte Pharmaceutical, Inc. on the human UniGEM V microarray. One EST tag was identified and used in 5′ RACE (SMART RACE, Clontech) to obtain the *TIGAR* cDNAs.

RNA Analysis

Northern blot analysis was carried out as previously described (Nakano et al., 2000) using probes described in the Supplemental Experimental Procedures.

Chromatin Immunoprecipitation Assay

U2OS cells treated for 24 hr with 0.2 μg/ml adriamycin and p53-inducible SAOS-2 cells treated with 2.0 μg/ml doxycycline were subjected to chromatin immunoprecipitation (ChIP) assay (EZ ChIP kit, Upstate Biotechnology) as detailed in the Supplemental Experimental Procedures.

Electrophoretic Mobility Shift Assay

The binding of p53 to DNA fragments derived from regions upstream of the start codon and in the first intron of *TIGAR* was analyzed by electrophoretic mobility shift assay (EMSA) as described in the Supplemental Experimental Procedures.

Plasmids

Flag-tagged TIGAR and TIGAR mutants were generated by RT-PCR using primers described in the Supplemental Experimental Procedures. The pcDNA3-PFK-2/FBPase-2 plasmid expressing the rat liver PFK-2/FBPase-2 enzyme and the pcDNA3-HA-tagged FBPase-2 plasmid expressing only the bisphosphatase domain have been described previously (Perez et al., 2000).

Cell Lines, Transfections, and Inhibition of TIGAR Expression

Cell lines used in this study have been previously described and are referenced in the Supplemental Experimental Procedures. Transfections were carried out using the Lipofectamine 2000 reagent (Invitrogen), and cells were harvested for flow cytometry (to allow sorting for transfected cells based on CD20 or Flag expression) or protein analysis at the indicated times. The sequences of siRNAs used to knock down TIGAR are described in the Supplemental Experimental Procedures.

Measurement of Fru-2,6-P_2 Levels and Glycolysis

Fru-2,6-P_2 levels were determined by activation of pyrophosphate-dependent PFK-1, and glycolysis was measured by monitoring the conversion of 5-^{3}H-glucose to ^3H_2O, as described previously (Liang et al., 1997) and detailed in the Supplemental Experimental Procedures.

Protein Analysis and Generation of Anti-TIGAR Antibodies

Rabbit polyclonal antibody to TIGAR was raised against two 15 amino acid peptides corresponding to the exon 6-encoded region in the human TIGAR protein (ADQKEQFSQGSPSNC and CMNLQDHLNGLTETR). The second of these peptides was also used to generate a mouse monoclonal antibody to TIGAR. Human p53, p21$^{WAF1/CIP1}$, PIG3, cdk-4, and β-actin proteins were detected using the antibodies DO-1, OP64 (Calbiochem), PC268 (Calbiochem), sc-260 (Santa Cruz Biotechnology), and #4967 (Cell Signaling), respectively. Rat PFKFB1 was detected using E-16 (Santa Cruz). Wild-type and mutant Flag-tagged TIGAR were detected using the anti-Flag M2 antibody (Sigma). HA-tagged FBPase-2 was detected using anti-HA F-7 antibody (Santa Cruz Biotechnology).

Flow Cytometry

To study the effect of overexpression of TIGAR on apoptosis, cells were transfected with plasmids of interest and, in some cases, co-transfected with a plasmid expressing CD20. Cells were harvested at 24, 48, or 72 hr after transfection, stained for CD20 or Flag expression, and analyzed by flow cytometry (FACScan, Becton Dickinson) as previously described (Rowan et al., 1996). Transfection efficiencies were determined by the percentage of cells positive for CD20 or Flag, and, within each cell line, minimal variation between different transfections was observed. Apoptosis was assessed using sub-G1 DNA content, the Annexin-V-FITC Apoptosis Detection Kit (BD Pharmingen), or caspase activity (using a CaspACE FITC-VAD-FMK in situ marker from Promega) according to the manufacturer's instructions.

Redox-State Analysis and ROS Measurement

Reduced and oxidized glutathione levels and ROS levels were measured as detailed in the Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and seven figures and can be found with this article online at http://www.cell.com/cgi/content/full/126/1/107/DC1/.

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for GFP and Flag-tagged TIGAR, HA-tagged FBPase-2, Flag-tagged TIGAR-TM, or empty vector pcDNA3.1, measured by flow cytometry after DCF treatment. GFP fluorescence was used as a marker to gate on transfected cells. The results are expressed as the mean and standard deviation of the mean intensity of cell fluorescence.

(F) Basal (left) and adriamycin-induced (0.15 μg/ml, 10 hr; right) ROS levels in U2OS cells in the presence of either scrambled siRNA or TIGAR siRNA1, measured by flow cytometry after DCF treatment. FL1-H, fluorescence level. One representative experiment is shown.

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