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Modulation of intracellular ROS levels by TIGAR controls autophagy

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The p53-inducible TIGAR protein functions as a fructose-2,6-bisphosphatase, promoting the pentose phosphate pathway and helping to lower intracellular reactive oxygen species (ROS). ROS functions in the regulation of many cellular responses, including autophagy—a response to stress conditions such as nutrient starvation and metabolic stress. In this study, we show that TIGAR can modulate ROS in response to nutrient starvation or metabolic stress, and functions to inhibit autophagy. The ability of TIGAR to limit autophagy correlates strongly with the suppression of ROS, with no clear effects on the mTOR pathway, and is p53 independent. The induction of autophagy in response to loss of TIGAR can function to moderate apoptotic response by restraining ROS levels. These results reveal a complex interplay in the regulation of ROS, autophagy and apoptosis in response to TIGAR expression, and shows that proteins similar to TIGAR that regulate glycolysis can have a profound effect on the autophagic response through ROS regulation.

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Introduction

Autophagy—a mechanism that results in lysosomal degradation of cytoplasmic constituents—is a critical response to metabolic stress (Meijer and Codogno, 2004; Mizushima, 2007). Limited autophagy in response to nutrient starvation has been shown to provide a survival function, and specific removal of damaged mitochondria by autophagy can also help prevent the activation of apoptotic pathways (Zhang *et al*, 2008). However, in some systems, the induction of autophagy has been shown to contribute to, or enhance, the apoptotic response (Crighton *et al*, 2006). The contribution of autophagy to tumour progression is complex, although evidence from animal studies suggests that autophagy can have an important tumour suppressive function (Qu *et al*, 2003; Yue *et al*, 2003; Marino *et al*, 2007).

Although autophagy in response to nutrient deprivation or metabolic stress is mediated through the regulation of the TSC-mTOR pathway (Reiling and Sabatini, 2006), recent studies have also highlighted the important contribution of mitochondrially generated reactive oxygen species (ROS) to this response (Scherz-Shouval *et al*, 2007; Chen and Gibson, 2008; Chen *et al*, 2008). ROS are produced as a normal by-product of cellular metabolism and function as signalling molecules that are involved in numerous pathways regulating cell proliferation, senescence, apoptosis, necrosis and autophagy (Martindale and Holbrook, 2002; Balaban *et al*, 2005). ROS have been shown to induce autophagy through several distinct mechanisms involving the Atg4 family of protein proteases, the mitochondrial electron transport chain and catalase (Yu *et al*, 2006; Chen *et al*, 2007; Scherz-Shouval *et al*, 2007).

p53 is a tumour suppressor protein that has a critical function in inhibiting cancer development, and mutation in the p53 pathway is an extremely common event in most human cancers. p53 induces many responses—including cell-cycle arrest, senescence and apoptotic cell death—each of which may contribute to tumour suppression (Murray-Zmijewski *et al*, 2008). However, in addition to the ability to block cell proliferation, several activities of p53 that contribute to cell survival have also been described. These include functions of p53 as an anti-oxidant (Sablina *et al*, 2005) and in the regulation of metabolism (Matoba *et al*, 2006; Bensaad and Vousden, 2007). Although the induction of survival signals seems to be inconsistent with the well-understood apoptotic function of p53, it has been suggested that this response may contribute to repair and recovery under conditions of mild stress, whereas more severe damage elicits the apoptotic response (Vousden and Lane, 2007). It is not clear how this switch in p53 responses is regulated, but this is likely to be an important factor in determining the success of p53-based therapies (Vousden and Prives, 2009). Intriguingly, the p53 tumour suppressor gene has recently also been shown to function to both induce and inhibit autophagy (Crighton *et al*, 2006; Tasdemir *et al*, 2008), although the contribution of this response to tumour suppression is not fully resolved.

A key mechanism of function of p53 is as a transcription factor, and the DRAM proteins have been identified as important mediators of the induction of autophagy by p53 (Crighton *et al*, 2006). However, other p53-target genes that contribute to the regulation of metabolic pathways and oxidative stress may also have a function in the regulation of autophagy. Several p53-inducible genes encode proteins that can function as anti-oxidants, and the constitutive p53-dependent expression of these anti-oxidant proteins under

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normal growth conditions *in vivo* helps to protect cells from the accumulation of ROS-associated DNA damage (Sablina *et al*, 2005). This can help to prevent the accumulation of mutations that might not only lead to genomic instability and cancer development, but has also been associated with a role for p53 in preventing premature ageing (Matheu *et al*, 2007). One of the p53-target genes that contributes to the regulation of intracellular ROS levels encodes TIGAR (TP53-induced glycolysis and apoptosis regulator), which indirectly affects ROS through the modulation of the glycolytic pathway (Bensaad *et al*, 2006). The TIGAR protein shows similarity to the bisphosphatase domain of PFK-2/FBPase-2 (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase), an enzyme that has an essential function in the regulation of glycolysis. Recently, TIGAR has been shown to function to hydrolyse fructose-2,6-bisphosphate and fructose-1,6-bisphosphate (Li and Jogl, 2009), two activities that lead to the same effects on glycolysis. Expression of TIGAR results in a decreased levels of Fru-2,6-P_2 and a decreased glycolytic rate, which in some cells was shown to be pro-apoptotic. However, dampening of flux through the glycolytic pathway by TIGAR also leads to the redirection of glycolytic metabolic intermediates to the oxidative branch of the pentose phosphate pathway. One consequence of this function of TIGAR is an increased NADPH production, which contributes to the scavenging of ROS by reduced glutathione. Induction of this pathway by TIGAR results in decreased intracellular ROS levels and a lower sensitivity of cells to oxidative stress-associated apoptosis, including that induced by p53 (Bensaad *et al*, 2006). However, ROS levels will also impact autophagy, so we have investigated the effects of TIGAR expression on the autophagic and apoptosis response in non-stressed cells and after conditions of nutrient starvation or metabolic stress.

Results

TIGAR regulates intracellular ROS levels in response to nutrient starvation or metabolic stress

We have shown previously that TIGAR expression can modulate intracellular ROS levels in response to oxidative stress inducing signals such as DNA damage or p53 activation (Bensaad *et al*, 2006). We extended these studies to examine the effect of TIGAR on intracellular ROS levels after nutrient starvation or metabolic stress in U2OS cell lines that constitutively over-expressed ectopic TIGAR, or after siRNA-mediated inhibition of endogenous TIGAR expression (Figure 1). Consistent with our earlier observations, we found that in these tissue culture systems even background levels of ROS were lower in cells constitutively expressing TIGAR compared with control cells (Figure 1A). Interestingly, nutrient starvation or metabolic stress strongly elevated ROS levels and over-expression of TIGAR effectively inhibited this enhancement of ROS (Figure 1A). Conversely, knockdown of TIGAR expression resulted in an increase in ROS levels, and the increase in ROS induced by nutrient starvation and metabolic stress was further elevated after inhibition of the endogenous TIGAR protein by siRNA knockdown (Figure 1B).

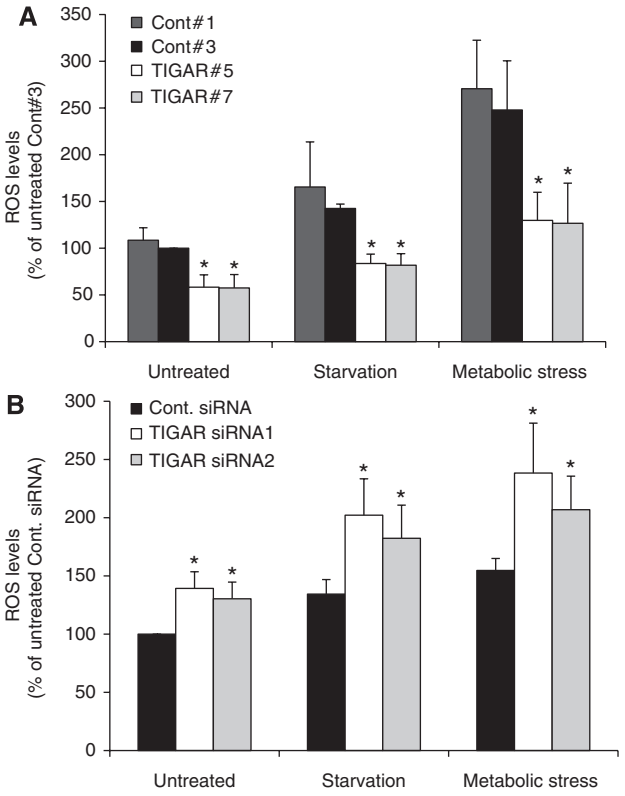
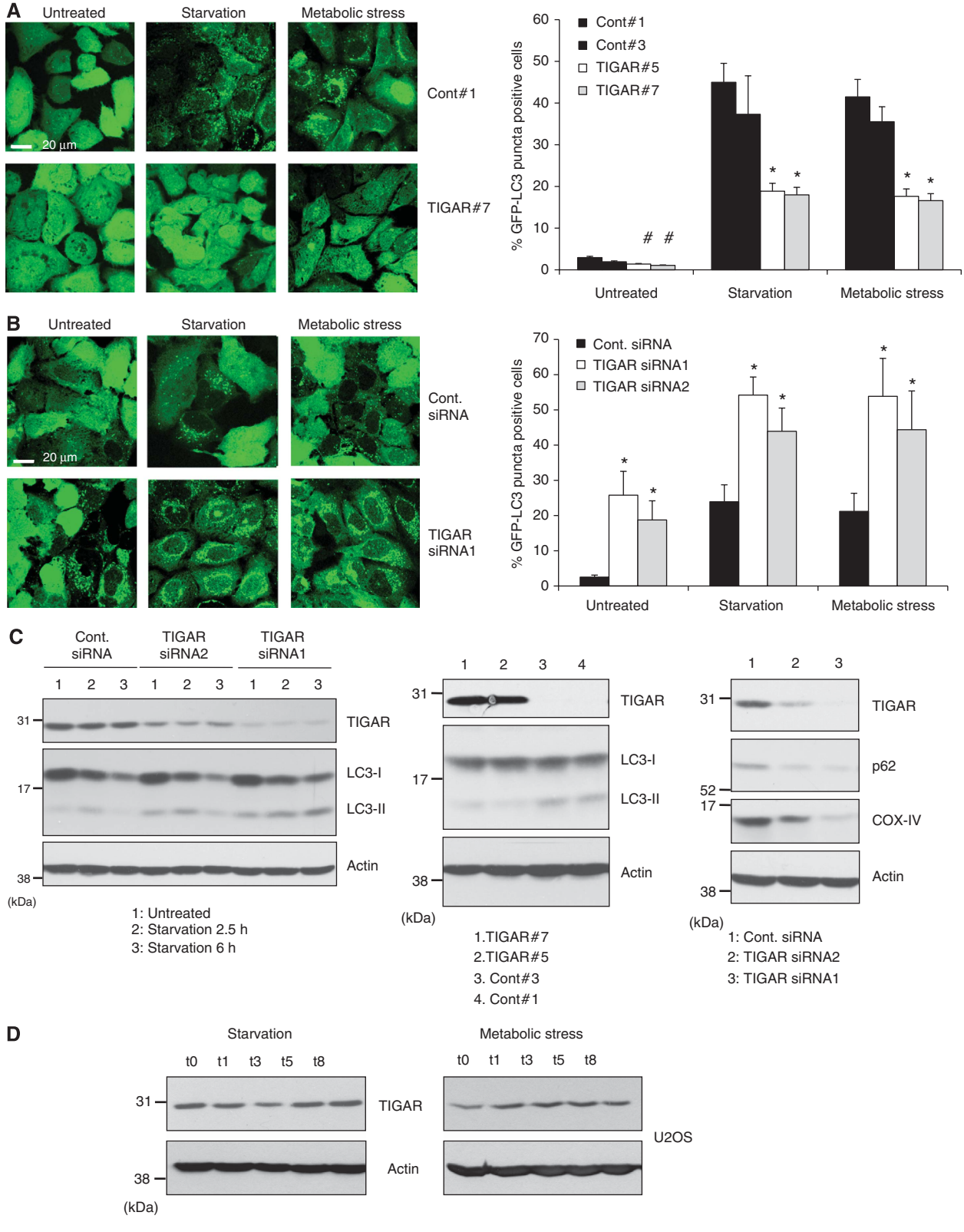


Figure 1 TIGAR regulates intracellular ROS levels in response to nutrient starvation or metabolic stress. (**A**) ROS levels in U2OS cells stably over-expressing Flag-tagged-TIGAR (clones TIGAR#5 and TIGAR#7) or control cells (clones Cont#1 and Cont#3) left untreated, after 6 h of nutrient starvation or 24 h of metabolic stress. ROS levels were measured by flow cytometry after DCF treatment. The results are expressed as the mean DCF fluorescence (and standard deviation), from three independent experiments. (**B**) Basal, nutrient starvation-induced (5 h) or metabolic stress-induced (18 h) ROS levels in U2OS cells in the presence of either scrambled, TIGAR siRNA1 or TIGAR siRNA2, measured by flow cytometry after DCF treatment. The results are expressed as the mean intensity of cell fluorescence (and standard deviation). * represents significant difference from control conditions ($P<0.05$).

Figure 2 TIGAR expression modulates autophagy in response to nutrient starvation or metabolic stress. (**A**) (Left panel) Confocal microscopic images of the fluorescence in U2OS cells stably over-expressing Flag-tagged-TIGAR (clone TIGAR#7) or control cells (clone Cont#1) and infected with an adenovirus expressing GFP-LC3 for 16 h. Cells were then left untreated, exposed to nutrient starvation for 6 h or to metabolic stress for 24 h. (Right panel) Quantitation of the percentage of GFP-LC3–positive cells displaying GFP puncta from three independent experiments. The mean values with standard deviation are presented. (**B**) (Left panel) Confocal microscopic images of the fluorescence in U2OS cells stably expressing GFP-LC3 and transfected with scrambled or TIGAR siRNAs. After 48 h transfection, cells were then left untreated, exposed to nutrient starvation for 5 h or to metabolic stress for 18 h. (Right panel) Quantitation of the percentage of GFP-LC3–positive cells displaying GFP puncta from three independent experiments. The mean values with standard deviation are presented. (**C**) (Left panel) Western blot showing the expression levels of endogenous LC3-I, LC3-II and TIGAR in U2OS cells transfected with scrambled or TIGAR siRNAs, and 48 h later exposed to nutrient starvation for 0, 2.5 and 6 h. (Middle panel) Western blot showing the expression levels of endogenous LC3-I, LC3-II and TIGAR in U2OS stably over-expressing Flag-tagged-TIGAR (clones TIGAR#5 and TIGAR#7) or control cells (clones Cont#1 and Cont#3) and left untreated. (Right panel) Western blot showing the expression levels of p62, COX-IV and TIGAR in U2OS cells transfected with scrambled or TIGAR siRNAs and left untreated. Actin expression was examined as a loading control. (**D**) Western blot showing the expression levels of endogenous TIGAR in U2OS cells after exposure to nutrient starvation or metabolic stress for 0, 1, 3, 5 and 8 h; * represents significant difference from control conditions ($P<0.05$); # represents a lack of significant difference from control conditions ($P>0.05$).

TIGAR expression modulates autophagy

Recent results have shown that the autophagic response to nutrient starvation or metabolic stress involves ROS (Scherz-Shouval *et al*, 2007). To determine whether changes in TIGAR expression and the consequent modulation of ROS levels can affect autophagy, we examined the response of cells to nutrient starvation or metabolic stress, two signals that have been shown to effectively induce an autophagic response (Munafo and Colombo, 2001; Jin and White, 2007). Autophagy was monitored by measuring the formation of autophagosomes, as measured in cells by the accumulation of GFP-tagged LC3 puncta by fluorescence microscopy (Klionsky *et al*, 2008) (Figure 2). As expected, either nutrient starvation or metabolic stress resulted in a strong activation



of autophagy, which was significantly reduced in the TIGAR over-expressing cells (Figure 2A). We have found previously that siRNA depletion of TIGAR expression in U2OS cells sensitized them to ROS-dependent apoptotic signals, and therefore we investigated the effect of TIGAR knockdown on the induction of autophagy. Interestingly, removal of TIGAR enhanced autophagy in unstressed cells, as well as in response to nutrient starvation or metabolic stress (Figure 2B). There was a very close correlation between the activation of autophagy and the elevation of ROS levels after knockdown of TIGAR (Figures 1B and 2B). The increase in autophagy after siRNA-mediated inhibition of TIGAR expression was seen in various cell lines, including other transformed cells and untransformed primary epithelial cells (Supplementary Figure 1).

To further validate the effects of TIGAR expression on autophagy, we analysed several other parameters of this process (Klionsky *et al*, 2008). The lipidation of the ubiquitin-like protein LC3 during the process of autophagy can also be used as a marker. The modification of LC3-I to form LC3-II during autophagy was measured by western blot, in which the increase in modification and conversion to LC3-II (indicative of autophagy) correlated with levels of TIGAR expression after treatment with different siRNAs (Figure 2C). Conversely, less LC3-II was formed in cells over-expressing TIGAR (Figure 2C). The degradation of p62, which serves as a link between LC3 and ubiquitinated substrates, and the mitochondrial protein COX-IV also serve as markers of autophagy (Klionsky *et al*, 2008). A decrease in the levels of both of these proteins was observed after inhibition of endogenous TIGAR (Figure 2C).

To determine whether TIGAR expression is regulated after starvation or metabolic stress, we examined protein levels in various cell lines over a time course of treatment (Figure 2D; Supplementary Figure 2). These results did not show a clear difference in overall levels of TIGAR expression, consistent with our observation that TIGAR depletion enhances autophagy even under control conditions (Figure 2B). Glucose starvation has been shown to induce a p53-dependent cell-cycle arrest (Jones *et al*, 2005), which might be expected to induce TIGAR expression. However, under the short-time course examined here, in which a clear autophagic response was seen, we did not note strong activation of p53 as measured by increase in p53 levels (Figure 3C), or enhanced expression of another p53-target gene, p21 (data not shown). However, the expression of TIGAR in these cells is, to some extent, dependent on basal levels of p53, as siRNA-mediated reduction of p53 leads to a drop in TIGAR levels (Figure 3C).

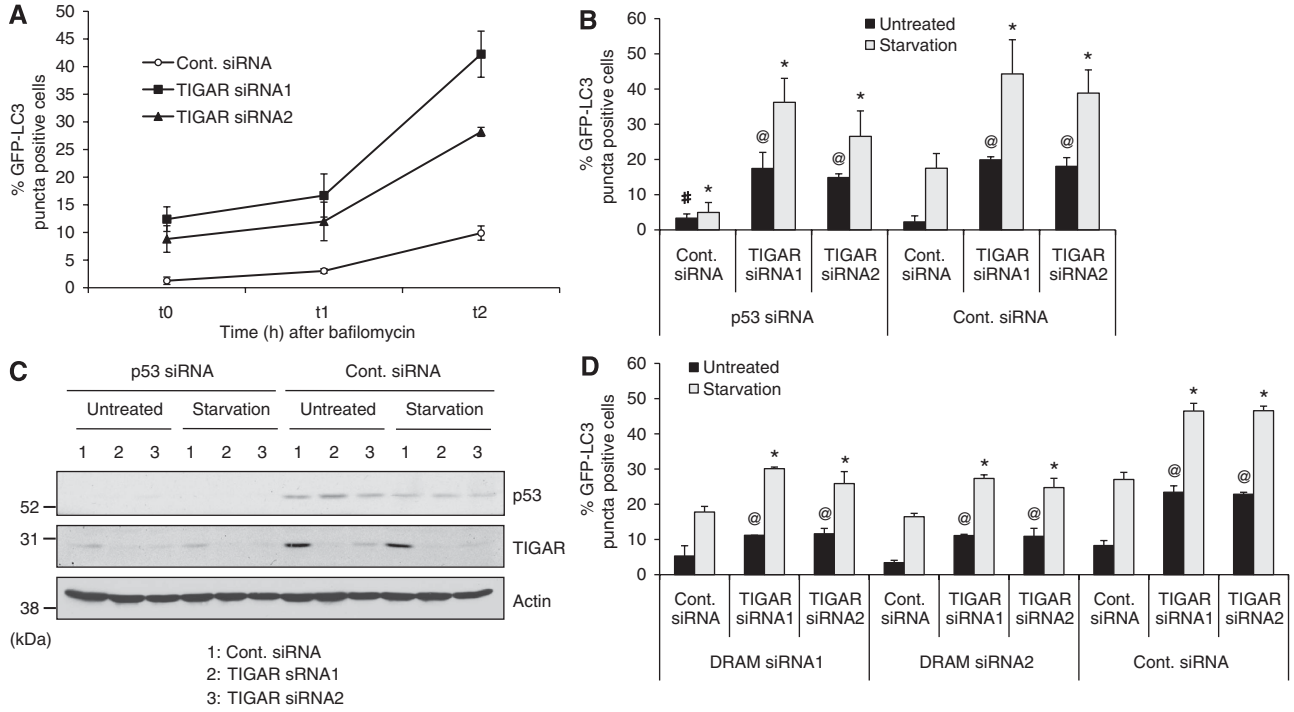


Figure 3 TIGAR expression modulates autophagy independently of p53. (**A**) Quantitation of the percentage of GFP-LC3–positive cells displaying GFP puncta. U2OS cells stably expressing GFP-LC3 were transfected with scrambled or TIGAR siRNAs, and 48 h after transfection, cells were left untreated (t0) or treated with Bafilomycin A1 (100 nM) for 1 or 2 h (t1 and t2). The percentage of cells with GFP-LC3 puncta was calculated at the indicated time points. Data are shown as the mean and standard deviation from three independent experiments. (**B**) Quantitation of the percentage of GFP-LC3–positive cells displaying GFP puncta. U2OS cells stably expressing GFP-LC3 were cotransfected with scrambled or TIGAR siRNAs, and scrambled or p53 siRNA. After 48 h transfection, cells were left untreated or exposed for 5 h to nutrient starvation. The percentage of cells with GFP-LC3 puncta was calculated, and data are shown as the mean and standard deviation from three independent experiments. (**C**) Western blot showing the expression levels of endogenous p53 and TIGAR in U2OS cells cotransfected with scrambled or TIGAR siRNAs, and scrambled or p53 siRNA, and 48 h later exposed to nutrient starvation for 5 h. Actin expression was examined as a loading control. (**D**) Quantitation of the percentage of GFP-LC3–positive cells displaying GFP puncta. U2OS cells stably expressing GFP-LC3 were cotransfected with scrambled or TIGAR siRNAs, and scrambled or DRAM siRNA1/2. After 48 h transfection, cells were left untreated or exposed for 5 h to nutrient starvation. The percentage of cells with GFP-LC3 puncta was calculated, and data are shown as the mean and standard deviation from three independent experiments; * represents significant difference from starved control conditions ($P<0.05$); @ represents significant difference from untreated control conditions ($P<0.05$); # represents a lack of significant difference from control conditions ($P>0.05$).

TIGAR expression modulates autophagy independently of p53

The formation of GFP-LC3 vesicles is a convenient way to measure autophagy, but could result from either the increased rate of autophagosome formation or an inhibition in their turnover (Klionsky *et al*, 2008). To determine whether TIGAR expression is promoting the degradation or inhibiting the formation of autophagosomes, we examined the accumulation of LC3 vesicles in cells treated with bafilomycin A1, which prevents degradation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes (Yamamoto *et al*, 1998). If the effect of TIGAR knockdown is to drive accumulation of vesicles through inhibiting their maturation, we would expect bafilomycin A1 treatment to neutralize the effect of TIGAR. However, even in the presence of bafilomycin A1, there was still a clear increase in autophagosome formation after inhibition of TIGAR expression (Figure 3A). Taken together, these results suggest that TIGAR can have a function in inhibiting the formation of autophagosomes rather than activating their degradation, and that removal of TIGAR promotes the autophagic response.

Earlier studies have shown that p53 can contribute to both the induction and inhibition of autophagy, and that nutrient starvation/metabolic stress can induce p53. The ability of p53 to regulate autophagy has been shown to be dependent both on direct cytoplasmic activities of p53, as well as on the activity of p53-inducible genes such as DRAM, which promotes autophagy (Crighton *et al*, 2006; Tasdemir *et al*, 2008). As TIGAR is a p53-target gene that seems to have a function in limiting autophagy, we were interested to determine the interplay between p53 and TIGAR, or DRAM and TIGAR, in the regulation of this process. The enhanced autophagic response to TIGAR inhibition was clearly retained in cells depleted of p53, indicating that p53 is not required for TIGAR-dependent modulation of autophagy (Figure 3B). However, p53 was clearly required for the autophagic response to nutrient starvation in cells that retain TIGAR expression (Figure 3B). These results suggest a balance in which p53 has a function in enhancing autophagy in these cells (possibly through regulation of DRAM expression), with TIGAR serving to dampen this response by decreasing ROS levels. As p53 regulates TIGAR expression, TIGAR levels were significantly lower after siRNA-mediated depletion of p53 (Figure 3C). However, even these reduced TIGAR levels were sufficient to limit autophagy in both untreated cells or in response to starvation, as shown by the enhanced autophagy after inhibition of TIGAR expression in p53 siRNA-treated cells (Figure 3B). To more directly assess the function of DRAM in the regulation of autophagy, we used siRNA to deplete cells of DRAM expression (Crighton *et al*, 2006) (Figure 3D). As expected, inhibition of DRAM expression reduced the autophagic response under all conditions, although starvation still enhanced autophagy in the absence of DRAM, suggesting that other p53-dependent genes may have a function in promoting autophagy under these conditions. Knockdown of TIGAR enhanced autophagy regardless of the presence or absence of DRAM, showing that these two proteins function independently to promote and inhibit autophagy, respectively.

Modulation of ROS by TIGAR correlates with modulation of autophagy

To determine whether the autophagy induced in our cell systems was dependent on increased ROS, we modulated ROS levels directly by treatment with *N*-acetyl cystein (NAC) and L-ascorbic acid, direct scavengers of ROS (Figure 4A) or hydrogen peroxide (H_2O_2) to enhance intracellular ROS levels (Figure 4B). Both nutrient starvation and metabolic stress-induced autophagy were lowered by the anti-oxidant treatment (Figure 4A). We were, however, unable to completely prevent the autophagic response by NAC and ascorbate treatment, suggesting that some ROS-independent autophagy was also being induced in these cells after these treatments. Treatment of cells with increasing concentrations of H_2O_2 enhanced intracellular ROS (Figure 4B) to levels comparable with those seen after knockdown of TIGAR (Figure 1B). Interestingly, enhanced ROS in response to H_2O_2 also promoted autophagy, even in the absence of further stresses, to levels very similar to those seen after TIGAR depletion (Figure 2B).

These results suggest that the changes in ROS levels seen after alterations in TIGAR expression may be responsible for the effects on autophagy. Further support for this model was provided by the observation that although anti-oxidant treatment with NAC and ascorbate effectively lowered autophagy in response to nutrient starvation or metabolic stress, this treatment had little further effect on decreasing autophagy in TIGAR over-expressing cells, in which autophagy in response to either nutrient starvation or metabolic stress was already lower compared with control (Figure 4C). The earlier described effects of TIGAR in lowering intracellular Fru-2,6-P_2 levels, promoting the pentose phosphate pathway and decreasing intracellular ROS levels, are consistent with the ability of TIGAR to carry out the bisphosphatase function of the bifunctional enzyme PFK-2/FBPase-2 (Bensaad *et al*, 2006; Li and Jogl, 2009). We have shown previously that the activities of TIGAR can be mimicked by the expression of the isolated bisphosphatase domain (FBPase-2) from PFK-2/FBPase-2. Accordingly, expression of the isolated bisphosphatase domain also inhibited starvation or metabolic stress-induced autophagy to levels comparable to that seen after over-expression of TIGAR (Figure 4C). Furthermore, the effect of expression of the isolated FBPase-2 domain was also lost after anti-oxidant treatment, as seen for TIGAR (Figure 4C). Similar results were obtained in cells stably over-expressing TIGAR (data not shown) and in cells treated with a number of other anti-oxidants (Figure 4D). These results are, therefore, consistent with a function for TIGAR in preventing autophagy by lowering ROS levels, as the effect of TIGAR is greatly diminished when ROS are removed by another mechanism (NAC and ascorbate). Conversely, the autophagic response induced after inhibition of endogenous TIGAR expression by treatment with siRNA was reduced after anti-oxidant treatment (Figure 4E), which correlated with a decrease in ROS levels after anti-oxidant treatment.

TIGAR does not clearly affect the mTOR signalling pathway

Although we have concentrated on a function for TIGAR in regulating autophagy through the control of ROS, it is possible that other functions of TIGAR might control other pathways important in the regulation of autophagy. The most

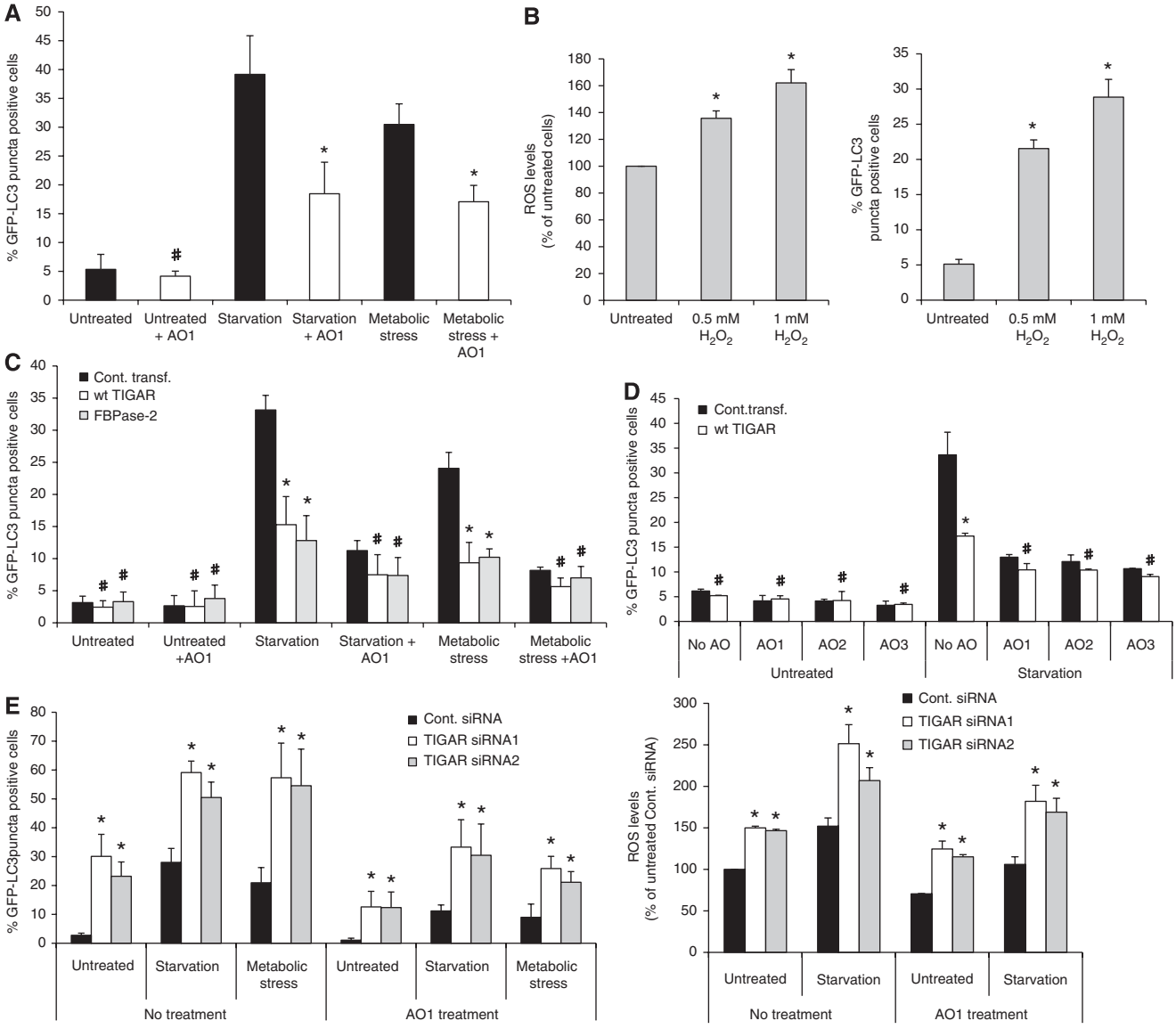


Figure 4 Modulation of ROS by TIGAR correlates with the modulation of autophagy. (**A**) U2OS cells were left untreated, exposed to nutrient starvation for 6 h or to metabolic stress for 24 h, with or without treatment with AO1 (NAC (2 mM) and L-ascorbic acid (2 mM)) for 24 h. The percentage of cells with GFP-LC3 puncta was calculated, and data are shown as the mean and standard deviation from three independent experiments. (**B**) (Left panel) ROS levels in U2OS cells left untreated or treated with 0.5 or 1 mM of H_2O_2 for 24 h. ROS levels were measured by flow cytometry after DCF treatment. The results are expressed as the mean DCF fluorescence (and standard deviation) from three independent experiments. (Right panel) Quantitation of the percentage of GFP-LC3 puncta positive cells for cells treated as described above. Data are shown as the mean and standard deviation from three independent experiments. (**C**) Quantitation of the percentage of GFP-LC3 puncta positive cells. U2OS cells stably expressing GFP-LC3 were transfected with vector pCHER1A expressing the mCherry gene as control, or expression plasmids for Flag-tagged-TIGAR or HA-tagged-FBPase-2. After 48 h transfection, cells were left untreated, exposed to nutrient starvation for 6 h or to metabolic stress for 24 h, with or without treatment with AO1 (NAC (2 mM) and L-ascorbic acid (2 mM)) for 24 h. The percentage of cells with GFP-LC3 puncta was calculated, and data are shown as the mean and standard deviation from three independent experiments. (**D**) Quantitation of the percentage of GFP-LC3 puncta positive cells. Cells were left untreated, exposed to nutrient starvation for 6 h with or without treatment with AO1 (NAC (2 mM) and L-ascorbic acid (2 mM)), AO2 (glutathione ethyl ester (4 mM)) or AO3 (ethyl pyruvate (4 mM)) for 24 h. U2OS cells stably expressing GFP-LC3 were transfected with vector pCHER1A expressing the mCherry gene as control, or expression plasmid for Flag-tagged-TIGAR. After 48 h transfection, cells were treated. The percentage of cells with GFP-LC3 puncta was calculated, and data are shown as the mean and standard deviation from three independent experiments. (**E**) (Left panel) Quantitation of the percentage of GFP-LC3 puncta positive cells. Cells were left untreated, exposed to nutrient starvation for 5 h or to metabolic stress for 18 h, with or without treatment with AO1 (NAC (2 mM) and L-ascorbic acid (2 mM)) for 24 h. U2OS cells stably over-expressing GFP-LC3 in the presence of either scrambled, TIGAR siRNA1 or TIGAR siRNA2. (Right panel) Basal or nutrient starvation-induced (5 h) ROS levels in U2OS cells in the presence of either scrambled, TIGAR siRNA1 or TIGAR siRNA2 with or without treatment with AO1 (NAC (2 mM) and L-ascorbic acid (2 mM)) for 24 h, measured by flow cytometry after DCF treatment. The results are expressed as the mean intensity of cell fluorescence (and standard deviation). * represents significant difference from control conditions ($P<0.05$); # represents a lack of significant difference from control conditions ($P>0.05$).

obvious of these is signalling through mTOR, the inhibition of which has been shown to be a critical component driving the activation of autophagy in response to nutrient starvation (Meijer and Codogno, 2004). A decrease in mTOR signalling in response to nutrient deprivation can be assessed by a reduction in phosphorylation of the downstream target

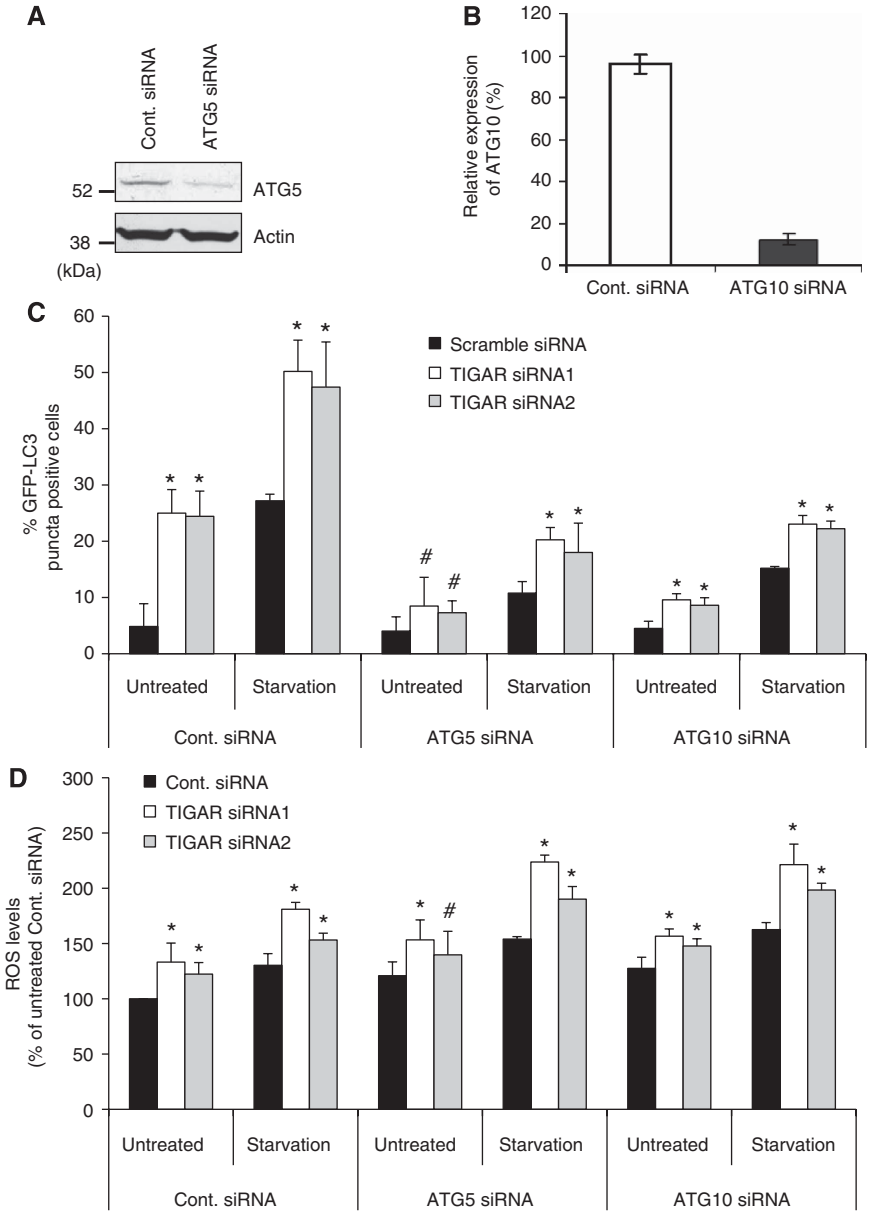


Figure 5 TIGAR modulates ROS levels upstream of the autophagic response. (**A**) Western blot showing knockdown of ATG5 protein expression by ATG5 siRNA. (**B**) RT–PCR showing knockdown of ATG10 mRNA expression by ATG10 siRNA. (**C**) Quantitation of the percentage of GFP-LC3 puncta positive cells. U2OS cells stably over-expressing GFP-LC3 in the presence of either scrambled, TIGAR siRNA1 or TIGAR siRNA2, and either scrambled, ATG5 siRNA or ATG10 siRNA. After 48 h, cells were left untreated or exposed to nutrient starvation for 5 h. (**D**) ROS levels in U2OS cells in the presence of either scrambled, TIGAR siRNA1 or TIGAR siRNA2, and scrambled, ATG5 siRNA or ATG10 siRNA, measured by flow cytometry after DCF treatment. After 48 h, cells were left untreated or exposed to nutrient starvation for 5 h. The results are expressed as the mean intensity of cell fluorescence (and standard deviation). * represents significant difference from control conditions ($P<0.05$); # represents a lack of significant difference from control conditions ($P>0.05$).

proteins p70 S6 kinase and S6 ribosomal protein (Averous and Proud, 2006) (Supplementary Figure 3A). Depletion of TIGAR expression, which results in enhanced autophagy, had no effect on the overall levels of p70 S6 kinase or S6 ribosomal protein. Furthermore, knockdown of TIGAR expression did not result in any clear reduction in the basal phosphorylation levels of either S6 kinase or S6 protein (Supplementary Figure 3A), and did not change the kinetics of the disappearance of the phosphorylated forms. Similarly, inhibition of TIGAR expression did not prevent the reappearance of phosphorylated S6 kinase and S6 after nutrient restimulation (Supplementary Figure 3A). The lack of effect of TIGAR knockdown on mTOR signalling was also noted in H1299 and HeLa cells (Supplementary Figure 3B and C), as well as RKO and RPE cells (data not shown). Finally, over-expression of TIGAR did not clearly delay the loss of S6 kinase or S6 phosphorylations (data not shown). These results suggest that there is no clear effect of TIGAR on mTOR signalling and indicate that this is not the principal mechanism by which TIGAR prevents autophagy.

TIGAR modulates ROS levels upstream of the autophagic response

Our model suggests that TIGAR lowers ROS levels and as a consequence decreases the levels of ROS-dependent autophagy. However, it is clear that autophagy itself can lower

intracellular ROS levels (mainly by eliminating dysfunctional mitochondria (Zhang *et al*, 2008)), creating a loop in which autophagy and ROS can modulate each other (Azad *et al*, 2009). We therefore wished to investigate the effect of inhibition of autophagy on the ability of TIGAR to regulate ROS. The autophagic process can be blocked by removal of the ATG5 or ATG10 proteins, whose expression is essential for autophagosome formation (Suzuki *et al*, 2001; Xie and Klionsky, 2007). ATG5 and ATG10 expression was reduced by using previously described siRNAs (Boya *et al*, 2005; Crighton *et al*, 2006) and validated in our cells (Figure 5A and B). Knockdown of ATG5 or ATG10 strongly decreased the formation of GFP-LC3 vesicles in response to starvation and/or TIGAR depletion (Figure 5C). As seen earlier, ROS levels were increased in starved cells, and this effect was further enhanced by loss of TIGAR (Figure 5D). Interestingly, the inhibition of autophagy by ATG5 or ATG10 knockdown further enhanced ROS levels under the same conditions, consistent with a function for autophagy in the removal of mitochondria (the main source of ROS generated during starvation) and the protection of cells from increased ROS levels (Figure 5D). These results show that the increased ROS levels seen after TIGAR depletion are not dependent on the ensuing autophagy—rather that the induction of autophagy limits, to some extent, this accumulation of ROS in the cells.

TIGAR modulation of autophagy influences apoptosis

A complex interplay between apoptosis and autophagy has been described (Suzuki *et al*, 2001; Maiuri *et al*, 2007). In some systems, the induction of autophagy can enhance apoptotic cell death, although the mechanisms underlying this cooperation are not well understood (Crighton *et al*, 2007). More straightforward is the ability of autophagy to decrease apoptosis, an effect that seems to be a reflection of the ability of autophagy to modulate ROS levels—as shown above—and remove damaged mitochondria. This prevents the release of apoptogenic factors such as cytochrome c from the mitochondria and the activation of the apoptotic cascade (Colell *et al*, 2007; Zhang *et al*, 2008). Our earlier studies have shown that TIGAR can function to inhibit apoptosis by limiting ROS levels in response to p53 activation or genotoxic stress, although TIGAR was not effective in modulating apoptosis that was not dependent on ROS (Bensaad *et al*, 2006). Treatment of cells with a caspase inhibitor to block apoptosis had no effect on the formation of autophagosomes after nutrient starvation, and TIGAR was equally efficient in inhibiting autophagy in these cells in the presence or absence of caspase inhibitor (Figure 6A). The induction and modulation of autophagy was, therefore, not dependent on apoptosis. On the other hand, however, we found that the apoptotic response was affected by the modulation of autophagy. Consistent with a model in which autophagy can limit ROS levels by preventing the generation of ROS by mitochondria, and in agreement with the results shown in Figure 5, inhibition of autophagy by depletion of ATG5 or ATG10 resulted in significantly enhanced apoptosis in response to either nutrient starvation or metabolic stress (Figure 6B and C), correlating with the observed elevation of ROS levels (Figure 5B). In keeping with an anti-oxidant function for TIGAR, ectopic expression of TIGAR inhibited apoptosis, and this anti-apoptotic effect of TIGAR was even more profound after inhibition of autophagy (Figure 6B). The reduction in autophagy after TIGAR expression was not compensated by an increase in other forms of cell death, as overall cell survival was also enhanced by TIGAR expression in both the absence and presence of a caspase inhibitor (Supplementary Figure 4), which reduced the apoptotic rate to background levels in these cells (data not shown). These results suggest that TIGAR, through lowering ROS, reduces both apoptotic and necrotic cell death. Conversely, knockdown of TIGAR expression enhanced apoptosis under all conditions (Figure 6C). Taken together, our results suggest that the ability of TIGAR to down-regulate ROS levels can limit both apoptosis and autophagy, although autophagy itself can also function to decrease ROS levels and so lower apoptosis.

Discussion

Our earlier work described an ability of TIGAR to decrease ROS levels through modulation of the glycolytic pathway, and an anti-apoptotic effect of TIGAR expression through this mechanism (Bensaad *et al*, 2006). We have now found that the ability of TIGAR to modulate ROS levels also has a profound effect on autophagy, with loss of TIGAR dramatically increasing autophagy, even in otherwise unstressed cells. This regulation of autophagy by TIGAR seems to be a consequence, rather than a cause, of the control of ROS by TIGAR, an effect similar to that seen in response to the modulation of the levels of catalase, a major ROS scavenger (Yu *et al*, 2006). Changes in TIGAR expression do not clearly alter the levels of p70 S6 kinase or the ribosomal S6 protein phosphorylation in the short term, suggesting that the control of autophagy by TIGAR through ROS regulation is not directly mediated by the mTOR signalling pathway. Interestingly, in this system autophagy can limit apoptosis, and so the two activities of TIGAR in reducing both autophagy and apoptosis would seem to be contradictory. This is apparent from the observation that direct inhibition of autophagy by knockdown of ATG5 or ATG10 further enhanced the increase in apoptosis seen in response to inhibition of TIGAR expression. These results further highlight the complex interplay of responses to modulation of ROS levels in determining the outcome of cell death or survival in response to stress.

The identification of an autophagy-regulating function for TIGAR further increases the intricacy of possible responses to p53 activation. Both TIGAR and DRAM can be transcriptionally activated by p53, and we show here that the autophagy inhibition function of TIGAR and the autophagy promoting activity of DRAM are independent, with both influencing the ultimate outcome. The ability of p53 to function in the cytoplasm to limit autophagy adds more complexity to the response. Furthermore, a number of other p53-regulated genes, in addition to TIGAR, are involved in the control of ROS. This anti-oxidant activity of p53 is important even in the absence of acute stress and functions to control tumour progression by preventing DNA damage and genetic instability. The Sestrin proteins have also been identified as an important component of the regulation of ROS by p53 (Budanov *et al*, 2004). This family of proteins was initially shown to function through the reduction, and thereby regeneration, of sulphinylated peroxiredoxins—which can catalyse the reduction of hyperperoxides. The expression of Sestrins in response to p53 was shown to be important to lower ROS levels in cells with activated Ras and so control genetic

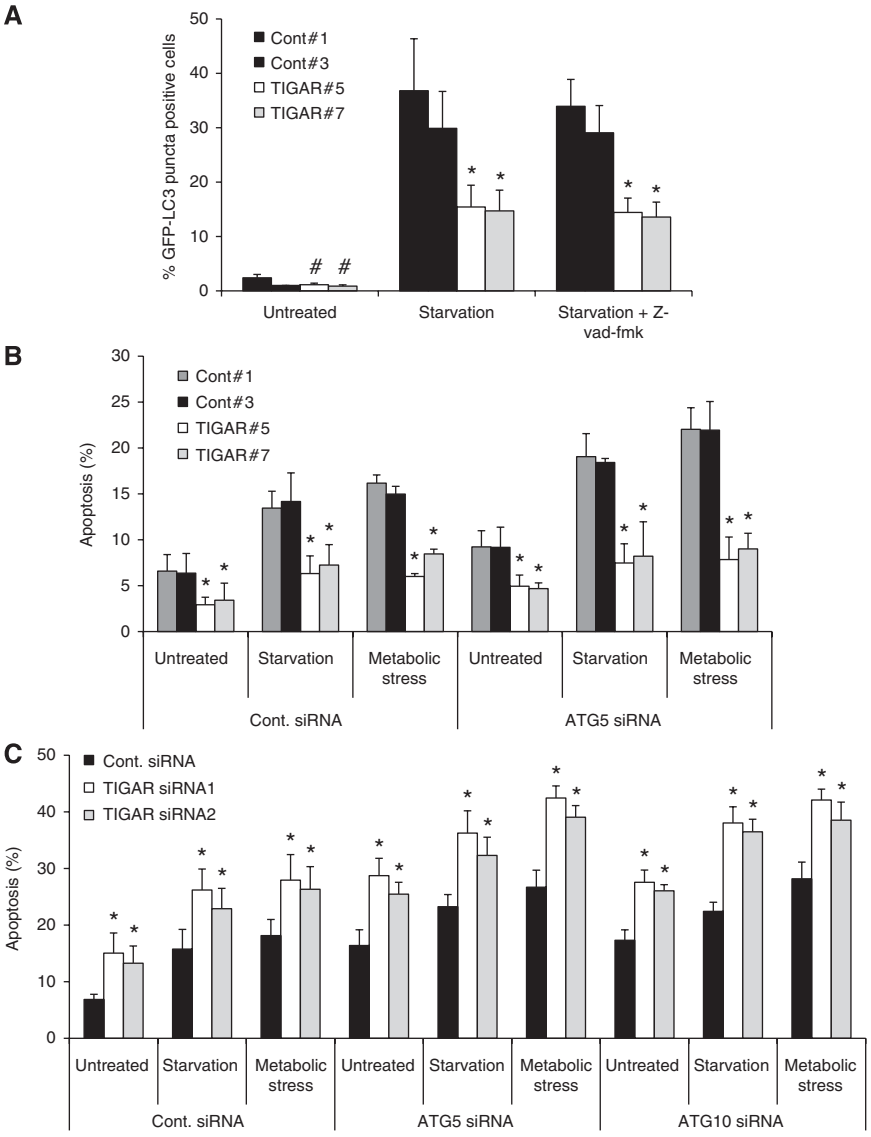


Figure 6 TIGAR modulation of autophagy influences apoptosis. (**A**) Quantitation of the percentage of GFP-LC3 puncta positive cells. U2OS cells stably over-expressing Flag-tagged-TIGAR (clones TIGAR#5 and TIGAR#7) or control cells (clones Cont#1 and Cont#3) were left untreated or exposed to nutrient starvation for 6 h, with or without treatment with Z-VAD-FMK for 24 h. The percentage of cells with GFP-LC3 puncta was calculated, and data are shown as the mean and standard deviation of the mean from three independent experiments. (**B**) Apoptosis in U2OS cells stably over-expressing Flag-tagged-TIGAR (clones TIGAR#5 and TIGAR#7) or control cells (clones Cont#1 and Cont#3), as measured by cells with a sub-G1 DNA content. Cells were transfected with either scrambled, TIGAR siRNA1 or TIGAR siRNA2. Cells were left untreated, exposed to nutrient starvation for 6 h or to metabolic stress for 24 h. (**C**) Apoptosis in U2OS cells cotransfected with scrambled or TIGAR siRNAs, and scrambled, ATG5 siRNA or ATG10 siRNA, as measured by cells with a sub-G1 DNA content. After 48 h, cells were left untreated, exposed to nutrient starvation for 6 h or to metabolic stress for 24 h. Data are shown as the mean and standard deviation from three independent experiments. In each case, the increase in apoptosis after knockdown of ATG5 or ATG10, compared with the matched control, was statistically significant; * represents significant difference from control conditions ($P<0.05$); # represents a lack of significant difference from control conditions ($P>0.05$).

instability (Kopnin *et al*, 2007). Given the similarity of function between the Sestrins and TIGAR, it might be predicted that Sestrins expression would also inhibit autophagy by removing ROS. However, more recently, Sestrin1 and Sestrin2 have been shown to activate activated protein kinase (AMPK), leading to the inhibition of mTOR (Budanov and Karin, 2008). This function of the Sestrins, which seems to be unrelated to the anti-oxidant activity, would be expected to have the opposite effect on autophagy, as inhibition of mTOR activity promotes autophagy. Although the ability of Sestrins to regulate autophagy has not been tested directly, it seems likely that there will be a balance between the positive regulation through mTOR inhibition and the negative regulation through anti-oxidant activity. A more recent study suggesting that Sestrin2 does not function as a peroxiredoxin reductase (Rhee *et al*, 2009) also raises the possibility that the anti-oxidant activity of at least this member of the Sestrin family may be mediated through a different mechanism. In conclusion, TIGAR does not clearly impact the mTOR signalling pathway, and so the opposing function on autophagy that may be shown by the Sestrins is not shared by TIGAR. Importantly, our results also provide a link between the direct

regulation of glycolysis by TIGAR and the control of autophagy through ROS, rather than the mTOR pathway.

Our results also suggest that the basal levels of TIGAR expression seen in our cell culture models (that are p53 dependent) are sufficient to modulate the autophagic response and that no increase in TIGAR expression is necessary. These results do not, however, preclude the existence of other mechanisms that control TIGAR function in response to nutrient starvation, such as alteration of subcellular localization, post-translational modification or interaction partners.

Apoptosis has a clear function in preventing tumour development, although the function of autophagy is less clear. Although the survival functions of autophagy suggest that this response may contribute to tumour development, *in vivo* studies of mice deficient in the autophagic response suggest a tumour suppressive function (Botti *et al*, 2006). Overall, a tumour suppressive function of TIGAR would be consistent with its activity as a mediator of the p53 response, and could reflect multiple consequences of TIGAR contributing to the control of ROS levels, preventing the accumulation of genetic damage and protecting cells from apoptosis to allow repair of genotoxic damage. Loss of TIGAR leads to enhanced ROS-dependent apoptosis (Bensaad *et al*, 2006), although this is balanced to some degree by an increase in autophagy, which dampens the increase in ROS and the extent of the apoptotic response. Several activities of p53 have been described that function to lower ROS levels, alter metabolism and promote cell survival. Although these may be entirely legitimate functions in response to p53 activating signals, to either help prevent damage or allow for repair, these activities would require close control, as their inappropriate expression could help to assist malignant transformation. Indeed, there is evidence that some of the survival and anti-oxidant responses to p53 are down-regulated under conditions of sustained stress when the cells shift to apoptosis (Sablina *et al*, 2005; Bensaad *et al*, 2006). It therefore seems possible that deregulated and sustained activation of these p53-response pathways (e.g. the inappropriate maintenance of expression of TIGAR in cells that are switching to an apoptotic response) may contribute to tumour development. Given these potentially opposing functions of TIGAR in suppression and promotion of tumourigenesis, it is difficult to predict the effect of modulation of TIGAR expression on cancer development. Most clearly, inhibition of TIGAR expression promotes ROS-dependent apoptosis, and it seems likely that cancer cells will be particularly susceptible to such a response. The recently reported structure of TIGAR (Li and Jogl, 2009) will help in the development of small molecule modulators of TIGAR activity that will ultimately allow direct testing of the effect of TIGAR inhibition on tumour cell growth and survival.

Materials and methods

Plasmids

Flag-tagged-TIGAR was obtained by RT–PCR using sense primer CGGGATCCCGCACCATGGACTACAAG and antisense primer CGGGATCCTTAGCGAGTTTCAGTCAGTCC, then subcloned into pcDNA3.1 to create pcDNA3.1-Flag-tagged-TIGAR. The pcDNA3-HA-tagged-FBPase-2 plasmid expressing only the bisphosphatase domain of the rat liver PFK-2/FBPase-2 enzyme has been described earlier (Perez *et al*, 2000). U2OS cells were infected with an adenovirus expressing LC3 fused to GFP (Ad5CMVGFP) to follow the progression of autophagy (Bampton *et al*, 2005).

Cell lines, transfections and siRNAs

U2OS, H1299 and Hela cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO), 10% foetal bovine serum, 2 mM L-glutamine, 50 U of penicillin/ml and 50 μg of streptomycin/ml. RPE cells were cultured in a 1:1 mixture of DMEM and nutrient mixture F12 (DMEM/F12, Gibco), 10% foetal bovine serum, 2 mM L-glutamine, 50 U of penicillin/ml and 50 μg of streptomycin/ml. Cells were treated with 2 mM NAC and 2 mM L-ascorbic acid, 4 mM glutathione ethyl ester or 4 mM ethyl pyruvate for 24 h to scavenge intracellular ROS. Cells were treated with 10 μM Z-VAD-FMK for 24 h to inhibit apoptosis. Cells were treated with 100 mM bafilomycin A1 for the indicated time points to prevent degradation of autophagic vacuoles. Transfections were carried out using the Lipofectamine 2000 reagent from Invitrogen, and the cells were harvested for flow cytometry or protein analysis at the indicated times. To inhibit TIGAR expression, two small-interfering RNAs (siRNA) matching region 115–133 in exon 3 (GCAGCAGCTGCTGGT ATAT; TIGAR siRNA1) and region 565–583 in exon 6 (TTAGCAGCC AGTGTCTTAG; TIGAR siRNA2) of the human *TIGAR* cDNA sequence were synthesized as an antisense, and a scramble sequence (TTACCGAGACCGTACGTAT) was synthesized as a control. To inhibit p53 expression, the sequence GACTCCAGTGGTAA TCTAC of the human p53 cDNA was synthesized as an antisense. To inhibit ATG5 expression, the sequence CATCTGAGCTACCCGGAT ATT of the human ATG5 cDNA was synthesized as an antisense. To inhibit ATG10 expression, the sequence GGAGUUCAUGAGUGCU AUA of the human ATG10 cDNA was synthesized as an antisense. To inhibit DRAM expression, the two sequences CCACGATGTATACAA GATA (1) and CCACAGAAATCAATGGTGA (2) were synthesized as an antisense.

Induction, detection and quantitation of autophagy

U2OS cells stably expressing GFP-LC3 were transfected with either scrambled or TIGAR siRNAs, and U2OS cells stably over-expressing Flag-tagged-TIGAR (clones TIGAR#5 and TIGAR#7) or control cells (clone Cont#1 and Cont#3) were infected for 16 h with an adenovirus expressing GFP-LC3. Autophagy was induced by nutrient starvation or metabolic stress. For nutrient starvation, cells were washed three times with phosphate buffered saline (PBS) and incubated with Earle's balanced salts solution (GIBCO) at 37°C for 5/6 h. For metabolic stress, cells were washed three times with PBS and incubated with DMEM without glucose (GIBCO) in a hypoxia chamber at 1% oxygen at 37°C for 18/24 h. In some experiments, cells were pre-treated with the indicated drugs before induction of autophagy. Autophagy was quantified by the percentage of GFP-LC3–positive cells displaying GFP puncta, and fluorescence was monitored by confocal microscopy (Olympus FV1000). Five-hundred cells were evaluated for the formation of GFP-LC3 punctas for each experiments at each time point.

Measurement of apoptosis and cell death

To study the effect of knockdown of TIGAR on apoptosis, cells were transfected with either 100 nM of a single siRNA or 50 nM each of two different siRNAs at 0 and 24 h; 72 h later, cells were harvested, fixed in methanol and analysed by flow cytometry (FACScan, Becton Dickinson). Cell with a sub-G1 DNA content was identified as apoptotic. Overall cell death was measured by propidium iodide exclusion assay.

Protein analysis and generation of anti-TIGAR antibody

Mouse monoclonal antibody to TIGAR was raised against a 15-amino-acid peptide corresponding to the exon COOH-terminal region of human TIGAR protein (CMNLQDHLNGLTETR). Human p53, LC3, total S6 ribosomal protein, phosphorylated S6 ribosomal protein, total p70 S6 kinase, phosphorylated p70 S6 kinase, p62, COX IV and b-actin proteins were detected using the antibodies DO-1, NB100-2331 (NOVUS BIOLOGICALS), #2317 (Cell Signaling Technology), #2211 (Cell Signaling Technology), #9202 (Cell Signaling Technology), #9206 (Cell Signaling Technology), 610833 (BD Biosciences), ab16056-100 (abcam) and MAB1501 (Millipore), respectively.

Measurement of ROS

ROS levels were determined by incubating the cells in PBS containing 10 mM 2′,7′-dichloro-dihydrofluorescein diacetate (H_2-DCFDA, Molecular Probes) for 30 min at 37°C. H_2-DCFDA was metabolized by non-specific esterases to the non-fluorescence product, 2′,7′-dichloro-dihydrofluoresceine, which was oxidized to the fluorescent product, DCF, by ROS. Then, the cells were washed twice in PBS, trypsinized, resuspended in PBS and measured for their ROS content by FACS (FACScan, Becton Dickinson).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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