TRIB3 enhances cell viability during glucose deprivation in HEK293-derived cells by upregulating IGFBP2, a novel nutrient deficiency survival factor

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A R T I C L E   I N F O

Article history:
Received 24 March 2015
Received in revised form 12 June 2015
Accepted 17 June 2015
Available online 18 June 2015

Keywords:
TRIB3
IGFBP2
Cell death
Glucose deprivation
Gene expression

A B S T R A C T

Glucose deprivation occurs in several human diseases, including infarctions and solid tumors, and leads to cell death. In this article, we investigate the role of the pseudokinase Tribbles homolog 3 (TRIB3) in the cellular stress response to glucose starvation using cell lines derived from HEK293, which is highly glycolytic under standard conditions. Our results show that TRIB3 mRNA and protein levels are strongly upregulated in glucose-deprived cells via the induction of activating transcription factor 4 (ATF4) by the endoplasmic reticulum (ER) stress sensor kinase PERK. Cell survival in glucose-deficient conditions is enhanced by TRIB3 overexpression and reduced by TRIB3 knockdown. Genome-wide gene expression profiling uncovered approximately 40 glucose deprivation-responsive genes that are affected by TRIB3, including several genes involved in signaling processes and metabolism. Based on transcription factor motif analysis, the majority of TRIB3-downregulated genes are target genes of ATF4, which TRIB3 is known to inhibit. The gene most substantially upregulated by TRIB3 is insulin-like growth factor binding protein 2 (IGFBP2). IGFBP2 mRNA and protein levels are downregulated in cells subjected to glucose deprivation, and reduced IGFBP2 expression aggravates cell death during glucose deficiency, while overexpression of IGFBP2 prolongs cell survival. Moreover, IGFBP2 silencing abrogates the pro-survival effect of TRIB3. Since TRIB3 augments IGFBP2 expression in glucose-starved cells, the data indicate that IGFBP2 contributes to the attenuation of cell death by TRIB3. These results implicate TRIB3 and IGFBP2, both of which are known to be overexpressed in several types of cancers, as pro-survival modulators of cell viability in nutrient-deficient microenvironments.

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1. Introduction

Glucose deprivation is a cell death-inducing condition that occurs during diseases such as cerebral and myocardial infarctions, due to a blockage of blood flow, and in the central regions of solid tumors, due to insufficient vascularization. When having the opportunity, malignant cells tend to consume glucose at a particularly high rate and predomi-

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ATF4, activating transcription factor 4; eIF2α, eukaryotic translation initiation factor 2α; ER, endoplasmic reticulum; IGF, insulin-like growth factor; IGFBP2, IGF binding protein 2; TCA, trichloroacetic acid; Tet, tetracycline; TRIB3, Tribbles homolog 3.

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http://dx.doi.org/10.1016/j.bbamcr.2015.06.006
0167-4889/© 2015 Published by Elsevier B.V.
as opposing effects of TRIB3 on cell viability have been reported for different cell types and stress situations (for example, a pro-survival function in [7,10–14] and a pro-death function in [15–17]). Further investigation of the biology of TRIB3 may increase the comprehension of its dichotomous effects on cell viability and help to bring TRIB3 closer to clinical applications.

IGFBP2 is one of the six members of the insulin-like growth factor (IGF) binding protein family, which bind circulating IGF-I or IGF-II, thereby limiting the bioavailability of IGF and increasing IGF half-life in plasma [18]. By modulating IGF activity, IGFBPs can affect cell functions that are mediated by the IGF receptor; for example, overexpression of IGFBP2 in cell culture can inhibit IGF-induced cell proliferation in low-serum medium [19]. Additionally, IGFBPs have also IGF-independent functions, which may be exerted intracellularly as well as extracellularly. In an IGF-independent manner, IGFBP2 is able to inhibit apoptosis by repressing the expression of procaspase-3 [20] and increase cell motility by interacting with integrin α5 and activating integrin signaling [21]. IGFBP2 is overexpressed in multiple malignancies, including colorectal, breast, gastric, pancreatic and ovarian cancer, leukemia and glioma [22–28]. Moreover, a high in situ level of IGFBP2 is associated with poor prognosis in glioma and leukemia patients [29,30], and a high serum level of IGFBP2 is a biomarker for poor prognosis in colorectal cancer and glioma patients [31,32].

In the current work, we study the expression and function of human TRIB3 during glucose deprivation using cells lines derived from HEK293, which in standard growth conditions exhibits a highly glycolytic (“glucose-addicted”) metabolic phenotype that is comparable to tumor cells, consuming glucose at a rate 7-fold greater than that of the next most utilized carbon source, glutamine, and converting approximately 80% of the intracellular pyruvate pool into lactate [33]. We determine the effect of TRIB3 on glucose-starved cell survival, and perform genome-wide gene expression profiling to shed light on TRIB3-induced changes to the glucose deprivation transcriptional response. Based on the results of transcriptional profiling, further experiments are performed to determine the effect of IGFBP2 on glucose deprivation-induced cell death and to uncover the relationship between IGFBP2 and TRIB3 in the cellular response to glucose deficiency.

2. Materials and methods
2.1. Cell culture and treatment

T-REx–293 cells (HEK293–derived cell line stably expressing tetracycline (Tet) repressor protein) were obtained from Invitrogen. TRIB3–293 and Vector–293 cells were created by stable transfection of T-REx–293 cells with a Tet-inducible human TRIB3 expression construct and the corresponding empty vector, respectively [10]. Cells were grown in IMDM supplemented with 10% FCS and 1× penicillin/streptomycin (all purchased from PAA) in an atmosphere of 5% CO2 at 37 °C.

For glucose deprivation experiments, cells were seeded onto poly-L-lysine-coated tissue culture plates. For plasmid transfection, cells were incubated in Opti-MEM I reduced-serum medium (Invitrogen) according to the manufacturer’s reverse transfection protocol. During transfection, cells were incubated in Opti-MEM I reduced-serum medium (Invitrogen) supplemented with 3% FCS (unless specified otherwise) on poly-L-lysine-coated tissue culture plates. For plasmid transfection, 500 ng of DNA was used per 24-well plate well (containing 500 μl of growth medium), and for siRNA transfection, the concentration of siRNA was 80 nM (unless specified otherwise). For both siRNA and plasmid DNA transfection, 3 μl of transfection reagent was used per milliliter of culture medium. The siRNAs targeting TRIB3 mRNA and ATF4 mRNA have been described previously [10], and the sequence of the siRNA targeting IGFBP2 mRNA was 5′-CCUCUAAACAGUGCAAGDdTdT-3′ (sense strand shown; based on [34]). The AllStars Negative control siRNA (Qiagen) was used as a non-targeting negative control siRNA. The IGFBP2 and BiP overexpression plasmids used in this study are described below.

2.3. Plasmid construction

To generate the human IGFBP2 expression plasmid IGFBP2-pCG, the IGFBP2 coding sequence was PCR-amplified from HEK293 cell line cDNA using the primers 5′-GGCTGTACCATGCGCGAGATGGGCT-3′ and 5′-GGGGGTACCTCTAGCTCTCCGGGTGTG-3′ (sense and anti-sense, respectively). Cells were grown in IMDM supplemented with 10% FCS and 1× penicillin/streptomycin (all purchased from PAA) in an atmosphere of 5% CO2 at 37 °C. After 24 h of growth, glucose was added to the culture medium to a final concentration of 4.5 g/l. Wortmannin (Calbiochem) and tunicamycin (Sigma-Aldrich) were used at 1 μM and 2.5 μg/ml concentrations, respectively, in the glucose-free or glucose-containing experimental treatment medium, respectively. To prepare methionine-deficient growth medium, methionine-free DMEM was obtained from BioSera and supplemented with dialyzed FCS and antibiotics as described above for the preparation of glucose-free medium. To inhibit PERK (EIF2AK3) activity, cells were pre-incubated with the indicated concentration of PERK inhibitor GSK2606414 (Axon Medchem) for 45 min in fresh growth medium before the application of the indicated experimental treatment medium supplemented with the indicated concentration of PERK inhibitor.

2.4. Real-time PCR quantification of gene expression

For RT-qPCR, total RNA was extracted from cells using the TRIzol reagent (Invitrogen) according to the manufacturer's recommendations and quantified spectrophotometrically using NanoDrop 1000 (Thermo Scientific). Total RNA was treated with DNase I and used for first strand cDNA synthesis as described previously [11,36]. Real-time PCR was performed as reported previously [11] and ribosomal protein L7a (RPL7A) mRNA was used as the endogenous reference for expression level normalization. The sequences of the primers used for RT-qPCR are as follows: TRIB3 mRNA: described in [37], TRIB3 unspliced pre-mRNA: described in [38], IGFBP2 mRNA: 5′-AAGCATGGCCTGTAACACT-3′ (sense) and 5′-GGTTCACACACCAGCATC-3′ (anti-sense), ATF4 mRNA: 5′-CCTGCCGAAGAGATCCAGTACCT-3′ (sense) and 5′-ACATTGACGCTCCTGAATCT-3′ (anti-sense), and RPL7A mRNA: described in [37].

2.5. Trypan blue exclusion assay

To determine cell viability, the trypan blue dye exclusion method was used as described previously [10]. In each independent experiment, viable and non-viable (dye-accumulating) cells were counted from two
RNA quality was verified with the Agilent 2100 Bioanalyzer system (RNA integrity number: 9.90). RNA from different cell treatments was isolated from the cells using the RNeasy Mini kit (Qiagen). For the detection of TRIB3 protein, horseradish peroxidase-conjugated anti-human TRIB3 antibody was used (1:3000 dilution) [10]. Unconjugated primary antibodies used for immunoblotting were the following: rabbit anti-human IGFBP2 polyclonal antibody (1:4000 dilution; Santa Cruz Biotechnology sc-200), rabbit anti-phospho-Akt (Thr308) monoclonal antibody (1:1000 dilution; Cell Signaling Technology #3292), rabbit anti-human AT4 polyclonal antibody (1:1000 dilution; Cell Signaling Technology #2965), rabbit anti-phospho-Akt (Ser473) polyclonal antibody (1:3000 dilution; Cell Signaling Technology #9271), and rabbit anti-human β-Tubulin monoclonal antibody (1:6000 dilution; Abcam ab6046). For the unconjugated primary antibodies, goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was used (1:5000 dilution; Cell Signaling Technology #7074). After incubation with antibody, membranes were treated with Amersham ECL Plus enhanced chemiluminescent detection reagent and proteins were detected by medical X-ray film exposure.

2.7. Genome-wide gene expression profiling

For gene expression microarray analysis, TRIB3-293 and Vector-293 cells were subjected to the indicated treatments in duplicate and total RNA was isolated from the cells using the RNeasy Mini kit (Qiagen). RNA quality was verified with the Agilent 2100 Bioanalyzer system (RNA integrity number: 9.90–10.00). RNA from duplicates was pooled in equal proportions based on concentration and analyzed with the Illumina HumanHT-12 v4 BeadChip genome-wide gene expression microarray according to the manufacturer’s instructions. The microarray data were log2-transformed and quantile-normalized using the Bioconductor package lumi [39] (version 1.14.0). The probe signal detection P value cutoff was 0.01, and probes not classified as detected in any of the samples were discarded. To obtain a high-sensitivity set of gene probes regulated by glucose deprivation in the T-REx-293 lineage of HEK293 cells, the results from Tet-treated and -untreated Vector-293 and Tet-untreated TRIB3-293 samples were selected for analysis, while Tet-induced TRIB3-293 samples were excluded to prevent possible TRIB3 overexpression-dependent effects (which are the focus of subsequent analyses). For each probe, data from the cell line demonstrating less modulation by glucose starvation was retained, and a cut-off of 2-fold change (either up- or downregulation) was applied. Thus, glucose deprivation-regulated gene probes were defined as probes regulated at least 2-fold in response to glucose withdrawal in Vector-293 as well as Tet-untreated TRIB3-293 cells. This approach lowers the amount of false-positives arising from cell line-specific effects and signal measurement error, which would not be robustly offset solely by averaging, due to the small number of samples in the microarray analysis. The annotations of gene products were obtained from the Gene Ontology project [41] (database release 2014-09-25). Functional profiling analysis of gene sets was performed with the g:Profiler online tool [41] (accessed 2015-02-12) by searching for Gene Ontology terms using an ordered query and applying hierarchical filtering (best per parent) to the enriched terms. Transcription factor motif enrichment analysis of gene sets was carried out with iRegulon software [42] (version 1.2) using the default settings. The microarray data from the current study has been deposited into the ArrayExpress public database (http://www.ebi.ac.uk/arrayexpress/) under the accession code E-MTAB-3318.

2.8. Measurement of protein synthesis by 35S-methionine/cysteine incorporation

Cells were grown in 6-well tissue culture plate wells and incubated in glucose-free medium or complete medium (control), as described above. After the indicated amount of time, the cells were incubated in 1 ml medium containing 20 μCi 35S-methionine/cysteine (Perkin-Elmer) per well for 30 min at 37 °C and 5% CO2. The labeling medium was prepared using glucose-free DMEM without cystine and methionine (BioSera) but was otherwise identical in composition to the glucose deprivation medium. After pulse-labeling, the cells were washed with PBS and lysed with 200 μl RIPA buffer. Twenty microliters of lysate was applied to Whatman 3MM paper blocked with 0.1% methionine and 0.1% cysteine, treated with 10% trichloroacetic acid (TCA) for 20 min on ice, boiled in 5% TCA for 15 min, washed once with 5% TCA, washed once with 96% ethanol, and allowed to dry. 35S incorporation was measured by liquid scintillation counting using a Tri-Carb 2800TR instrument (Beckman Coulter) and Optiphase HiSafe 3 scintillation cocktail (Perkin-Elmer). For each sample, the number of disintegrations per minute was normalized to the total protein content of the cell lysate, which was determined using the BCA protein assay kit (Pierce).

2.9. ATP quantification

ATP levels were measured using the luciferase-based ATP Bioluminescence Assay Kit CLS II (Roche) as described previously [10].

2.10. Differential interference contrast microscopy

Cells were transfected with siRNAs and deprived of glucose, as described above. Cells were incubated on poly-L-lysine-coated tissue culture plates and visualized directly on the tissue culture plates by differential interference contrast microscopy using an Olympus IX81 inverted microscope with a 10× objective. Images of the cells were acquired with an ORCA-ER CCD digital camera (Hamamatsu).

2.11. Statistical analysis

Data are presented as the mean ± SD, where applicable, and the number of independent experiments performed is stated in the figure legends. Statistical significance of gene ontology term enrichment in gene lists was evaluated using the default settings of the analysis tool [41]. Other statistical analyses were conducted using R software (version 3.0.2; The R Foundation for Statistical Computing) and the statistical methods used for each analysis are stated in the figure legends. One-way ANOVA and two-tailed t tests were used to compare group means, and P values were corrected for multiple testing using the Holm–Bonferroni method. For all analyses, P < 0.05 was considered statistically significant.

3. Results

3.1. TRIB3 is transcriptionally induced in response to glucose deprivation

To characterize the regulation of TRIB3 gene expression in response to glucose deprivation, we incubated T-REx-293 cells (HEK293 cells expressing Tet repressor) in glucose-free growth medium and quantified TRIB3 mRNA and pre-mRNA abundance using real-time PCR. The results show that the TRIB3 mRNA level is rapidly induced following glucose withdrawal, exhibiting approximately 2-fold upregulation after 3 h of incubation without glucose, and subsequently, TRIB3 expression continues to increase, reaching greater than 6-fold induction after 6 h, compared to cells grown in complete medium (Fig. 1A). Next, we utilized the quantification of unspliced pre-mRNA abundance to study gene transcription rate [43]. As demonstrated in Fig. 1B, the level of TRIB3 unspliced pre-mRNA is substantially upregulated in glucose-starved
cells, and furthermore, the induction of TRIB3 pre-mRNA precedes that of TRIB3 mRNA (Fig. 1A), with pre-mRNA induction reaching approximately 4.5- and 6-fold after 3 and 6 h of glucose deprivation, respectively. Thus, TRIB3 gene expression is promptly activated in response to glucose deficiency by transcriptional regulation.

To elucidate the effects of elevated TRIB3 expression in glucose-deficient conditions, we employed the TRIB3-293 cell line, which is derived from T-REx-293 cells by stable transfection with a Tet-inducible expression vector. As shown in Fig. 1C, the amount of TRIB3 protein is greatly increased in cells that have been deprived of glucose for 24 h, and the addition of Tet into the culture medium further augments the TRIB3 protein level in TRIB3-293 cells. To confirm that Tet does not affect TRIB3 expression by unspecific mechanisms, the control cell line Vector-293, which is derived from T-REx-293 cells by stable transfection with an empty Tet-regulated gene expression vector, was utilized. Vector-293 cells incubated for 24 h in growth medium lacking glucose display an approximately 7-fold increase in TRIB3 mRNA abundance, compared to cells incubated in complete medium, and no significant difference in TRIB3 expression is evident in response to the presence of Tet in complete or glucose-deprived cells (Fig. 1D).

3.2. The PERK–ATF4 axis mediates the upregulation of TRIB3 during glucose starvation

It has been shown that in cells treated with tunicamycin or thapsigargin, pharmacological inducers of ER stress, as well as during arsenite-induced oxidative stress, the TRIB3 gene is activated by the binding of ATF4 to a C/EBP–ATF composite site located in the proximal promoter region [16,44]. To determine if the activation of TRIB3 during glucose deprivation involves ATF4, we analyzed ATF4 expression in glucose-deprived T-REx-293 cells and performed ATF4 silencing experiments. As shown in Fig. 2A, the ATF4 protein level is greatly increased in cells subjected to glucose starvation for 8 or 24 h, and the transfection of siRNA targeting ATF4 substantially reduces the abundance of ATF4 protein in glucose-deprived cells. Real-time PCR quantification of ATF4 mRNA expression reveals that ATF4 transcript abundance is also upregulated in response to glucose withdrawal, and confirms the knockdown of ATF4 by siRNA (Fig. 2B). Importantly, the silencing of ATF4 expression leads to significantly decreased TRIB3 mRNA (Fig. 2C) as well as TRIB3 unspliced precursor mRNA (Fig. 2D) levels in glucose-starved cells, indicating that ATF4 participates in the transcriptional activation of TRIB3 in response to glucose deficiency.

ATF4 protein production is controlled by the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which can be carried out by four stress-sensing kinases (PERK, GCN2, PKR and HRI) that each recognizes a different set of stress conditions, forming a converging pathway termed the integrated stress response [45]. The characteristic activator of PERK is ER stress [45], and since ER stress can occur as a consequence of glucose deficiency [3], we wondered whether this was the mechanism leading to the induction of ATF4 and TRIB3 in glucose-deprived cells. It has been previously shown that the overexpression of BiP (GRP78, HSPA5), a major ER chaperone protein, is able to alleviate pharmacologically induced ER stress [46]. As depicted in Fig. 2E, BiP overexpression in T-REx-293 cells suppresses the upregulation of the ATF4 protein level in response to glucose withdrawal as well as in response to treatment with ER stress inducer tunicamycin (positive control), implicating ER stress in the glucose deprivation-induced upregulation of ATF4. To elucidate whether PERK activation is the signal leading to ATF4 induction in glucose-deprived cells, we utilized the highly selective PERK inhibitor GSK2606414 [47]. The results reveal that treatment with PERK inhibitor substantially reduces the ATF4
protein level in glucose-deprived cells as well as in tunicamycin-treated cells (Fig. 2F). At the same time, ATF4 protein induction in response to essential amino acid deprivation (specifically, methionine deprivation), which is known to be mediated by the eIF2α kinase GCN2 [45], is unaffected by the presence of PERK inhibitor (Fig. 2F). Comparing different doses of PERK inhibitor, a robust impairment of glucose starvation-induced ATF4 upregulation is evident at concentrations as low as 150 nM (Fig. 2G). In agreement with the inhibition of ATF4 induction, treatment with PERK inhibitor completely prevents the increase of TRIB3 mRNA level, as well as the activation of TRIB3 gene transcription.

Fig. 2. TRIB3 induction in glucose-deprived cells is mediated by the activation of the PERK–ATF4 endoplasmic reticulum stress response pathway. (A) ATF4 protein level in T-REx-293 cells transfected with siRNA targeting ATF4 or non-targeting negative control (NC) siRNA and incubated in either glucose-free growth medium (Glc−) or complete medium (Glc+) for the indicated amount of time. The immunoblot presented is representative of three independent experiments. (B–D) RT-qPCR quantification of ATF4 mRNA (B), TRIB3 mRNA (C) and TRIB3 unspliced pre-mRNA (D) levels in T-REx-293 cells transfected with ATF4 siRNA or NC siRNA and incubated in growth medium with (+) or without (−) glucose for 8 h. The means ± SD from three independent experiments are presented relative to the expression levels in NC siRNA-transfected cells grown in glucose-containing medium. (E) Effect of BiP overexpression on ATF4 protein abundance in T-REx-293 cells incubated in either glucose-containing control medium (Glc+) or glucose-free medium (Glc−) or glucose-containing medium supplemented with the ER stress inducer tunicamycin (Tun; 2.5 μg/ml). Prior to the experimental treatment, the cells were transiently transfected with either the expression plasmid for human BiP (BiP-pCG) or the corresponding empty plasmid vector. The immunoblot shown is representative of two independent experiments. (F) ATF4 protein level in T-REx-293 cells subjected to stressful growth conditions in the absence (−) or presence (+) of 0.8 μM PERK inhibitor GSK2606414. The growth medium compositions designated Glc+, Glc− and Tun are as in panel E, and Met− indicates glucose-containing growth medium lacking the essential amino acid methionine. The results presented are representative of two independent experiments. In panels F and G, gel stained with Coomassie Brilliant Blue is shown below the ATF4 immunoblot to confirm equal protein loading. (G) Efficacy of different concentrations of PERK inhibitor to suppress the glucose deprivation-induced upregulation of ATF4 protein level. The results shown are representative of two independent experiments. (H and I) TRIB3 mRNA (H) and TRIB3 unspliced pre-mRNA (I) expression levels in T-REx-293 cells that were either treated with PERK inhibitor or mock-treated and incubated in either control medium, glucose-free medium or growth medium supplemented with 2.5 μg/ml tunicamycin. The expression analyses were performed using RT-qPCR and the mean expression levels ± SD from three independent experiments are presented relative to the levels in control medium-grown cells without PERK inhibitor treatment. *p < 0.05 comparing NC siRNA- and ATF4 siRNA-transfected samples (B–D) or PERK inhibitor-treated and -untreated samples (H, I) in matching growth conditions (two-tailed t test followed by Holm–Bonferroni correction).
(as measured by the abundance of TRIB3 unspliced pre-mRNA), in cells subjected to glucose starvation or tunicamycin treatment (Fig. 2H and I). Thus, the induction of TRIB3 in glucose-deprived T-REx-293 cells is mediated by the ER stress sensor kinase PERK via the upregulation of its downstream transcriptional effector, the transcription factor ATF4.

3.3. TRIB3 expression increases the survival of glucose-deprived cells

Since TRIB3 mRNA and protein were upregulated by glucose deficiency, we proceeded to determine the influence of TRIB3 on cell survival. Tet-treated and -untreated TRIB3-293 and Vector-293 cells were deprived of glucose, and the trypan blue dye exclusion method was used to monitor cell culture viability. After 24 h in glucose-free medium, the incidence of cell death is low and approximately equal in all of the different cell populations (Fig. 3A). However, as the duration of the stress increases, elevated TRIB3 expression is able to promote cell survival: starting from 48 h, Tet-treated TRIB3-293 cells display significantly less cell death compared to TRIB3-293 cells without Tet, with the difference between Tet-treated and -untreated populations reaching nearly 2-fold at 72 h (36% and 63% cell death, respectively) (Fig. 3A). Results from the Vector-293 control cell line verify that the addition of Tet does not affect sensitivity to glucose starvation by unspecific mechanisms (Fig. 3A).

To determine the effect of endogenous TRIB3 on glucose-deprived cell viability, siRNA knockdown experiments were performed in T-REx-293 cells. At the onset of glucose starvation (0 h), there is no measurable difference in cell death between cultures transfected with siRNA targeting TRIB3 and cultures transfected with non-targeting negative control siRNA (Fig. 3B). During glucose deprivation, however, TRIB3 knockdown leads to a significant increase in cell death after 48, 72 and 96 h in glucose-free medium, compared to control siRNA transfection (Fig. 3B). Verification of TRIB3 knockdown was carried out with Western blotting and real-time PCR. The results, presented in Fig. 3C and D, demonstrate that TRIB3 siRNA reduces the TRIB3 protein and mRNA levels, respectively, in glucose-deficient cells. Thus, in HEK293-derived cells suffering from glucose deprivation stress, elevated TRIB3 expression increases resistance to cell death.

TRIB3 has been reported to interact with the protein kinase Akt and block its activation [48]. In certain contexts, Akt activity increases sensitivity to glucose withdrawal, as revealed by cell viability analysis of glioblastoma cells expressing constitutively active Akt [49]. Therefore, we studied Akt activation (phosphorylation at positions Thr308 and Ser473) in Tet-treated and -untreated TRIB3-293 cells at different timepoints during glucose starvation. As demonstrated by the immunoblots presented in Supplementary Fig. S1A, the phosphorylation of Akt at Thr308 as well as Ser473 is similar in glucose-deprived cells with and without Tet-induced TRIB3 expression. Moreover, treatment with the PI3K-Akt pathway inhibitor wortmannin does not influence the incidence of glucose deprivation-induced cell death in either Tet-treated or -untreated TRIB3-293 cells (Supplementary Fig. S1B). The intracellular ATP level can be influenced by Akt [50] as well as glucose deficiency [3]. As shown in Supplementary Fig. S1C, Tet-induced TRIB3 expression does not alter the ATP level in complete medium-grown TRIB3-293 cells. As a result of 24 h glucose deprivation, the ATP level in Tet-treated as well as -untreated TRIB3-293 cells decreases approximately 4.5-fold, indicating the occurrence of severe bioenergetic stress and a lack of effect of elevated TRIB3 level on the intracellular ATP level (Supplementary Fig. S1C). Taken together, these data suggest that the effect of TRIB3 on cell survival during glucose starvation is not mediated by Akt.

![Fig. 3. TRIB3 enhances the viability of cells subjected to glucose starvation. (A) Cell death in glucose-deprived TRIB3-293 and Vector-293 cell cultures treated with tetracycline (Tet+) or left tetracycline-untreated (Tet−). After the indicated amount of time in growth medium lacking glucose, cell culture viability was determined by the trypan blue exclusion method. The mean cell death percentage ± SD from eight (in the case of 48 and 72 h time-points) or four (24 h time-point) independent experiments is presented. (B) Glucose deprivation-induced cell death in T-REx-293 cells transfected with siRNA targeting TRIB3 or non-targeting negative control (NC) siRNA. The incidence of cell death was quantified as in A, and the data presented is the mean ± SD from four independent experiments. Transfection was performed in IMDM supplemented with 10% FCS and the concentration of siRNA was 60 nM. (C) Confirmation of TRIB3 knockdown at the protein level by immunoblot analysis of NC or TRIB3 siRNA-transfected T-REx-293 cells starved of glucose for 24 h. Cells were transfected as in B. The results are representative of three independent experiments. (D) RT-qPCR quantification of TRIB3 knockdown efficiency T-REx-293 cells during glucose deprivation. The level of TRIB3 mRNA expression was analyzed in cells transfected with NC or TRIB3 siRNA and incubated in growth medium lacking glucose for 24 h. The mean expression level ± SD from three independent transfection experiments is shown (presented relative to the level in NC siRNA-transfected cells). *P < 0.05 comparing Tet-treated and -untreated TRIB3-293 samples (A) or NC siRNA- and TRIB3 siRNA-transfected samples (B, D) at the indicated time-point (two-tailed t test followed by Holm–Bonferroni correction).]
3.4. Genome-wide transcriptional response to glucose deprivation in HEK293-derived cells

TRIB3 is present in the nucleus and has been shown to interact with several diverse transcription factors [9]. Therefore, we sought to investigate the influence of TRIB3 on the transcriptional response to glucose deprivation by performing genome-wide gene expression profiling of TRIB3-293 and Vector-293 cells that were incubated in either glucose-free medium or complete medium, either in the presence or absence of Tet.

In order to provide context for the effects of TRIB3, we set out by evaluating the landscape of transcriptional changes that occur in glucose-starved HEK293-derived cells. Overall, approximately 19,000 probes yielded detectable signals from a total of more than 47,000 different gene probes on the microarray. Based on microarray samples without Tet-induced TRIB3 expression (Vector-293 and Tet-untreated TRIB3-293), the effect of glucose deprivation was calculated for all detectable probes. The results reveal that TRIB3 (endogenous) ranks as the most highly regulated gene in response to 24 h of glucose deprivation, displaying an upregulation of more than 10-fold (Fig. 4A; the microarray probe for TRIB3 is limited to detecting endogenous TRIB3 mRNA by targeting the 3′-UTR). In total, approximately 300 gene probes are induced and 270 are repressed in response to the glucose deficiency, using a cut-off of 2-fold change (Supplementary Tables S1 and S2, respectively).

To functionally characterize the transcriptome-level response to glucose starvation, we performed Gene Ontology term enrichment analysis. As shown in Supplementary Table S3, glucose deprivation-induced genes are significantly enriched for ER stress response and amino acid metabolism genes, in agreement with reports that glucose deprivation leads to ER stress and the activation of the ATF4-mediated amino acid/nutrient-sensing response [51,52]. Meanwhile, glucose starvation-repressed genes are significantly enriched for cell cycle progression-associated genes (Supplementary Table S3), which is consistent with the cessation of cell proliferation in glucose-free medium.

To characterize the regulatory mechanisms behind the glucose starvation-associated gene expression patterns, we performed transcription factor binding motif enrichment analysis using iRegulon software [42]. The results reveal that in glucose deprivation-upregulated genes, the greatest over-representation occurs for the binding motif shared by ATF and C/EBP transcription factors, and predict that members of these families are major direct activators of transcription in the glucose deprivation response (Supplementary Table S4). In the genes that are downregulated in response to glucose starvation, the most over-represented putative regulatory motifs are the binding motifs for E2F transcription factors (Supplementary Table S5), a family of regulators that is known to have a crucial role in the control of cell cycle progression [53].

3.5. Glucose deprivation-regulated genes that are downregulated by TRIB3

To uncover the effect of TRIB3 on glucose deprivation-regulated genes, a series of filtering steps, depicted in Fig. 4B, were performed. For all genes regulated by glucose deprivation (Supplementary Tables S1 and S2), the effect of TRIB3 overexpression on gene expression level in glucose-deprived TRIB3-293 cells was assessed, and gene probes demonstrating at least 1.5-fold difference between Tet-treated and -untreated cells were selected. According to this analysis, approximately 8% of glucose deprivation-regulated gene probes are affected by TRIB3 in glucose-starved cells. Among the probes upregulated by glucose deprivation, 1 probe (1 gene) is further upregulated by Tet-induced TRIB3, while 20 probes (16 genes) are downregulated by exogenous TRIB3 (Fig. 4B). Conversely, among the probes downregulated by glucose deprivation, 3 probes (3 genes) are further downregulated by Tet-induced TRIB3, while 22 probes (19 genes) are upregulated by exogenous TRIB3 (Fig. 4B). These results indicate a trend towards the alleviation of glucose deprivation-induced transcriptional changes by increased TRIB3 expression.

We have previously shown that TRIB3 inhibits the activity of ATF4 [54], a central activator of the transcriptional control of amino acid availability [55]. In line with this, the current analysis identifies several genes involved in amino acid biosynthesis and transport (ASS1, ASNS, CTH, CBS, and SLC1A4) among the genes repressed by TRIB3 (Table 1). An over-representation of genes related to amino acid metabolism is also the main result of functional profiling of TRIB3-downregulated genes (Supplementary Table S6), and furthermore, transcription factor motif analysis indicates that the inhibition of ATF4 may be the predominant mechanism of TRIB3-mediated gene repression, as 84% of TRIB3-downregulated genes are predicted to be ATF4 target genes (Supplementary Table S7). It has been found that during ER stress, ATF4 activity...
induces cell death by leading to increased total protein synthesis [56]. Since we observed the activation of the ER stress response in glucose deprivation gene expression profile (Supplementary Table S3), we investigated whether TRIB3 affects total protein synthesis by performing 

Table 1

<table>
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<th>Gene</th>
<th>TRIB3 effect (fold)</th>
<th>Glc – effect (fold)</th>
<th>Definition</th>
<th>Annotation*</th>
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* Difference in gene expression between tetracycline (Tet)–induced and -uninduced TRIB3–293 cells incubated in glucose-free growth medium for 24 h. Gene probes regulated at least 1.5–2-fold by Tet-induced TRIB3 expression and at least 2-fold by glucose deprivation (in samples without Tet-induced TRIB3 expression) are presented.

** The effect of 24 h glucose deprivation (Glc–) on gene expression in the absence or presence of Tet (Tet− and Tet+, respectively) in TRIB3–293 cells.

* Annotation terms from the Gene Ontology database.

In addition to amino acid metabolism genes, TRIB3-repressed genes include several other metabolic enzymes, including FUT1, PM20D2, PK2, MTHFD2 and PM20D2, as well as several factors involved in signaling processes, including INHBE, SH3BGRL, TAC1 and CALCB (Table 1). The latter group includes the gene most substantially influenced by TRIB3, the TGF-β superfamily member INHBE, which is inhibited nearly 3-fold by Tet-induced TRIB3 during glucose starvation (Table 1).

3.6. Glucose deprivation-regulated genes that are upregulated by TRIB3

The genes upregulated by elevated TRIB3 expression in glucose-starved cells belong to diverse functional classes, including several genes involved in energy metabolism (such as PKM2, PGAM4, UC2, FABP5, and SLC6A8) and several factors involved in signaling processes (such as IGFBP2, BSG, and LY6E) (Table 1). PKM2 (which encodes pyruvate kinase isozyme M2) is the most highly glucose deprivation-downregulated gene in Tet-untreated TRIB3–293 cells, nearly 9-fold,
which is reduced to 3.8-fold repression in the presence of Tet-induced TRIB3 (Table 1). Among glucose deprivation–inhibited genes, the impact of TRIB3 is most prominent towards the level of IGFBP2 — an insulin-like growth factor binding protein that is linked to cancer progression [18] — which is repressed 4.3-fold by glucose deprivation in TRIB3-293 cells without Tet treatment and merely 1.8-fold when Tet is present (Table 1). DNA motif enrichment analysis for TRIB3–upregulated genes suggests several transcription factors as direct regulators that each possess approximately one-third of the gene set as predicted targets, with the transcription factors Pax6, Bcl6 and Hsf1 displaying the highest enrichment scores (Supplementary Table S8).

In our gene expression profiling experiments, we did not detect the influence of TRIB3 on genes that are central to the initiation and execution of cell death programs (such as Bcl-2 family members, death receptors, or caspases). The results obtained from genome-wide gene expression profiling show that the induction of TRIB3 is among the most significant gene expression changes that occurs in response to glucose starvation and reveals a subset of glucose deprivation–regulated genes that are modulated by TRIB3.

3.7. IGFBP2 mRNA and protein levels decrease in response to glucose deprivation

From the TRIB3–regulated genes revealed by expression profiling, we decided to study IGFBP2 further because of its significant clinical associations (see the Introduction section) and its relatively substantial TRIB3–dependence according to our microarray data. To determine the time course of IGFBP2 mRNA expression regulation in response to glucose deprivation, T-REx–293 cells were incubated in glucose-free growth medium for up to 72 h and the level of IGFBP2 mRNA was quantified by real-time PCR. As depicted in Fig. 5A, IGFBP2 mRNA abundance after 3 h of glucose starvation is unchanged from 0 h, however, after 6 h in medium lacking glucose, the level of IGFBP2 mRNA is decreased 2-fold compared to the level in complete medium–incubated cells. At the 12 h and 24 h time-points, IGFBP2 mRNA abundance remains relatively unchanged compared to 6 h, remaining at approximately 50% of its non-starved level (Fig. 5A). After 48 and 72 h without glucose, IGFBP2 mRNA expression level is more than 5-fold below the level in complete medium (Fig. 5A). The repression of IGFBP2 in response to glucose deprivation is also detectable at the protein level, as demonstrated in Fig. 5B.

3.8. Reduced IGFBP2 expression aggravates cell death during glucose starvation

Having determined that IGFBP2 is robustly downregulated in response to glucose deprivation, we decided to investigate whether IGFBP2 influences the viability of cells suffering from glucose deficiency. For this purpose, we transfected T-REx–293 cells with siRNA targeting IGFBP2 or a negative control siRNA, and monitored cell death in cultures with either glucose-free or complete growth medium. The results reveal that before the onset of glucose deprivation, the viability of IGFBP2 knockdown cells does not differ from that of control siRNA-transfected cells, whereas after 72 h of glucose deprivation, the incidence of cell death is significantly higher in cells transfected with IGFBP2 siRNA (52%) than in control siRNA-transfected cells (27%), and a similar effect is apparent at 96 h (Fig. 6A). The knockdown of IGFBP2 was confirmed at the mRNA and protein levels, as shown in Figs. 6B and 5B, respectively.

To examine whether IGFBP2 overexpression leads to increased cell survival during glucose starvation, T-REx–293 cells were transfected with different amounts of IGFBP2 expression plasmid (using empty vector to equalize the total amount of DNA transfection) and cell viability was monitored during incubation in glucose-free medium. After 72 h of glucose deprivation, cells that received only empty vector exhibit 48% cell death, while cells transfected with varying amounts of IGFBP2 expression construct display 27–37% cell death, depending on the amount of expression plasmid transfected, representing a significant decrease in cell death as a result of IGFBP2 overexpression (Fig. 6C). Similar results are obtained for cells deprived of glucose for 96 h, and at both time-points, transfecting very high amounts of IGFBP2 expression plasmid tends to lessen the pro-survival effect compared to moderate doses (Fig. 6C). Thus, the level of endogenous IGFBP2 significantly contributes to the survival of glucose-deprived T-REx–293 cells, and in agreement with this, IGFBP2 overexpression is able to prolong cell survival.

3.9. Elevated TRIB3 expression increases the level of IGFBP2 in glucose-deprived cells

Since we established that IGFBP2 is a determinant of cell survival during glucose deficiency and our gene expression microarray results indicated that TRIB3 affects IGFBP2 mRNA level in glucose-deprived cells, we decided to perform further experiments to examine the regulation of IGFBP2 expression by TRIB3. Western blotting was used to study IGFBP2 protein levels in TRIB3–293 and Vector–293 cells treated with Tet or left Tet-untreated. The results, presented in Fig. 7A, demonstrate that Tet treatment does not significantly alter the level of IGFBP2 protein expression in TRIB3–293 cells in the presence of glucose or after 12 h of glucose deprivation, however, after being deprived of glucose for 48 h, the abundance of IGFBP2 protein in TRIB3–293 cells is substantially greater in Tet-treated cells compared to cells not treated with Tet. Importantly, in Vector–293 cells subjected to glucose starvation for 48 h, the presence of Tet does not lead to a rise in IGFBP2 expression compared to cells without Tet (Fig. 7A), indicating that increased IGFBP2 level in TRIB3–293 cells is due to elevated TRIB3 expression and not caused by unspecific effects of Tet. Using real-time PCR, the level of IGFBP2 mRNA expression was quantified in TRIB3–293 and Vector–293 cells treated with Tet or left Tet-untreated and incubated in either glucose-free or complete medium. In the presence of glucose, Tet has no significant effect on IGFBP2 mRNA expression in either TRIB3–293 or Vector–293 cells (Fig. 7B and C). After approximately one day of glucose deprivation, IGFBP2 mRNA abundance declines approximately

Fig. 5. Glucose deprivation represses the expression of IGFBP2. (A) IGFBP2 mRNA expression level in T-REx–293 cells deprived of glucose for the indicated amount of time. For each time-point, mRNA expression was quantified by RT-qPCR, and the mean ± SD from two independent experiments is presented relative to the average expression level at the start of glucose starvation (0 h). (B) IGFBP2 protein level after 0 and 36 h of glucose deprivation in T-REx–293 cells transfected with either siRNA targeting IGFBP2 or non-targeting negative control (NC) siRNA, as indicated above the immunoblot. *P < 0.05 compared to 0 h glucose deprivation samples (one-way ANOVA followed by two-tailed comparisons of all time-points to 0 h, with correction for multiple testing using the Holm–Bonferroni method).
2.5-fold in Vector-293 cells as well as in Tet-untreated TRIB3-293 cells, however, IGFBP2 mRNA expression in Tet-treated TRIB3-293 cells is approximately 2-fold greater than in Tet-untreated TRIB3-293 cells (Fig. 7B and C). Thus, the elevation of IGFBP2 protein level in glucose-starved cells by increased TRIB3 expression involves the upregulation of IGFBP2 mRNA.

3.10. The pro-survival effect of TRIB3 is IGFBP2-dependent

To shed light on the relationship between TRIB3 and IGFBP2 in the determination of cell survival, we concurrently manipulated the levels of TRIB3 and IGFBP2, and tracked cell viability during glucose deficiency. In the first experiment, TRIB3-293 cells were transfected with either IGFBP2 siRNA or negative control siRNA, and either treated with Tet to induce TRIB3 expression or left Tet-untreated. In cells transfected with negative control siRNA, 72 h of glucose starvation resulted in significantly less cell death in Tet-treated cells, compared to Tet-untreated cells (Fig. 8A), which is in line with the pro-survival effect of TRIB3 demonstrated in Fig. 3A. At the same time, in cells transfected with IGFBP2 siRNA, the presence of Tet does not affect cell viability, and the percentage of cell death in both Tet-treated and -untreated cells is greater than in negative control siRNA-transfected cells (Fig. 8A). Thus, in cells with diminished IGFBP2 expression, TRIB3 overexpression is unable to convey enhanced viability.

In order to investigate the interaction of endogenously expressed TRIB3 and IGFBP2 on the viability of glucose-deprived cells, we transfected T-REx-293 cells with combinations of negative control siRNA, TRIB3 siRNA and IGFBP2 siRNA, and monitored cell survival and morphology. For each combination of siRNAs, the total amount of transfected siRNA was equalized by adjusting the amount of negative control siRNA. As shown in Fig. 5B, cell death in cultures with individual or concurrent knockdown of TRIB3 and IGFBP2 is similar at the start of glucose deprivation (0 h). After 72 h of incubation in glucose-free medium, the percentage of cell death is approximately 2-fold greater in cells transfected with TRIB3 or IGFBP2 siRNA, compared to cells transfected with negative control siRNA alone, and importantly, the simultaneous knockdown of TRIB3 and IGFBP2 does not further impair cell survival compared to the knockdown of the genes individually (Fig. 8C). When grown in complete medium, the knockdown of TRIB3 and/or IGFBP2 does not grossly alter the morphology of T-REx-293 cells, with the cells of all siRNA combinations displaying a similar appearance (attached to substrate, polygonal in shape) after 48 h, as determined by differential interference contrast microscopy (Fig. 8D). The withdrawal of glucose for 72 h causes the morphology of control siRNA-transfected cells to become more elongated in shape, and while some rounded and detached cells are also present among the cells transfected with control siRNA, cell rounding and detachment is much more prevalent among the cells subjected to individual or simultaneous knockdown of TRIB3 and IGFBP2 (Fig. 8D). Taken together, these results demonstrate that TRIB3-mediated repression of glucose deprivation-induced cell death is dependent on IGFBP2 expression.

4. Discussion

In the interior of solid tumors, cells suffer from glucose deficiency as a result of insufficient nutrient supply due to poorly developed vasculature, excessive proliferation and metabolic abnormalities [1,2,57]. For instance, in human colon tumors, the glucose concentration has been found to be on average 10-fold lower than in normal colon tissue, while the levels of free amino acids are unchanged or even moderately elevated in the tumor tissue [2]. An increased demand for glucose and a metabolic switch to aerobic glycolysis are considered hallmarks of malignant cells [58], and pharmacologically inhibiting glycolysis, effectively starving cancer cells of glucose, presents a promising therapeutic
Glucose deprivation indeed results in substantially increased ATF4 protein level, which in turn leads to the induction of TRIB3 in glucose-deprived cells. Furthermore, we uncovered that the upregulation of ATF4 in response to glucose starvation requires the activity of the ER stress sensor kinase PERK, indicating that ER stress is the factor which evokes the upregulation of ATF4 and TRIB3 in cells suffering from glucose deficiency. Intriguingly, in cell cultures, TRIB3 is also upregulated in response to hypoxia [4,7], which generally accompanies glucose deficiency in the intra-tumor microenvironment [57]. Therefore, the overexpression of TRIB3 in tumors [4,5] may be a convergent response to multiple stressful environmental factors.

By performing silencing of endogenous TRIB3 expression as well as TRIB3 overexpression experiments, we uncovered that elevated TRIB3 expression in glucose-deficient conditions conveys a substantial survival advantage in HEK293-derived cells. The increased nutritional stress resistance afforded by TRIB3 expression could underlie the association between high tumor TRIB3 expression level and poor prognosis in colorectal cancer patients [6], and may also have implications for the variety of other tumor types that have been found to overexpress TRIB3 compared to normal tissue [4,5]. Cell death rescue mechanisms of “glucose-addicted” cells subjected to glucose deprivation may also be relevant in the context of cancer therapies that aim to inhibit glycolysis, a field that is currently actively investigated [3]. To clarify how TRIB3 augments glucose-deprived cell survival, we examined the effect of elevated TRIB3 expression on the transcriptional response to glucose starvation using genome-wide gene expression profiling. Our analysis revealed a diverse set of approximately 40 genes that are regulated by glucose deprivation and further regulated by TRIB3 during glucose starvation. The fraction of TRIB3-sensitive genes among glucose deprivation-sensitive genes is less than 10%, indicating that TRIB3 is not a broad regulator of the transcriptional response to glucose starvation but rather it modulates the expression levels of a specific group of genes, a large portion of which are controlled by ATF4.

In our microarray experiment, one of the genes downregulated in response to glucose starvation is IGFBP2, and further experiments confirmed the repression of IGFBP2 in glucose-deprived cells at both the mRNA and protein level. The molecular mechanisms that underlie the regulation of IGFBP2 abundance by TRIB3 are currently unclear. Our analysis of transcription factor motifs in the subset of glucose deprivation-regulated genes that are upregulated by TRIB3 revealed BCL6, a transcriptional repressor [60], as one of the most highly enriched transcription factors, and predicted that IGFBP2 is a BCL6 target gene. Interestingly, a mass spectrometry-based search for BCL6-interacting proteins has found that TRIB3 forms a complex with BCL6 [61]. Several other transcription factors are known to bind to TRIB3, leading to the inhibition of their activity [16,54,62,63]. It is currently not known whether and how the formation of a complex between BCL6 and TRIB3 affects the transcriptional properties of BCL6, and whether an interaction with TRIB3 could lead to the de-repression of BCL6 target genes. Further studies using the silencing and overexpression of BCL6 are necessary in order to elucidate the possible role of BCL6 in the regulation of IGFBP2 expression and to characterize the effect of BCL6 on cell viability during glucose starvation. Additionally, our regulatory motif enrichment analysis implicated several other transcription factors in glucose deficiency-induced gene expression regulation, and the further characterization of their roles is imperative to increasing our understanding of the transcriptional control of the stress response to glucose deprivation.

Previous research has revealed that IGFBP2 has remarkably diverse modes of action (e.g. endocrine, paracrine, and intracellular/nuclear; IGF receptor-dependent and –independent) [18] and, in different biological contexts, IGFBP2 is able to either suppress [20,64] or facilitate [65] cell death. In the case of glucose starvation, our results show that endogenously produced IGFBP2 is a substantial positive modulator of
cell survival. Therefore, the marked downregulation of IGFBP2 expression in response to glucose deficiency represents a mechanism for evoking stress-induced cell death. Intriguingly, our results revealed that increased expression of TRIB3 is able to alleviate the repression of IGFBP2 during glucose deprivation, thereby providing a mechanism to defer cell death. Colorectal and breast tumors have been shown to overexpress IGFBP2 as well as TRIB3 [6,7,22,23], raising the possibility that, similar to our results from cells subjected to glucose deprivation, high TRIB3 expression in these types of tumors may contribute towards the observed overexpression of IGFBP2, which could support malignant cell survival. The mechanism employed by IGFBP2 protein, upregulated by TRIB3, to increase the viability of glucose-deprived cells is currently obscure. IGFBP2 has been found to downregulate the level of procaspase-3 protein and caspase-3 activity, and in this way to contribute to the inhibition of apoptosis [20]. Interestingly, Shimizu et al. [14] have recently reported that TRIB3 prevents cytoplasmic activation of caspase-3 by promoting procaspase-3 entry into the nucleus, thereby inhibiting apoptosis. Thus, the characterization of TRIB3 and IGFBP2 effects on (pro)caspase-3 level, location and activity may uncover an additional level of association between TRIB3 and IGFBP2 in deferring the instigation of cell death in glucose-starved cells.

In conclusion, we have shown that TRIB3 is highly induced in response to glucose deprivation in cells of the glycolysis-inclined HEK293 lineage, and that increased expression of TRIB3 enhances cell viability in glucose-deficient conditions. Further, we established that IGFBP2 is a positive modulator of glucose-deprived cell survival that undergoes marked downregulation during glucose deficiency. Elevated expression of TRIB3 is able to augment IGFBP2 level in cells suffering from glucose starvation, and the silencing of IGFBP2 abolishes the prosurvival effect of TRIB3, implicating IGFBP2 in the mechanism of TRIB3-mediated cell survival.

Competing interests

The authors declare that they have no conflicts of interest related to the research reported.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

We thank Margus Leppik, Dmitri Lubenets, and Raivo Kolde for advice regarding radioisotope procedures, microscopy, and microarray data analysis, respectively. We are grateful to Kersti Lillevali, Kristina Mäemets-ALLAS and Viljar Jaks for the gift of antibodies. This work was...
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