Tp53-induced glycolysis and apoptosis regulator (TIGAR) protects glioma cells from starvation-induced cell death by upregulating respiration and improving cellular redox homeostasis.*

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Background: Tp53-induced glycolysis and apoptosis regulator (TIGAR) is a p53-target gene that has been shown to inhibit glycolysis and activate the pentose phosphate pathway (PPP).

Results: TIGAR regulates mitochondrial respiration and intracellular reactive oxygen species (ROS) levels.

Conclusion: TIGAR improves cellular redox homeostasis.

Significance: TIGAR may be a target for metabolic therapies aiming to enhance tumor cell sensitivity towards hypoxia.

SUMMARY:
Altered metabolism in tumor cells is increasingly recognized as a core component of the neoplastic phenotype. Since p53 has emerged as a master metabolic regulator, we hypothesized that the presence of wild-type p53 in glioblastoma cells could confer a selective advantage to these cells under the adverse conditions of the glioma microenvironment.

Here, we report on the effects of the p53-dependent effector Tp53-induced glycolysis and apoptosis regulator (TIGAR) on hypoxia-induced cell death. We demonstrate that TIGAR is overexpressed in glioblastomas, and that ectopic expression of TIGAR reduces cell death induced by glucose and oxygen restriction. Metabolic analyses revealed that TIGAR inhibits glycolysis and promotes respiration. Further, generation of ROS levels was reduced whereas levels of reduced glutathione were elevated in TIGAR-expressing cells. Finally, inhibiting the transketolase isoenzyme transketolase-like 1 (TKTL1) by siRNA reversed these effects of TIGAR.

These findings suggest that glioma cells benefit from TIGAR expression by i) improving energy yield from glucose via increased respiration and ii) enhancing defense mechanisms against ROS. Targeting metabolic regulators such as TIGAR may therefore be a valuable strategy to enhance glioma cell sensitivity towards spontaneously occurring or therapy-induced starvation conditions or ROS-inducing therapeutic approaches.

Glycolysis and mitochondrial respiration are the two main energy sources in eukaryotic cells to fuel biological functions. During the oxygen-independent glycolytic pathway, glucose is metabolized to pyruvate, which can then either be fermented to lactate to regenerate NAD+, or be metabolized via the citric acid cycle, whereby the generated NADH and succinate can be oxidized through the mitochondrial electron transport chain to provide large amounts of ATP. Unlike normal cells, cancer cells preferentially utilize the glycolytic pathway instead of oxidative phosphorylation even in the presence of oxygen (1,2). Oxidative phosphorylation is by far more efficient in ATP generation per Mol glucose, but it also leads to raised intracellular ROS levels (3,4). While ROS can play important roles in
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regulating cell signaling and homeostasis when present in moderate quantity (5-11), excessive amounts can damage cellular components like proteins or DNA (12-15). ROS homeostasis is dependent on NADPH generation through the pentose phosphate pathway (PPP) and subsequent production of reduced glutathione. In the last few years, regulation of PPP has been found to be more and more complex and versatile (16-20). For example, it was revealed that the tumor suppressor p53 can inhibit the PPP by binding to glucose-6-phosphate dehydrogenase (G6PD), preventing the formation of the active dimer of the enzyme (20), which ultimately results in decreased NADPH levels. Other results suggest that p53 may also regulate genes that function to lower ROS levels, indicating important functions of p53 in preventing DNA damage and tumor development (21,22).

Underscoring the influence of p53 on metabolism, TIGAR has recently been discovered as a new p53 target gene (17). Analyses of TIGAR’s structure and functions revealed interesting aspects: TIGAR, which shows similarity to fructose-2,6-bisphosphatase (FBPase-2), was demonstrated to inhibit glycolysis and activate PPP in U2OS cells, correlating with the ability to protect cells from oxidative (17) or metabolic stress-induced cell death (16). Primary or de novo glioblastomas (GBM) are highly aggressive and hypoxic human tumors (23,24) which typically retain p53 wild-type (wt) status (24,25). Oxygen concentrations in these tumors often reach levels of profound hypoxia as low as 0.1% O2 (26,27). Further, the availability of nutrients, i.e. glucose, is also severely impaired in some regions of solid tumors (28-30). We recently showed that wt p53 can limit glucose demands under tumor microenvironment conditions by inducing expression of Synthesis of cytochrome C oxidase 2 (SCO2), ultimately promoting cellular survival (31). Resistance mechanisms towards these hypoxic and nutrient-starved conditions are considered to be important for the survival of tumor cells within a solid tumor and for the resistance to radiotherapy, surgery and targeted therapy (32).

However, whereas suppression of p53 sensitized cells to metabolic stress even under severe hypoxia, protection by SCO2 required the presence of sufficient oxygen (e.g., 1-5% O2) consistent with its function at the respiratory chain. Therefore, we speculated that other p53-dependent target genes would be functional even under severe hypoxia. For that reason, we investigated whether the p53-target gene TIGAR could be involved in metabolic regulation in glioma cells. Here we describe a mechanism implicating TIGAR as a regulator of redox metabolism under hypoxic conditions and an activator of the mitochondrial respiratory chain in the oxygenated tumor fraction.

Because i) TIGAR exhibits an antioxidant function through the PPP (16,17), ii) the PPP plays an important role in cancer (33-36), and iii) transketolase-like 1 (TKTL1), an isoenzyme of the transketolase, has been found to be overexpressed in different tumor types (18,37-39) and was suggested to be important for PPP function and protection of tumor cells against oxidative stress (40), we also assessed a possible link between TIGAR and TKTL1.

EXPERIMENTAL PROCEDURES

Cell lines - LNT-229 cells were described previously (41). T98G cells were obtained from the ATCC (Rockville, MD). LNT-229 cells stably expressing a temperature-sensitive murine p53V135A possessing dominant-negative properties at 38.5°C and hygromycin-resistant control cells transfected with the empty vector (LNT-229hygro) were described previously (42). Cells, if not otherwise specified, were maintained in Dulbecco’s modified Eagle’s medium (DMEM, PAA, Coelbe, Germany) containing 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin. LNT-229hygro and LNT-229p53V135A cells and derived transfectants were cultivated at 38.5°C. In experiments requiring defined glucose conditions, Dulbecco’s modified Eagle’s glucose free medium (PAA) was used without FCS, and glucose was added as required. Cells were seeded at a density of 5.7x10^4 cells/cm² if not otherwise specified.

Constructs - The hygromycin control vector and p53V135A vector were obtained from M. Clarke The plasmids pcDNA3.1-TIGAR and pcDNA3.1-TIGAR-TM, which encodes a triple mutant of TIGAR lacking glycolysis inhibitory properties, were generously provided by K. Vousden (16,17). The control pcDNA3.1 vector
was purchased from Invitrogen. The p53-luc reporter gene vector PathDetect p53 was purchased from Stratagene (Cedar Creek, TX), pRL-CMV Renilla vector was obtained from Promega (Mannheim, Germany). All stable and transient transfections of plasmids were done using Metafectene pro (Biontex).

To inhibit TIGAR expression, small interfering RNA (siRNA) of the human TIGAR cDNA sequence published in (17) was used (matching region 115–133 in exon 3 5’-GCAGCAGCTGCTGGTATAT-3’). To inhibit TKTL1 expression, a small interfering RNA matching region 2175-2195 in the 3’-UTR region (5’-AAGTGTTTCCTTCGTGAATAA-3’ described in (40)) was used. A scrambled siRNA was used as control (Allstars negative siRNA, Qiagen, Hilden, Germany). siRNA was transfected using HiPerfect (Qiagen) according to the manufacturer’s protocol (3µl Hiperfect: 100 nM for TIGARsiRNA or 20 nM for TKTL1siRNA).

**Luciferase assay** - Cells were seeded at 10,000 cells per well into 96 well plates, cotransfected using Metafectene pro with the p53-luc reporter gene vector and pRL-CMV (Renilla) vector at a ratio of 7.5 : 1 and exposed to 0, 170, 250, 345, 500 or 1000 ng/ml adriamycin for 20 h. Experiments were conducted in triplicates. Activities of renilla luciferase and firefly were determined using a luminometer (Mithras). Background was subtracted from all values and the counts obtained from the measurement of firefly luciferase were normalized to renilla luciferase (43,44).

**Immunoblot analysis** - Cells were seeded in 6 well plates and exposed to 0, 170, 250, 345, 500 or 1000 ng/ml for 20 h. Thereafter, cells were washed with cold phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Tris-HCl pH 8, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40) containing protease inhibitors (Roche, Mannheim, Germany). Cellular lysates were prepared as described (45) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Membranes were probed with antibodies to human p53 (sc-263, Santa Cruz, San Diego, CA) and GAPDH (MAB374, Chemicon, Nuernberg, Germany). Secondary antibody was purchased from Santa Cruz. The chemiluminescence solution used for detection was composed of 1 ml solution A (200 ml 0.1 M Tris-HCl pH 8.6, 50 mg luminol), 100 µl solution B (11 mg p-hydroxycumarinacid, 10 ml DMSO) and 0.3 µl H2O2 (30%).

**RNA extraction and quantitative RT-PCR (qRT-PCR)** - Total RNA was extracted using Trizol and RNaseasy Kit (Invitrogen, Karlsruhe, Germany). First strand cDNA was synthesized using the Vilo cDNA synthesis kit (Invitrogen) for 10 min at 25°C and 2 h at 42°C. Subsequently, the enzyme was inactivated at 85°C for 10 min. To determine changes in gene expression, qRT-PCR was performed in the IQ5 real-time PCR detection system (Biorad, Muenchen, Germany) using Absolute Blue Q-PCR Mastermix with SybrGreen+Fluorescein (Thermo Fisher Scientific, Hamburg, Germany) and the following primer pairs: TIGAR bw 5´-CCATGTGCAATCCAGAGATG-3´, TIGAR fw 5´-CCTTACCAGCCACTCTGAGC-3´ (recognizes both TIGAR and TIGAR-TM sequences), TKTL1 bw 5´-CATCCTAACAAGCTTTCGCTG-3´, TKTL1 fw 5´-TAACCACTATGGCCTACTGC-3´, 18S fw 5´-CGGCTACCACATCCAAGGAA-3´, 18S bw 5´-GCTGGAATTACCGCGGCT-3´. Cycle threshold (Ct) values were normalized for amplification of the 18S ribosomal RNA and the data were analyzed using the Vandesompele method (46).

**Cell death analysis** - Cell death was assessed by propidium iodide (PI)-FACS (31). Experiments were performed in triplicates and are presented as mean ± standard deviation (SD).

**Induction of hypoxia** - Profound hypoxia (0.1% O2) was induced by incubating cells in Gas Pak pouches for anaerobic culture (Becton-Dickinson, Heidelberg, Germany) (45). Moderate hypoxia (5% O2) was induced in a Labotect incubator (Goettingen, Germany).

**Measurement of glucose and lactate** - Cell-free supernatant was collected and glucose and lactate concentrations were measured using the biochemistry analyzer Hitachi 917.

**Oxygen consumption** - Oxygen concentration in the medium was measured using the ABL-80 FLEX Blood Gas Analyzer (Radiometer, Willich, Germany) as previously described (31).

**ATP assay** – Cells were treated as indicated. Immediately after treatment, plates were placed
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**ROS analysis** - ROS levels were determined by H$_2$DCFDA-FACS (31). The membrane permanent molecule 2',7' dichlorodihydrofluorescein diacetate (H$_2$DCFDA-AM) passes through the cell membrane. In the cytoplasm, cellular esterases hydrolyse H$_2$DCFDA-AM to form the non-fluorescent moiety H$_2$DCFDA. Oxidation of H$_2$DCFDA by intracellular ROS leads to the formation of 2',7' dichlorodihydrofluorescein (DCF), which is highly fluorescent and can be assessed by FACS-analysis. Experiments were performed in triplicates and are presented as mean ± standard deviation (SD).

**GSH measurement** - Cellular GSH levels were measured with GSH-Glo™Glutathione assay from Promega according to the supplier protocol. Experiments were performed in triplicates and are presented as mean ± standard deviation (SD).

**LDH measurement** - LDH assay (Roche, Mannheim, Germany) according to manufacturer’s instructions.

**RESULTS**

**p53 regulates TIGAR expression in human glioma cells** - To our knowledge, TIGAR expression in vivo has hitherto only been assessed in invasive breast cancer. A high expression of TIGAR was noted in almost 75% of the examined breast tumors (47). To assess if TIGAR may be regulated in GBM tumors, an in silico analysis was performed with data from (48), using the Oncomine database (www.oncomine.org/), a cancer microarray database allowing gene expression analysis in different tumor types from genome-wide expression analyses of patient material. TIGAR expression was significantly elevated in GBM tumors (n=81) vs control samples from normal brain (n=23), (fold change: 1.407, p-value: 1.3x10^{-5}, Fig. 1A). As TIGAR was described as a p53 target gene (17), we investigated a possible regulation of TIGAR expression by p53 in glioma cells. TIGAR expression was decreased in LNT-229 cells in which the p53 transactivation activity was inhibited by stable expression of the temperature-sensitive dominant-negative p53V135A mutant (Fig. 1B).

To further explore whether expression of TIGAR is regulated by p53, cells were treated by adriamycin (17), a DNA-damage-inducing drug, and transcriptional activity of p53 and expression of TIGAR were analyzed. Adriamycin dose-dependently increased p53 accumulation and activity (Fig. 1C). TIGAR expression was upregulated up to 5-fold in adriamycin treated cells, supporting the assumption of TIGAR being a p53-dependent gene in the examined glioma cell line (Fig. 1D).

Further, TKTL1 expression, like TIGAR expression, was enhanced by adriamycin (Fig. 1E). TKT expression itself remained unchanged (data not shown).

**TIGAR protects glioma cells from hypoxia-induced cell death** - In different areas of solid tumors, oxygen concentrations often fluctuate between 5% O$_2$ (49-53) and 0.1% O$_2$ (26,27). We therefore studied the function of TIGAR under conditions characteristic for the tumor microenvironment in a well-established paradigm (0.1% O$_2$ and 2 mM glucose (31)). For this purpose, three models were defined: (i) LNT-229 cells (p53 wild-type) in which TIGAR expression was transiently suppressed by siRNA (Fig. 2A), ii) T98G cells (p53 mutant) stably transfected with TIGAR and corresponding control transfectants (Fig. 2B), and iii) LNT-229p53V135A cells lacking p53 wt transactivational activity in which TIGAR was stably reexpressed (Fig. 1C). We found that suppression of TIGAR in LNT-229 cells enhanced hypoxia-induced cell death (Fig. 2D and G), whereas TIGAR reexpression protected T98G and LNT-229p53V135A cells against hypoxia-induced cell death (Fig. 2E, H, F and I). As expression of TIGAR-TM, a triple mutant of TIGAR which is unable to lower Fru-2,6-P$_2$ levels and to inhibit glycolysis (17), did not lead to protection towards hypoxic cell death (Fig. 2B and C), TIGAR-mediated rescue seemed to depend on its functionality in glycolysis inhibition and PPP activation.

**TIGAR protects glioma cells from ROS-induced cell death** - As ROS seem to be important for hypoxia-induced cell death in the model described here (31), and TIGAR has previously been shown to confer protection against apoptosis induced by H$_2$O$_2$ in human osteosarcoma (U2OS) and non-small cell lung carcinoma (H1299) cells (17), we analyzed whether TIGAR modulates ROS-induced cell death.
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date in glioma cells as well. Suppression of TIGAR expression sensitized LNT-229 cells against hydrogen peroxide-induced cell death (Fig. 3A), whereas reexpression of TIGAR in LNT-229p53V135A or T98G cells efficiently mediated resistance to oxidant stress (Fig. 3B and C), suggesting that TIGAR enhances ROS detoxification mechanisms. We therefore examined whether TIGAR influenced ROS levels under metabolic stress conditions. Analysis of ROS levels by H2DCFDA-FACS showed decreased levels of ROS in TIGAR reexpressing cells (Fig. 3E and F), whereas suppression of TIGAR increased ROS concentrations (Fig. 3D). Again, TIGAR-TM did not recapitulate the effects of TIGAR. One possible mechanism for enhanced ROS detoxification by TIGAR might be an increased flux through the PPP resulting in elevated levels of NADPH and subsequently of reduced glutathione (GSH). In agreement with this hypothesis, luminometric analysis of GSH showed increased levels of GSH in TIGAR expressing cells (suppl. Fig. 1).

Inhibition of TKTL1 expression antagonizes TIGAR-mediated protection - To further characterize the function of the PPP for TIGAR-mediated safeguard functions, it was investigated whether gene suppression of TKTL1 by siRNA would abolish the protective effects of TIGAR in LNT-229p53V135A-TIGAR and T98G-TIGAR cells (Fig. 4A). First, we could confirm that depletion of TKTL1 leads to sensitization of glioma cells towards oxidative stress-induced cell death (data not shown), as previously reported in colon carcinoma cells (40). Analysis of ROS levels by H2DCFDA-FACS showed an increased amount of ROS under hypoxia in TKTL1si cells even in the presence of TIGAR (Fig. 4B). Second, gene suppression of TKTL1 mimicked loss of TIGAR function and enhanced sensitivity towards hypoxic conditions (Fig. 4C). These data indicate that TIGAR function is linked to PPP activation and, at least in the examined cell lines, depends on the presence of TKTL1.

TIGAR inhibits glycolysis and promotes cellular respiration in glioma cells - Considering the fluctuant oxygen concentrations in solid tumors, we analyzed the role of TIGAR on cell survival at 5% oxygen, too. In a first step, we evaluated glucose consumption and lactate production in TIGAR transfected p53 mutant cells (LNT-229p53V135A and T98G) when exposed to limited glucose (5 mM) and physiologic oxygen conditions (5% O2). In TIGAR-expressing cells, decreased glucose consumption and lactate production were observed (Fig. 5A and E), indicating inhibition of glycolysis by TIGAR as shown previously (17). Because pharmacological inhibition of glycolysis by the glucose analog 2-deoxyglucose (2DG) has recently been demonstrated to inhibit anaerobic glycolysis by forcing glioma-derived cells into mitochondrial metabolism under low oxygen conditions (54), we analyzed whether reducing glycolytic activity would also modulate respiration in the LNT-229 glioma cell line. Treatment of glioma cells with 2DG similarly conferred a more oxidative phenotype characterized by lower glucose consumption and lactate production accompanied by increased mitochondrial respiration (suppl. Fig. 2A and B), indicative of at least some metabolic flexibility of glioma cells. We therefore hypothesized that TIGAR, similar to 2DG, could also increase mitochondrial respiration in tumor cells. Expression of TIGAR, but not TIGAR-TM, increased oxygen consumption in LNT-229p53V135A and T98G cells (Fig. 5B and F) whereas mitochondrial respiration was reduced in TIGARsi cells (data not shown).

Together, these data indicate that TIGAR shifts cellular metabolism towards oxidative phosphorylation. We therefore wished to define this phenotype in more detail by pharmacological perturbing oxidative phosphorylation (OXPHOS) in T98G-neo and TIGAR cells. Inhibition of ATP synthase by oligomycin (31) did not significantly decrease ATP content of neo cells, whereas it strongly suppressed ATP levels in TIGAR-reexpressing cells (suppl. Fig. 3A). Similarly, oligomycin’s effect on oxygen consumption was more pronounced in T98G-TIGAR cells (suppl. Fig. 3B). Oligomycin also abolished the protective effect of TIGAR on cell death (suppl. Fig. 3C). These data confirm that TIGAR-reexpressing cells employ OXPHOS to enhance energy homeostasis and to resist cell death under conditions of moderate starvation. Interestingly, the mitochondrial substrate methyl-pyruvate mimicked the effect of TIGAR in neo cells, enhancing oxygen consumption, increasing
ATP levels and conferring protection from cell death (suppl. Fig. 3C).

DISCUSSION
Solid tumors are characterized by areas of heterogeneous blood supply resulting in regions with varying supply of oxygen and nutrients, thus forcing tumor cells to survive under different metabolic conditions (28-30). Although increased glucose consumption is a hallmark of tumors, metabolic versatility therefore could be useful for tumor cells. Indeed, the assumption of Warburg that the increased aerobic glycolysis is a direct consequence of defects in cellular respiration does not seem to adequately reflect tumor cell metabolism. It is now clear that mitochondria of tumor cells are still capable of oxidative phosphorylation but that oncogenic alterations redirect metabolism away from cellular respiration towards metabolic pathways important for anabolic processes such as glycolysis and the PPP (55). For example, we and others could previously show that the p53-target gene SCO2 is capable of activating oxidative phosphorylation in tumor cells, leading to a more energy-efficient metabolism of glucose, thus delaying glucose depletion and prolonging tumor cell survival under starvation conditions (31,56).

In accordance with the assumption that SCO2-mediated alterations in metabolism involve oxidative phosphorylation, this protective effect was no longer present under severe hypoxia (0.1 % O2). We however still observed increased sensitivity of p53-mutant cells towards starvation under severe hypoxia and therefore hypothesized that another metabolic target of p53, TIGAR, could be responsible for the observed phenotype.

Indeed, we were able to demonstrate that under severe hypoxia, expression of TIGAR protected p53-mutant cells against cell death (Fig. 2). Considering the elevated levels of ROS during hypoxia (31), we speculate that this effect is possibly mediated by increased defense mechanisms towards ROS by activation of the PPP and subsequent generation of NADPH and GSH. It has been previously shown that TIGAR activates the PPP by its fructose-2,6-bisphosphatase activity (17). These results are confirmed in our experiments by the demonstration of lower levels of ROS in TIGAR-expressing cells (Fig. 3), the protective effect of TIGAR towards exogenous ROS (Fig. 3) and increased levels of GSH in TIGAR-proficient cells (suppl. Fig. 1).

Further, suppressing the expression of TKTL1 antagonized the protective effects of TIGAR towards ROS and starvation (Fig. 4C). Due to its homology to transketolase, a role for TKTL1 in the PPP is likely, and the loss of TIGAR’s protective effect by suppression of TKTL1 would therefore be compatible with the importance of the PPP for the protection by TIGAR. Important functions of TKTL1 in cancer biology have been proposed based on different observations: first, TKTL1 was shown to be expressed in a variety of tumor cells including glioma (37,38,57,58) and to be associated with malignant grade, presence of metastasis and prognosis (57,59,60). Second, as putative mechanisms for these associations, TKTL1 has been demonstrated to increase glycolysis and hypoxia inducible factor-1α (HIF-1α) expression, proliferation (61,62) the generation of NADPH and resistance towards ROS (40). The presented results of increased sensitivity of TKTL1-suppressed cells against ROS and hypoxia (Fig. 4) are in agreement with these observations.

In addition to TIGAR’s function at severe hypoxia, protective effects of TIGAR in the presence of oxygen were also detected. These were associated with reduced glucose consumption and lactate generation, elevated oxygen consumption, enhanced ATP levels and increased vulnerability towards OXPHOS inhibition, indicative of a less glycolytic and more oxidative phenotype (Fig. 5 and suppl. Fig. 3).

The way TIGAR acts on respiration remains unclear. It appears possible that metabolites from the PPP are redirected to the tricarboxylic acid cycle and subsequently to OXPHOS. Our finding that the mitochondrial substrate methyl-pyruvate mimics the effect of TIGAR on OXPHOS (suppl. Fig. 3C) suggests that indeed the supply with mitochondrial substrates is the critical regulator for OXPHOS.

Despite increased respiration, however, endogenous ROS levels were not elevated (data not shown). Gruning et al. recently revealed new insights into the function of the glycolysis regulator pyruvate kinase (PYK) in yeast. They showed that lowering PYK expression, on the one side, activates PYK expression, on the one side, activates mitochondrial energy metabolism. Surprisingly, however, this was not
TIGAR coordinates glycolysis and cellular respiration accompanied by increased ROS levels, and the authors identified activation of the PPP by phosphoenolpyruvate-induced inhibition of triosephosphate isomerase as responsible for increased NAPDH generation, leading to suppression of ROS formation (19). Our results indicate that inhibition of glycolysis and activation of the PPP by TIGAR has similar effects in tumor cells. Inhibition of glycolysis by TIGAR could impair anabolic metabolism by limiting availability of substrates necessary for macromolecular synthesis. However, under conditions of limited glucose availability as present in significant areas of gliomas (64-67), the switch to a more oxidative phenotype, as characteristic for quiescent cells (55), could result in a more efficient energy production and prolonged tumor cell survival. Recently, this assumption has been supported by the observation that glucose oxidation is used in glioblastoma cells to meet energetic and biosynthetic demands (63). Intriguingly, modulating glucose metabolism indeed may alter fundamental characteristics of tumor cells as suggested by the results of Pistollato et al. (54), who observed that antagonizing glycolysis in glioma cells by 2DG at 2% O\textsubscript{2} not only forces cancer cells into a mitochondrial metabolism but also induces cellular differentiation. An additional advantage tumor cells could derive from TIGAR are increased defense mechanisms against potentially toxic levels of ROS which occur during hypoxia or anticancer therapies, e.g., radiotherapy (68). Another important mechanism by which TIGAR could mediate cytoprotection involves inhibition of autophagy through modulation of ROS under nutrient starvation or metabolic stress conditions (16). These effects of TIGAR were not assessed here but certainly could also play a role in our paradigm.

In summary, our results indicate that TIGAR is a major metabolic regulator which serves to increase survival of glioma cells under hypoxia and improves energy yield from glucose by activation of respiration, while suppressing formation of ROS possibly by PPP activation. As TKTL1 seems to be indispensable for these effects of TIGAR, strategies targeting the PPP or TKTL1 might reduce tumor viability and increase sensitivity towards hypoxia- and ROS-inducing therapies.
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FOOTNOTES

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The abbreviations used are: GSH, reduced glutathion; H2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; hygro, hygromycin; luc, luciferase; mutant, mt; OXPHOS, oxidative phosphorylation; PI, propidium iodide; PPP, pentose phosphate pathway; ROS, reactive oxygen species; scr si, scrambled
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siRNA; siRNA, small interfering RNA; TIGAR, Tp53 inducer and regulator of glycolysis; TKTL1, transketolase-like 1; wt, wildtype.

FIGURE LEGENDS

FIGURE 1. p53 regulates TIGAR expression in human glioma. (A) An in silico analysis was performed with the Oncomine database. TIGAR expression in normal brain samples was compared to glioblastoma patients samples (GBM), fold change: 1.407, p-value: 1.3x10^{-5}. (B) TIGAR expression was assessed by qRT-PCR in LNT-229p53V135A cells carrying the temperature-sensitive dominant-negative mutant p53V135A and hygro control cells (mean ± SD, * p<0.05). (C) LNT-229 cells were treated with increasing concentrations of adriamycin for 20 h. p53 transactivation activity was determined by luciferase assay (shown is mean ± SD of triplicates, one experiment out of two independent experiments with similar results is shown, ** p<0.01, unpaired student t-test compared to untreated cells). Expression of endogenous human p53 was confirmed by western blot. GAPDH expression was employed as loading control. Expression of (D) TIGAR and (E) TKTL1 was determined by qRT-PCR in LNT-229 cells exposed to 345 ng/ml adriamycin for 20 h (shown is mean ± SD of triplicates, one experiment out of two independent experiments with similar results is shown, ** p<0.01, unpaired student t-test).

FIGURE 2. TIGAR protects glioma cells from hypoxia-induced cell death. Expression of TIGAR was assessed by qRT-PCR in (A) LNT-229 transiently transfected with 100 nM scrambled siRNA (scr si) or TIGARsiRNA, (B) T98G stably transfected with TIGAR-pcDNA3.1 or pcDNA3.1 plasmid (neo) and (C) LNT-229p53V135A stably transfected with TIGAR-pcDNA3.1 or pcDNA3.1 plasmid (neo), and hygro control cells transfected with pcDNA3.1. (D-F) These cells were exposed to serum-free media containing 2 mM glucose and 0.1% O_2 for 20 h, and cell death was analyzed by PI-FACS. T98G- and LNT-229-TIGAR-TM-pcDNA3.1 cells were used as respective control (shown is mean ± SD of triplicates, one experiment out of ≥3 independent experiments with similar results is shown, * p<0.05, ** p<0.01, unpaired student t-test). (G-I) Cells were monitored by microscopy (x10) under these conditions.

FIGURE 3. TIGAR protects glioma cells from ROS-induced cell death. (A) LNT-229 transiently transfected with 100 nM scrambled siRNA (scr si) or TIGARsiRNA, (B) T98G stably transfected with TIGAR-pcDNA3.1 or pcDNA3.1 plasmid (neo) and (C) LNT-229p53V135A cells stably transfected with TIGAR-pcDNA3.1 or pcDNA3.1 plasmid (neo) were exposed to 1 mM H_2O_2 for 4 h (LNT-229) or 24 h (T98G), and cell death was assessed by PI-FACS (shown is mean ± SD of triplicates, one experiment out of ≥3 independent experiments with similar results is shown, ** p<0.01, unpaired student t-test). (D-F) Similarly, ROS were measured in the same cells by H_2DCFDA-FACS, T98G- and LNT-229-TIGAR-TM-pcDNA3.1 cells were used as respective control (shown is mean ± SD of triplicates, one experiment out of ≥3 independent experiments with similar results is shown, * p<0.05, ** p<0.01, unpaired student t-test).

FIGURE 4. TKTL1 is required for TIGAR-mediated protection against hypoxia. (A) TKTL1 expression was suppressed by 20 nM TKTL1siRNA in T98G-TIGAR and LNT-229p53V135A-TIGAR cells; scrambled siRNA was used as control. (B) Cells were grown in 5 mM glucose in serum-free media and exposed to 21% or 0.1% O_2 for 20 h. ROS levels were determined by H_2DCFDA-FACS (shown is mean ± SD of triplicates, one experiment out of ≥3 independent experiments with similar results is shown, * p<0.05, unpaired student t-test). (C) T98G and LNT-229p53V135A cells transfected with neo or TIGAR were transiently transfected with scrambled siRNA (100 nM), TKTL1siRNA (20 nM) or TIGARsiRNA (100 nM). After 16 h, cells were exposed to 0.1% O_2 for 20 h in the presence of serum-free media containing 2 mM glucose and cell death was assessed by PI-
FACS (shown is mean ± SD of triplicates, one experiment out of ≥3 independent experiments with similar results is shown, * p< 0.05, ** p<0.01, unpaired student t-test).

**FIGURE 5.** TIGAR inhibits glycolysis and promotes cellular respiration. (A and E) T98G and LNT-229p53^{V135A} cells carrying TIGAR or empty pcdna3.1 vector (neo) were exposed to serum-free media containing 5 mM glucose and 5% O\textsubscript{2} for 20 h. Glucose consumption and lactate production were determined (shown is mean ± SD of triplicates, one experiment out of two independent experiments with similar results is shown, * p< 0.05, unpaired student t-test). (B and F) Oxygen consumption was analyzed in T98G and LNT-229p53^{V135A} neo control, TIGAR or TIGAR-TM cells. A representative experiment out of 3 independent experiments with similar results is shown. (C and G) Cell death induced by moderate hypoxia (5% O\textsubscript{2}) was analyzed by PI-FACS (shown is mean ± SD of triplicates, one experiment out of 3 independent experiments with similar results is shown, * p< 0.05, ** p<0.01, unpaired student t-test). (D and H) Phase contrast microscopy (x10) under the same conditions as in C and G.

**FIGURE 6.** Hypothetical schematic drawing showing the TIGAR-dependent dual regulation of energy homeostasis and antioxidant production. TIGAR induces PPP activation, promoting NADPH production and therefore protection against ROS (e.g. produced in mitochondria). (A) In the presence of oxygen, enhanced mitochondrial respiration and ATP production by yet unknown mechanisms might contribute to maintain energy homeostasis and viability. (B) Under severe hypoxia (0.1% O\textsubscript{2}), PPP is sustained whereas ATP production by oxidative phosphorylation is irrelevant due to limited oxygen availability. Enhanced detoxification of intracellular ROS might therefore be more relevant for TIGAR’s capacity to promote cellular survival under hypoxia.
TIGAR coordinates glycolysis and cellular respiration

Fig. 1

A

TIGAR
(Oncomine)

\[
\begin{align*}
\text{log2 median correlated intensity} \\
\text{Brain} & \quad \text{GBM}
\end{align*}
\]

B

LNT-229

\[
\begin{align*}
\text{LNT-229 expression} [\text{qPCR}] \\
hygro & \quad \text{p53ts}
\end{align*}
\]

C

LNT-229

\[
\begin{align*}
\text{mL p53-luminescence} \\
\text{p53} & \quad \text{GAPDH}
\end{align*}
\]

D

TIGAR

\[
\begin{align*}
\text{mL expression} [\text{qPCR}] \\
adriamycin - & \quad +
\end{align*}
\]

E

TKTL1

\[
\begin{align*}
\text{mL expression} [\text{qPCR}] \\
adriamycin - & \quad +
\end{align*}
\]
TIGAR coordinates glycolysis and cellular respiration

**Fig. 2**

A. LNT-229

![Bar graph showing TIGAR expression](image)

B. T98G

![Bar graph showing TIGAR expression](image)

C. LNT-229

![Bar graph showing TIGAR expression](image)

D. Cell death [%]

![Graph showing cell death](image)

E. Cell death [%]

![Graph showing cell death](image)

F. Cell death [%]

![Graph showing cell death](image)

G. [Image of control and treatment groups]

H. [Image of control and treatment groups]

I. [Image of control and treatment groups]
TIGAR coordinates glycolysis and cellular respiration

Fig. 3

A

LNT-229

H₂O₂

Cell death [%]

0 mM

1 mM

0

20

40

60

80

scr si TIGARsi scr si TIGARsi

B

T98G

H₂O₂

Cell death [%]

0 mM

1 mM

0

20

40

60

80

neo TIGAR TIGAR-TM neo TIGAR TIGAR-TM

C

LNT-229ΔS59

H₂O₂

Cell death [%]

0 mM

1 mM

0

20

40

60

80

neo TIGAR TIGAR-TM neo TIGAR TIGAR-TM

D

0.1% O₂

DCF [mean]

0

200

400

600

800

1000

scr si TIGARsi

E

0.1% O₂

DCF [mean]

0

200

400

600

800

1000

neo TIGAR TIGAR-TM

F

0.1% O₂

DCF [mean]

0

200

400

600

800

1000

neo TIGAR TIGAR-TM
TIGAR coordinates glycolysis and cellular respiration

**Fig. 4**

**A**

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**B**

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<th>0.1%</th>
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<td></td>
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<tr>
<td>DCF [mean]</td>
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**C**

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<tbody>
<tr>
<td>Cell death [%]</td>
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**T98G**

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**TKTL1**

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**LNT-229**

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Fig. 5

TIGAR coordinates glycolysis and cellular respiration.
TIGAR coordinates glycolysis and cellular respiration

Fig. 6

A 5% O₂
oxic conditions

B 0.1% O₂
hypoxic conditions

glucose

TIGAR

PPP

NADPH

NADP⁺

GSH

GSSG

ROS

ATP

lactate

pyruvate

lactate

pyruvate

I II III IV