INHIBITION OF GLYCOLYTIC ENZYMES MEDIATED BY PHARMACOLOGICALLY ACTIVATED p53: TARGETING WARBURG EFFECT TO FIGHT CANCER
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Background: High dependence of cancer cells on glycolysis is a good target for cancer therapy

Result: Tumor suppressor p53 represses the expression of key regulators of metabolic genes HIF1A, c-Myc, and glucose transporters GLUT1 and GLUT12

Conclusion: Blocking ATP production network by pharmacologically activated p53 contributes to cancer cell death

Significance: Tumor-selective killing by reconstituted p53 might be in part due to inhibition of glycolysis

Unique sensitivity of tumor cells to the inhibition of glycolysis makes it a good target for anticancer therapy. Here, we demonstrate that the pharmacologically activated tumor suppressor p53 mediates the ablation of several glycolytic genes in normoxia, whereas in hypoxia downregulation of HIF1α contributed to this effect. We identified Sp1 as a transcriptional cofactor cooperating with p53 in the ablation of metabolic genes. Using different approaches, we demonstrated that glycolysis block contributes to the robust induction of apoptosis by p53 in cancer cells. Taken together, our data suggest that tumor-specific reinstatement of p53 function targets “Achilles hill” of cancer cells, i.e., their dependence on glycolysis, which could contribute to the tumor-selective killing of cancer cells by pharmacologically activated p53.

The metabolism of most solid tumors is significantly different from that of surrounding normal tissues, which derive their energy from the oxidative phosphorylation. In contrast, increased aerobic glycolysis occurs in a wide spectrum of human cancers and is considered as one of the most fundamental alterations during malignant transformation (1). High dependence of cancer cells on glycolysis for ATP production in the presence of oxygen, known as the Warburg effect (2), is recognized as the 7th hallmark of cancer (3).

High glucose uptake exploited in cancer diagnosis and monitoring of treatment using the glucose analogue tracer 18fluorodeoxyglucose (FdG) and positron...
emission tomography (PET) (4,5) occurs due to the overexpression of glucose transporters, especially the glucose transporter isoform 1 (GLUT1) (6). Upon uptake, glucose molecules are irreversibly phosphorylated by hexokinases 1 and 2 (HK1 and 2), also overexpressed in cancers. (7). Key glycolytic enzymes acting downstream of hexokinase include PFKFB3, PFK1, pyruvate dehydrogenase (PDH) and pyruvate dehydrogenase kinase (PDK) (2C).

The oncogenic networks, such as PI3K/Akt, c-Myc and HIF1 influence the metabolic shift during cancerogenesis and support growth and proliferation of cancer cells under metabolic stress and hypoxia. HIF1, a transcription factor (8) stabilized under hypoxia (9) triggers the upregulation of genes critical for the switch to glycolysis, including SLC2A1 (GLUT1), HKII, PDK1, PFK1 (10) and lactate dehydrogenase LDHA (11). Transcriptional factor c-Myc, one of the major oncogenes, cooperates with HIF1 in promoting glycolysis by activating PDK1, HKII (12) and LDHA genes (11). Aberrations in PI3K/Akt pathway constitute one of the most common sets of mutations in tumors (13). Enhanced PI3K/Akt signaling results in metabolic transformation via multiple pathways, including increased expression of genes involved in glycolysis and stimulation of HK and PFK activities (11).

Targeting aerobic glycolysis for anticancer treatment is a very promising approach. Several glycolysis inhibitors are in preclinical and clinical development, such as lactate dehydrogenase A inhibitor FX11 (14) or hexokinase inhibitor 2-deoxyglucose (2DG) which is now in clinical trials (15).

p53 is a transcriptional factor which suppresses tumor development by regulating the expression of genes inducing cell cycle arrest, apoptosis and senescence upon stress conditions (16). In order to survive, cancer cells render p53 inactive, either by point mutations (approximately 50% of human cancers) (17), or by increased degradation of wild type p53 due to the deregulation of E3 ubiquitin ligase MDM2 (18). Recently p53 has been implicated in metabolic control by influencing the balance between glycolysis and oxidative phosphorylation. Moreover, p53 inhibits the expression of glucose transporters GLUT1 and GLUT4 (21) indicating that p53 can impede metabolism by reducing glucose import. Additionally, wild-type p53 was shown to downregulate oncogenic phosphoglycerate mutase (PGM) (22). However, p53 involvement in metabolic regulation is rather complex; it may both inhibit and promote tumor growth (23,11). Determining the stimuli that trigger different p53 responses affecting cell metabolism is very important, especially in light of recent development of small molecules reactivating p53 function in cancer cells.

A number of strategies reactivating p53 (24) has been developed over the years. Our group has identified p53-reactivating compound RITA (Reactivation of p53 and Induction of Tumor cell Apoptosis) (25). RITA binds p53 N-terminal domain and disrupts the interaction with its negative regulator MDM2 which results in p53 activation and induction of apoptosis (25,26,27). Notably, we showed that RITA activates p53 in cells expressing oncogenes, whereas the effect in non-transformed cells is almost negligible (25,28). In addition, we found that the response of tumor cells to different doses of RITA (0.1 and 1 µM) was similar in terms of induction of p53 and transcriptional activation of its apoptotic targets, but transcriptional repression of oncogenes c-Myc, PI3K, IGFR, Mcl-1, survivin and others was triggered only by higher dose (28). Oncogene repression correlated with apoptosis induction, indicating that it contributes to cancer cell killing by p53.

In the present study we investigated whether pharmacological reconstitution of p53 can inhibit aerobic glycolysis in cancer cells in vitro and in vivo, using small molecule RITA as a research tool. We report a potent p53-dependent inhibition of the glucose transport and the first steps of glycolysis via the transcriptional repression of key players of these processes.

**EXPERIMENTAL PROCEDURE**

**Cell culture and transfection**

Tumor cells HCT116 (wtp53), MCF7 (wtp53), U2OS (wtp53), HCT116TP53 (p53 null), H1299 (p53 null) and Saos2 (p53 null) were maintained in Iscoves modified
Dulbeccos medium (IMDM) supplemented with 10% fetal calf serum, Penicillin/Streptomycine [10 U/ml] and L-Glutamine [2mM] (all purchased from Sigma Aldrich). The isogenic colon carcinoma cell lines HCT116 (p53 positive) and HCT116 TP53 -/- (p53 null) were provided by B. Vogelstein. siRNA (small interfering RNA) for HIF1α (sc-35561), c-Myc (sc-29226) and HK2 (sc-35621) were purchased from Santa Cruz Biotechnology, and GFP (green fluorescent protein) siRNA, which was used as a control, was purchased from Thermo Scientific Dharmacon. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions using 20 pmol siRNA Drug treatment was performed 32h after transfection.

MCF7 cells stably depleted for Sp1 using Sp1 shRNA lentivirus construct (Sigma) were treated with 1 µM RITA for 8h to detect mRNA levels by qPCR and microarray analysis, or for 48 h to assess survival.

Metabolic chip assay
HCT 116 and its negative counter part HCT 116 p53-null cells were grown on the metabolic chips in DMEM medium supplemented with 10% fetal calf serum, Penicillin/Streptomycine [10 U/ml] and L-Glutamine [2mM] under standard conditions for 24 h. Before analysis the cells were treated with 0.1 or 1 µM RITA reconstituted from the stock (0.1 M in 100% DMSO) for 12 h in a standard cell incubator. Afterwards, the chips were transferred to the Bionas® 2500 analyzing system for 4 days with running medium supplemented only with 2% FCS. Programming of the Bionas® 2500 analyzing system was performed according to the user manual. Just before the measurements started cell morphology was controlled microscopically and photographed for documentation (not shown). After the measurement the cells were killed by addition of 0.2 % Triton X-100 to the running medium (last grey field on the right side). The values from the cells killed after Triton X-100 addition were set to 0 %.

p53 ChIP-seq
Chromatin immuno precipitation (ChIP) library preparation, massive parallel sequencing and ChIP-Seq primary analysis were performed as previously published (29). Data has been archived at NCBI Sequence Read Archive (SRA) under Accession SRP007261. Anti-p53 mouse monoclonal antibody D01 (Genespin and Santa Cruz), and a non-specific Sc-2025 mouse IgG (Santa Cruz) antibodies were used to IP p53 from MCF7 cells treated with p53-activating compounds for 8h. More detailed information on the ChIP-seq experiment and data processing will be provided in the separate paper (Nikulenkov et.al, submitted,30).

Microarrays
Total RNA was isolated with RNEasy Mini kit (Qiagen). cDNA was synthesized with One-Cycle cDNA Synthesis kit from Affymetrix. cRNA was synthesized from cDNA by following the IVT Labeling kit (Affymetrix) and purified with the GeneChip Sample Cleanup Module from Affymetrix. Labeled cRNA was fragmented in fragmentation buffer (5× buffer: 200 mM Tris-acetate (pH 8.1)/500 mM KOAc/150 mM MgOAc) and hybridized to the microarrays in 200μl of hybridization solution containing 10μg labeled target in 1× Mes buffer (0.1 M Mes/1.0 M NaCl/20 mM EDTA 0.01%/Tween20) and 0.1mg/ml herring sperm DNA, 0.5mg/ml BSA, 50 pM control oligonucleotide B2 and 1x eukaryotic hybridization controls (bioB, bioC, bioD, cre). Both control oligonucleotide B2 and eukaryotic hybridization controls were purchased from Affymetrix. Samples were then hybridized on Human Genome U133A 2.0 Arrays or U219 (Affymetrix). The arrays were then stained with a streptavidin-phycoerythrin conjugate (Molecular Probes), followed by 10 washing cycles of 4 mixes/cycle with 6x SSPE-T. To enhance the signals, the arrays were further stained with Anti-streptavidin antibody solution for 10 min at 25°C followed by a 10 min staining with a streptavidin-phycoerythrin conjugate. After 15
washing cycles of 4 mixes/cycle, the arrays were scanned using a confocal scanner (Affymetrix). The image data were analyzed by GCOS 1.4 (GeneChip Operating Software, Affymetrix).

F-match analysis of selected metabolic genes
We performed F-Match analysis (31) using Explain 2.4.1 and geneXplain platform 1.0 software packages (32; www.genexpplain.com) to search for overrepresented transcription factor binding sites in promoter region. We used TRANSFAC® database (34) version 2010.4. As a background set we chose a set of 1000 genes that did not show expression changes after nutlin3a or RITA treatment. The profile that was used for analysis contains collection of vertebrate non-redundant transcription factor matrices. Promoter window was selected from −1000 to +100 from transcription start site (TSS) and only best supported promoters of analysed genes were used. To obtain only binding sites with high score we chose cut-off with p-value threshold of 0.01. Among matrices that were found we selected those with high overrepresentation of the site frequency in promoters under study versus the background promoters (Ratio > 1.3).

Key node analysis
We performed identification of potential master regulators in signal transduction network using Explain 2.4.1 and geneXplain platform 1.0 software packages (www.genexpplain.com). The signal transduction network was provided by the manually curated database, TRANSPATH® (34). The software applies an upstream analysis approach, which is based on implementation of machine learning and graph topological analysis algorithms in order to identify causality key nodes in the network of signal transduction (35). The algorithm starts from a set of transcription factors (found overrepresented in the promoters under study using F-Match tool, see above) and performs a graph-topological search in the signal transduction network upstream of the transcription factors in order to identify “key nodes” – nodes in the network that can play a crucial role in transducing intracellular signalling from various receptors to the considered set of transcription factors. Such key nodes may be considered as master regulators of the process under study.

Animal experiments
All animals studies were approved by the Northern Stockholm Animal Ethical Committee. The animal care was in accordance with Karolinska Institutet guidelines. Male SCID mice, 4–6 weeks old, were implanted subcutaneously with 1x10⁶ HCT116 or HCT116 TP53-/- cells in 90% Matrigel (Becton Dickinson). Palpable tumors were established 7 days after cell injection; at this point, we injected 1 mg/kg RITA in tumors in a total volume of 100 µl phosphate-buffered saline.

Hypoxia and drug treatments
RITA (2,5-bis(5-hydroxymethyl-2-thienyl)-furan) was obtained from the National Cancer Institute (NCI). RITA was dissolved to a concentration of 0.1 M in 100% dimethyl sulfoxide (DMSO). Afterwards, RITA was diluted in PBS (phosphate buffered saline) and used at a final concentration of either 0.1 µM or 1 µM for different time points (indicated in the figures). Physiological hypoxia was achieved by incubating cells in 1% O₂, 5% CO₂ and 94% nitrogen in an In Vivo Hypoxic Workstation 400 (Ruskinn Technology). Cells were put under hypoxic conditions for approximately 18 hours prior to treatment. The hypoxic mimetic agent CoCl₂ (Sigma-Aldrich) was used at a final concentration of 100µM for 24h in combination with 1µM RITA (8h). The glycolysis inhibitor 2-Deoxyglucose (Sigma-Aldrich) was used at a final concentration of 10mM. Cells were pretreated with Deoxyglucose 24 hours prior to RITA treatment (8h) in IMDM 2x diluted in PBS.

Real-time quantitative PCR
Total RNA was extracted and purified with a PerfectPure RNA kit (5 PRIME) using the manufacturer’s protocol. RNA (1 µg) was reverse transcribed using the Super-Script III First-Strand Synthesis SuperMix for quantitative qRT-PCR (Invitrogen). Real-time PCR was performed with SYBR green reagent (Bio-Rad) using 20 ng template in a 15 µl reaction mixture according to the manufacturer’s protocol. Results were normalized to the GAPDH gene.
qRT-PCRs using tumor tissue were based on xenografts-samples obtained from previously conducted experiments (28). RNA from xenografts was purified using PerfectPure RNA Tissue Kit from 5 PRIME according to the manufacturer’s protocol. All primers were designed using the PerlPrimer program (Marshall, 2004) with one primer overlapping an exon boundary. Primer sequences are listed in Supplementary Table 1.

Protein extraction and Western Blot
Cells were washed with ice-cold PBS and soluble proteins were extracted with cell lysis buffer (100mM Tris-HCl pH=8, 150mM NaCl, 1% NP-40, phosphatase and protease inhibitor cocktail tablets (Roche) according to the manufacturer’s protocol). Insoluble material was removed by centrifugation (13000 rpm, 30min). The protein concentration was determined using the Bio-Rad Bradford assay and BSA (bovine serum albumin) standards. An equal amount of protein was separated by SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) using Ready Made Gradient (4-20%) Gels (Bio-Rad). Transfer was performed at 4°C (180mA, 80V) and the following antibodies were used for Western Blotting: p53 (DO-1); HK2 (sc-6521), TIGAR (sc-74577) and c-Myc (sc-764) (all from Santa Cruz Biotechnology); ACL (Cell Signaling, 4332); PUMA (PC686) and Noxa (OP180) (from Calbiochem); HIF1α (BD Biosciences, 610959) and β-Actin (Sigma). The latter was used to verify equal loading. Primary antibody incubation (1:500 dilution in 5% nonfat dry milk in PBS) was overnight (4°C). Secondary HRP-conjugated antibodies (1:3000 dilution in 5% nonfat dry milk in PBS; 1h incubation at room temperature) and Super Signal West Dura Extended Duration Substrate were from Pierce.

Cell viability assays
For colony formation assays, cells were seeded at a density of 30% and treated with RITA for 48 hours. Afterwards, cells on the plate were fixed with 70% ethanol and stained with crystal violet. After staining, the absorbance of the different wells was measured in a microplate reader (Victor X3, Perkin Elmer) at a wavelength of 562nm. The expected additive effect was calculated adding the percentage of dead cells after siGFP combined with 0.1µM RITA to the percentage of dead cells after siHK2 alone.

Statistical analysis
The statistical significance of results was calculated by a parametric Student’s t test (for variances Fisher–Snedecor’s t test was applied and the normality was assessed with the Shapiro–Wilk’s test). p < 0.05 values were considered statistically significant. The statistical significance of qRT-PCRs results for tumor samples was calculated by a one way Anova. p < 0.05 values were considered statistically significant. Statistical analysis was done using SAS version 9.2 (Raleigh, NC, USA)

RESULTS

RITA inhibits cancer cells’ metabolism in a p53-dependent manner
We addressed the question whether reactivated p53 can mediate the inhibition of aerobic glycolysis, the key ATP-generating pathway in cancer cells. Using a pair of isogenic human colon cancer cell lines HCT116 and HCT116 TP53-/— varying only in p53 status we analyzed their metabolic state upon treatment with p53-activating molecule RITA. As we found previously p53 is differentially activated by 0.1 and 1 µM of RITA (28). While p53 induction and transcriptional activation of pro-apoptotic targets was similar, transcriptional repression of oncogenes as well as cell death, was achieved only upon 1 µM RITA. Therefore, we treated cells with 0.1 and 1 µM RITA and continuously monitored their metabolism via assessing the acidification and respiration activity over a period of 96 hours using metabolic chip (Bionas® metabolic chip SC 1000) (36). Upon treatment with 1 µM RITA of HCT116 cells the acidification rate, which reflects the export of the end product of glycolysis, lactate, was reduced to approx. 80% of the initial value (1A, green line). In contrast, non-treated cells increased acidification to nearly 500% after 3 days (1A, black line). Treatment with 0.1 µM RITA only slightly decreased acidification (1A, red line). Thus, although treatment with 0.1 µM RITA induces p53 (28 and data not shown), higher dose of RITA is required for the inhibition of cancer cell metabolism. These results are in
line with our previous study, showing that a higher threshold is required for the inhibition of survival genes by p53 compared to the induction of pro-apoptotic genes (1 μM RITA vs 0.1 μM RITA, respectively, 28).

1 μM RITA did not change the metabolism of p53-null cells (1B, dark blue line control and pink line 1 μM RITA). Thus, our data confirm that inhibition of cell metabolism as manifested by the levels of the end product of glycolysis, namely lactate, was p53-dependent.

Interestingly, 0.1 μM RITA increased the acidification rate of treated p53-null cells (1B, light blue line). We speculate that this phenomenon could be due to RITA-mediated induction of ROS levels (37), which might lead to activation of glycolytic enzymes resulting in higher acidification (see Discussion).

More detailed analysis at early time points allowed to detect that the effect of 1 μM RITA on cell metabolism was manifested within a few hours of treatment (data not shown) which indicates that the observed inhibition of cancer cells metabolism is not a consequence of apoptosis induction by RITA. Interestingly, p53-null cells seem to display a different metabolic rate compared to p53-positive cells, suggesting that p53 can modulate metabolism of cells at basal level, even in the absence of external stimuli, which is in line with published data (23).

The respiration rate, analyzed on the same chip (1C) was significantly reduced by 1 μM RITA compared to non-treated or 0.1 μM RITA-treated HCT116 cells (220% vs 370%), in line with results shown above. However, it was not reduced in p53-null cells (1D).

Taken together, data obtained using the metabolic chip analysis demonstrate the p53-dependent inhibition of cell metabolism upon reactivation by 1 μM RITA.

Genome-wide gene expression analysis reveals transcriptional repression of key metabolic genes upon p53 activation by 1 μM RITA

Next we assessed whether the changes in cell metabolism upon p53 activation occur at the transcriptional level. To estimate the expression levels of key enzymes driving glycolysis and oxidative phosphorylation (illustrated in 2C), we performed a microarray analysis of gene expression profiles in human breast cancer cell line MCF7 treated or non-treated with RITA. Our analysis revealed a substantial repression of a group of metabolic genes in MCF7 cells upon 1 μM RITA; among these set of glycolytic genes was clearly distinguishable (2A). Time- and dose-dependent repression was identified for the following metabolic genes: SLC2A1 (glucose transporter 1, Glut1), HKII (hexokinase 2), PFKFB3 (phosphofructokinase fructose-bisphosphate isozyme 3), PFK (phosphofructokinase isoforms P and M), PGM3 (pyruvate glycerate mutase); further, LDHA (lactate dehydrogenase A) and SLC16A1 (monocarboxylate transporter 1) were found to be downregulated.

mRNA levels of several factors known to be involved in the activation of some of the genes mentioned above were substantially repressed. These include oncogenic transcriptional factors c-Myc and HIF1α, as well as both catalytic subunits of PI3K, PIK3CA and PIK3CB (p110α and p110β, respectively) (2A). Notably, mRNA of PDHX (pyruvate dehydrogenase complex, component X), involved in oxidative phosphorylation, and its negative regulator PDK1, were also downregulated upon RITA treatment. Genes encoding oncogenic phosphoglycerate mutase, PGM and hexokinase 2 (HKII), whose expression has been shown to be ablated by wild-type p53 (22,38,39) were inhibited in cancer cells upon p53-activation (2A and B). Functional role in glycolysis of differentially expressed factors mentioned above is indicated in 2C.

Further, induction of p53 with yet another p53-activator, nutlin3a (40) also resulted in inhibition of HKII, PFKFB3, LDHA, SLC16A1 (2B). However, there were differences in the pattern of gene expression upon RITA and nutlin3a treatment – some genes such as MYC, HIF1A, PGM3 and SLC2A1 were not substantially inhibited by nutlin3a, or were even upregulated (SLC2A1, 2B). In contrast, we observed a more pronounced repression of PDK1 and PFKP, and robust activation of the p53-target gene C12orf5 encoding inhibitor of glycolysis TIGAR (19) upon nutlin3a treatment.

Notably, our microarray data suggest that the induction of p53 target genes involved in regulation of oxidative phosphorylation, C12orf5 (encoding TIGAR) and SCO2 depended on the type of p53-activating stimuli
C12orf5 was induced by nutlin3a, 5FU and 0.1 μM RITA, but not by 1 μM RITA (2B and 2A, respectively, and Supplementary Table 2). Induction of TIGAR correlated with the inefficient killing of MCF7 cells by nutlin3a and 0.1 μM RITA (26,28 and data not shown), which is in line with the previously published data that TIGAR can promote survival of cells in the absence of glycolysis (23). SCO2, on the other hand, was only marginally affected by RITA or 5-FU, but was suppressed by nutlin3a (Supplemental Table 2). These data suggest that distinct p53-activating agents differently affect transcriptional regulation of metabolic gene by p53s. This is in line with previously published studies on p53-induced gene expression profiles which show that the array of genes activated or repressed by p53 varies depending on the stimulus, the dose of the agent and cell type (41).

We compared the microarray data on expression profiles of metabolic genes upon RITA treatment of p53-positive and p53-null HCT116 cells and found that MYC, HIF1A, HKI, HKII, PGM1, PGM3, LDHA, SLC16A1, PDHX and PDK1 were downregulated in a p53-dependent manner (data not shown).

Thus, pharmacological activation of p53 affects the expression of a number of genes driving ATP-generating pathways; depending on the type of p53 activator, different regulators of cancer cell metabolism could be affected.

p53-mediated repression of metabolic genes confirmed by qPCR and analysis of protein levels

To further assess the p53-dependent inhibition of the metabolic genes upon RITA treatment and to validate the results of the microarray experiment we selected a group of 6 genes which were significantly repressed in wild type p53 carrying cancer cell lines HCT116, and MCF7 and tested their expression by quantitative RT-PCR (qPCR). As shown in 3A and B, a potent transcriptional inhibition of SLC2A1, HKII, PFKFB3, SLC2A12, PDK1 and HIF1A was detected by qPCR. In addition, we confirmed transcriptional repression of metabolic genes in wild type p53-expressing osteosarcoma cell line U2OS (Supplementary Figure 1A). Moreover, these genes were not repressed upon RITA treatment of the p53-null cells HCT116 TP53-/-, Saos-2 cells (3C and 3D) and H1299 (Supplementary Figure 1B), demonstrating that the observed effect was strictly p53-dependent.

To further assess p53-dependence, we evaluated the changes in mRNA levels of selected metabolic genes upon specific block of p53 activity by p53 inhibitor pifithrin-α (PFT-α) (42). Pifithrin-α is a superior p53 inhibitor compared to p53 shRNA, as it completely blocks p53 induction by RITA, whereas p53 shRNA, while depleting 70-80% of basal p53 levels, could not prevent its induction by RITA (28). Indeed, repression of the metabolic genes was successfully prevented by PFT-α pretreatment (3A, 3B black bars).

Importantly, protein analysis of HK2 and HIF1α revealed a strong depletion of these factors upon RITA treatment (3E). In accordance with the data obtained using qPCR, downregulation of these proteins was observed only in p53-positive HCT116, MCF7, U2OS, but not in p53-negative HCT116TP53-/-, Saos2 and H1299 cells (3E and data not shown).

RITA-activated p53 mediates the inhibition of selected metabolic genes in human tumor xenografts

We previously reported that RITA efficiently inhibits the growth of HCT116 and HeLa human tumor xenografts in mice (25,43). Therefore, we examined p53-induced inhibition of metabolic genes in a more physiological environment resembling clinical setting using HCT116 and HCT116 TP53-/- xenografts grown in immunodeficient mice. Animals were treated with 1 mg/kg intratumor injection of RITA for 18 hours and the status of metabolic genes was analyzed using qRT-PCR (Supplementary table 1). The expression of selected genes was repressed upon RITA treatment in at least some tumors. SLC2A12 (encoding glucose transporter type 12) was the most statistically significantly repressed gene upon p53 activation in tumors. Notably, this effect was p53-dependent, as it was not observed in p53-null xenografts (Figure 4).

Thus, our data demonstrate that RITA-activated p53 inhibited GLUT12, one of the
key metabolic factors crucial for providing ATP to cancer cells in vivo.

Assessment of p53 binding to metabolic genes using ChIP-seq

In order to assess whether p53 might play a direct role in regulation of expression of a set of metabolic genes we investigated whether p53 binds the promoter regions of these genes in vivo by analyzing p53 genome-wide chromatin occupancy upon 1 µM RITA treatment in MCF7 cells using chromatin immunoprecipitation coupled to deep sequencing (ChIP-seq). 6 million sequencing reads were mapped to the human genome (NCBI36) and used to calculate the height of the peaks. As a negative control IgG-precipitated sample was used. Pre-filtering of the obtained data according to the height of the p53 peak allowed to identify 21 000 high quality p53 peaks. Detailed information on this experiment will be published elsewhere (Nikulenkov et al, submitted, 30).

Among top score 10 957 peaks with the height >8 and p-values <0.05, significantly enriched over IgG samples (ratio p53/IgG>2) which were present upon p53 activation with RITA, several peaks were located within metabolic genes. We found that upon RITA treatment p53 bound DNA in vicinity to a number of genes regulating cell metabolism: PFKP, C12orf5, HIF1A, SLC2A1, SLC2A12 and MYC (Table 1). Binding of p53 to C12orf5 and MYC promoters is in line with previously reported data identifying these as direct p53 target genes (19, 44). In most of the genes we identified p53-bound DNA fragments in a proximity to transcriptional starting site (TSS), ±10kb (for example SLC2A1). HIF1A, on the other hand, had several sites where p53 was bound which were located much further, 100kbp from TSS (Table 1). This observation suggests that HIF1A might be regulated by p53 through distal interactions via so called “looping” mechanism (45).

We identified p53 consensus motifs within p53-bound DNA fragments using p53MH and p53Scan programs (46,47). The presence of p53 consensus sites in HIF1A, SLC2A1 and SLC2A12 was confirmed by both approaches, along with consensus sites in MYC and C12orf5 (Table 1). Taken together with expression profiles data in cells and in tumor xenografts, these results suggest that SLC2A12, SLC2A1 and HIF1A are directly repressed by p53.

Since HIF1α and c-Myc are transcriptional factors which can regulate the expression of several genes involved in metabolism (2C), we addressed their contribution in inhibition of metabolic genes by activated p53.

Involvement of c-Myc in p53-mediated ablation of metabolic genes

Several lines of evidence suggest that c-Myc can upregulate the expression of SCL2A1 (48), LDHA (49), PDK1, HKII (12), and HIF1α (50). c-Myc was downregulated by p53 upon treatment with 1 µM, but not 0.1 µM RITA, as we showed previously (28) and in Figure 5A.

Thus, we addressed the question whether downregulation of these genes upon RITA treatment is due to c-Myc inhibition. We determined the mRNA levels of metabolic genes upon MYC depletion by siRNA in HCT116 cells. c-Myc knockdown resulted in downregulation of SLC2A1, HKII and HIF1A, suggesting that transcriptional repression of these genes upon RITA treatment could be, at least partially, due to c-Myc ablation (5B).

Inhibition of HIF1α by reactivated p53 contributes to the ablation of metabolic genes in hypoxic but not normoxic conditions

Many tumors encounter hypoxic environment during their development which results in the overexpression of HIF1α (51). It has been previously shown that p53 acts as a potent negative regulator of HIF1α at protein level (52) and that upon hypoxic conditions HIF1α translation is inhibited by RITA-reactivated p53 (53). In the present study we show that p53 downregulated HIF1α mRNA (2-4), as well as HIF1α protein (5C) in MCF7 and HCT116 cells. To test whether the induction of HIF1α can rescue it from p53-dependent inhibition, we induced the levels of HIF1α by cobalt chloride (CoCl2), a widely used experimental hypoxia mimetic which upregulates the expression of HIF1α (54).

As expected, HIF1α-expression was increased in both HCT116 and MCF7 cell lines after treatment with CoCl2 (5D). Due to the potent induction of HIF1α upon CoCl2 treatment the membranes were exposed for a
shorter time, hence the basal level seems lower than in previous figure. However, CoCl$_2$ did not rescue HIF1α from downregulation by activated p53. Upon treatment with RITA even robustly accumulated HIF1α was almost completely ablated in MCF7 and HCT116 cells (5D).

Cell viability assays revealed that cells were killed by RITA to the same extent in the presence or absence of CoCl$_2$ (data not shown). This suggests that HIF1α can not confer survival to tumor cells upon p53 reactivation, even upon its massive induction by CoCl$_2$. However, inability of CoCl$_2$ to rescue HIF1α levels did not allow us to assess a possible contribution of HIF1α inhibition to the growth suppression effect of RITA.

Next, we studied whether the induction of HIF1α under hypoxic conditions might rescue cancer cells from p53-dependent inhibition of ATP-generating pathways.

The viability assay based on cell morphology analysis confirmed previous data (53) that RITA induces cell death in MCF7 and HCT116 cells to the same extent under hypoxia as under normoxia (Supplementary Figure 2A, 2B). Further, protein analysis demonstrated that upon RITA administration c-Myc and HK2 were decreased to comparable levels under hypoxia and normoxia (5E and 3E). HIF1α levels were downregulated somewhat differently in HCT116 and MCF7 cells: it was substantially ablated already by 1 µM RITA in HCT116 cells, whereas for MCF7 cells 5 µM RITA was required to achieve complete inhibition of HIF1α (5E). Thus, p53 activated by RITA is capable to inhibit HIF1α overexpressed under hypoxic conditions.

Next, we evaluated the efficiency of RITA to ablate the expression of metabolic genes under hypoxia and performed qRT-PCR analysis in MCF7 cells.

SLC2A1, PFKFB3, SLC2A12 and PDK1 mRNAs were upregulated in hypoxia (5F), along with HIF1α protein level, in line with being known HIF1 targets. Reactivation of p53 by RITA in hypoxia led to a significant repression of HIF1α mRNA (5F). Moreover, we observed a dramatic repression of SLC2A1, more than 5-fold, whereas in normoxia it was inhibited 2-fold. These data suggest the contribution of HIF1α inhibition in repression of SLC2A1 upon RITA treatment in hypoxia.

In spite of the induction of PFKFB3 and SLC2A12 in hypoxia, they were still downregulated by RITA approximately 2-fold similarly to normoxia (5F). Thus, our data indicate that downregulation of HIF1α contributes to the transcriptional repression of at least some metabolic genes observed upon p53 reactivation in hypoxia.

Identification of Sp1 as a transcriptional cofactor involved in p53-mediated repression of metabolic genes

In search for other candidate transcriptional factors involved in the regulation of the identified set of genes, we analyzed the transcription factor binding sites in the promoters of these genes based on the microarray data obtained for MCF7 cells. Using the F-Match program and TRANSFAC® database, we selected those transcription factors whose potential binding sites (one or more) were significantly overrepresented (frequency ratio > 1.3 and p-value < 0.01) in the promoters of metabolic genes differentially expressed upon RITA treatment (2A). Our analysis revealed several transcription factors-candidates for a common co-regulator(s) of the metabolic genes (Supplementary Table 3), including paired-box family of transcription factors (Pax-5, Pax-3), c-Myc associated zinc finger protein (Maz), Sp1, Ahr, HIF1α, ZNF148, Egr1 and Ap2. Data for two factors, ZNF148 and Sp1, demonstrated the most significant p-values exceeding the multiple testing correction threshold (10^-6) (Bonferroni correction). Interestingly, many of the factors co-occur in several promoters of the metabolic genes under study (Table 2).

In order to identify most important regulatory molecules crucial for the p53 transcriptional response we applied another bioinformatics method. We performed the key node analysis of our microarray data (as described in Materials and Methods) which revealed p53 as one of the top ranking key nodes (by the key node score p53 was ranked number 12 out of more than 2400 other factors). Importantly, Sp1 transcription factor was found to be tightly linked to the p53 in our key node diagram (see Supplementary Figure 3). Sp1 was the most significantly overrepresented transcriptional factor (Supplementary Table 3) that belongs to the
constructed p53 network (Supplementary Figure 3). Thus, two bioinformatics approaches suggest that Sp1 might be involved in p53-dependent transcriptional regulation upon RITA treatment. This is in line with previously published studies suggesting that Sp1 is p53’s co-repressor for several genes, including DNMT1 and Cdc25B (55, 56). Therefore, we selected Sp1 as a candidate p53 cofactor for regulation of metabolic genes and performed a series of experiments to address the question whether Sp1 plays a role in p53-mediated repression of glycolytic genes.

To study the impact of Sp1, we stably depleted it in MCF7 cells and performed microarray analysis. Expression profiling showed that depletion of Sp1 in MCF7 cells significantly protected from the repression of metabolic genes PDK1, HIF1A, HKII, SLC2A1, and SLC2A12 upon RITA treatment (6A). Assessment of mRNA levels by qPCR revealed that the repression of HIF1A, HKII, and SLC2A12 was significantly attenuated in the absence of Sp1 (6B), suggesting that Sp1 contributes to the p53-mediated inhibition of these genes. Interestingly, the repression of SLC2A1 gene was even more pronounced in Sp1-depleted cells which suggests a different mechanism of its transcriptional regulation.

Furthermore, Sp1 depletion partially prevented RITA-mediated growth suppression (6C), indicating that the contribution of Sp1 to the p53-mediated transcriptional repression of HIF1A and other glycolytic genes plays a role in the biological outcome.

Ablation of glycolysis contributes to the full scale induction of apoptosis by RITA-activated p53

There is currently a great interest in the development of inhibitors of glycolysis for treating cancer. Therefore, we addressed the question whether inhibition of glycolysis plays a role in growth suppression by RITA. In line with the absence of robust effects of 0.1 µM RITA on cell metabolism and viability, we did not detect downregulation of HKII protein at 0.1 µM RITA in both MCF7 and HCT116 cells (7A) in spite of p53 induction. Thus, we investigated whether the depletion of HK2 can facilitate growth suppression effect of p53 upon 0.1 µM RITA. HKII was depleted by corresponding siRNA (see 7C) and cells were treated with 0.1 µM RITA. Although downregulation of HKII by siRNA on its own had barely detectable effect on cell viability, it synergized with 0.1 µM RITA in apoptosis induction in MCF7 cells (7B and 7C). These data indicate that HK2 ablation is important for apoptosis induction by RITA-reactivated p53.

Besides gene silencing, hexokinase could be inhibited by the treatment with the inactive analog of glucose - 2-Deoxyglucose (2-DG). Treatment with 2-DG changed the morphology of MCF7, HCT 116 and HCT116 TP53-/- cells, but did not affect their viability (7D). However, the combination of 2-DG with 0.5 µM RITA resulted in stronger induction of apoptosis in MCF7 and HCT 116, but not in HCT116 TP53-/- cells (7D).

Taken together, our results suggest that inhibition of glycolysis contributes to the induction of apoptosis by RITA. These data show that the small molecule RITA combined with glycolytic inhibitors analogous to 2-Deoxyglucose could be a promising therapeutic approach to induce robust apoptosis in cancer cells.

DISCUSSION

Despite a high genetic diversity, cancer cells exhibit a common set of functional characteristics, one of them being “Warburg effect”, i.e., continuously high glucose uptake and higher rate of glycolysis than in normal cells. Extensive studies have provided evidence that a number of genes that initiate tumorigenesis are linked to the metabolic regulation, i.e., several oncogenes are very prominent inducers of glycolytic phenotype (reviewed in 57-59). Such altered tumor cells are uniquely sensitive to the inhibition of glycolysis suggesting the potential to exploit changes in tumor cell metabolism for anticancer therapy. Thus, development of molecules targeting metabolic pathways can open a new vista for cancer treatment.

A “triad” of transcriptional factors, c-Myc, HIF1α and p53, have been implicated in the control of transcription of genes involved in energy production and metabolism (3). Our previous work demonstrates that upon pharmacological activation of p53 in cancer cells by small molecule RITA, c-Myc oncogene is potently inhibited on
transcriptional and translation levels, as well as destabilized on protein level (28). Well-documented involvement of c-Myc in regulation of glycolysis prompted us to investigate whether the activation of p53 by small molecules can affect metabolism of cancer cells.

Indeed, metabolic chip data revealed p53-dependent inhibition of respiration and acidification rates in cells already a few hours upon RITA treatment. To elucidate the molecular mechanism of the observed phenotype, we used microarray analysis to study gene expression profiles in wild type p53-expressing MCF7 and HCT116 cancer cells upon pharmacological p53 reactivation and found the transcriptional repression of a set of key factors involved in ATP-generating pathways. Interestingly, changes in expression profiles of metabolic genes induced by nutlin3a, another p53 activating molecule, differed from those induced by RITA. It has been shown previously, that p53 transcriptional response can differ significantly upon induction by different stimuli (18). This might be due to different posttranslational modifications of p53 and/or different cofactors bound by p53.

Small molecules are most likely to have more than one target in cells, since they are generally too small to have as high specificity as, for example, antibodies. It is thus plausible that their effect on additional targets contributes to the overall response. We have previously shown that RITA binds and inhibits TrxR1, enzyme important for keeping ROS levels in check (37). Induction of ROS due to the effect of RITA on TrxR1 together with p53-dependent induction of DNA damage response (53, 61, 62), might contribute to the differential p53 modifications upon RITA treatment, compared to nutlin3a. Indeed, our collaborators showed p53 phosphorylation at Ser46 upon RITA, but not nutlin3a treatment, which can affect p53-mediated response (63). However, p53 induction by RITA is independent of DNA damage signaling and ATM, as we recently showed (64), therefore the additional effects of RITA are not the cause of p53 activation, although might contribute to it by amplifying the signal.

We validated our microarray data using qRT-PCR in p53-positive MCF7 and HCT116, U2OS as well as p53-null cells HCT116TP53-/-, Saos-2 and H1299 which demonstrated that 1 µM RITA significantly downregulated the expression of glucose transporters SLC2A1 and SLC2A12, enzymes HKII, PFKFB3, and PDK1 and transcriptional regulator of metabolic genes HIF1α in a p53-dependent manner. Thus, p53 activated by RITA ablates the first steps of glycolysis, namely, glucose uptake, primary phosphorylation of glucose molecules and the rate-limiting step of glycolysis - conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6BP) (2C). Additionally, microarray data pointed to the inhibition of lactate production and lactate/proton removal pathway.

Analysis of ChIP-seq data provided valuable insights into the p53-mediated regulation of metabolic genes upon RITA treatment. We found that p53 binds the promoters of several metabolic genes in vivo and identified p53-binding elements in the promoter regions of SLC2A12, SLC2A1, HIF1A and MYC, suggesting that they are directly repressed by p53.

Interestingly, downregulation of HIF1α by RITA under hypoxia has been previously reported (53,65), but it has been attributed to the decreased translation of HIF1α. p53 has been also shown to promote degradation of the HIF1α protein (52). In this study we found yet another level of negative regulation of HIF1α by p53, i.e., inhibition of its transcription. In contrast to the previous studies (53,65), downregulation of HIF1α upon RITA treatment was observed both in normoxia and hypoxia. We tested whether p53 might directly affect the transcription of HIF1α by binding to its promoter. Our ChIP-seq data indeed indicated that p53 binds to HIF1α promoter at two sites, within which we found p53 consensus binding sites (Table 1). Importantly, p53 binding to the same sites in HIF1α promoter was observed upon p53 activation with RITA, nutlin3a and 5-FU. Since p53 binding sites are located quite far from the transcriptional starting site, it is possible that DNA looping mechanism is involved in p53-mediated regulation of HIF1A.

In addition, indirect transcriptional inhibition of HIF1α upon RITA treatment might be attributed to the repression of c-Myc, since HIF1A was identified as the high affinity c-Myc target (50). This notion is supported by
our data showing that c-Myc depletion by siRNA results in transcriptional downregulation of HIF1α in MCF7 cells.

HIF1α is transcriptionally regulated through oncogenic PI3K acting upstream of Akt (66). We have shown previously potent p53-dependent ablation of IGFR/PI3K/Akt pathway upon RITA treatment (28), thus, inhibition of both subunits PI3KCA and PI3KCB might contribute to the repression of HIF1α as well. It appears that the combined inhibition of several pathways regulating HIF1α results in its potent repression.

Our results demonstrate that the ablation of both c-Myc and HIF1α, transcriptional factors which cooperate in positive regulation of expression of glycolytic genes (reviewed in 3, 38,67), further promote the inhibition of glycolytic genes, both in normoxia and hypoxia.

Bioinformatics analysis of transcription factor binding sites present in the promoters of the repressed genes and keynode analysis allowed to identify Sp1 as a p53 cofactor facilitating the repression of metabolic genes, including novel p53 target genes that we identified, HIF1A, and SLC2A12. We did not observe the induction of SCO2 and C12orf5 genes, shown to be activated by p53 (19,20) upon 1 μM RITA treatment, which suggests the p53-dependent ablation of both energy production pathways, aerobic glycolysis and oxidative phosphorylation. We reported previously that RITA induces ROS in a p53-dependent manner due to the inhibition of TrxR, which contributes to cancer cell killing (37). We speculate that the absence of TIGAR induction might contribute to ROS increase in RITA-treated cells, along with the inhibition of TrxR.

We observed transcriptional repression of C12orf5 (TIGAR) by 1 μM RITA, in contrast to its induction by 0.1 μM RITA, 5-FU and nutlin3a, while p53 was bound to C12orf5 promoter irrespective the type of treatment (Table 1). These data suggests that under certain conditions, p53 might be converted from transcriptional activator to a transcriptional repressor, as it has been shown, for example, in case of p53-mediated regulation of survivin gene (68). An interesting example of another transcription factor which can both activate and repress the same genes upon binding to their promoters is MyoD. Depending on its interaction with HDAC1 or LSD1 at the promoter of its target genes, MyoD can repress or activate genes, respectively (69,70).

Previous studies demonstrated that depending on the dose of activating agent p53 can either promote cell survival or cell death by differentially regulating anti-oxidant or pro-oxidant genes, respectively (71). Further, small molecule nutlin3a also can act in dose-dependent manner: high dose of nutlin promotes p53-dependent inhibition of mTOR, whereas lower dose – does not (72). It is plausible, that p53’s posttranslational modifications and cofactors that associate with p53 can serve as the bars of the ‘barcode’ that governs p53 transcriptional activity, thereby forming the underlying basis of the heterogeneity of p53 response, depending on the type and dose of stimuli as well as cell type (73). Since there are more than fifty known p53 partner proteins, which can modulate p53-mediated regulation of gene expression, high throughput approaches are required to identify the bars of the code which confer the differential gene regulation in response to different p53-activating molecules. In order to address this, we initiated a proteomics and functional genomics studies aimed to identify cofactors differentially bound to p53 upon nutlin3a and RITA treatment.

Based on our results, we propose a model suggesting that pharmacologically activated p53 triggers a set of events which ablate the network of key regulators of ATP production. Upon activation by RITA p53 inhibits c-Myc and HIF1α transcriptional factors regulating a number of genes involved in cell metabolism (7E). Combined inhibition of c-Myc and HIF1α results in downregulation of a whole set of metabolic genes. Further, p53 directly inhibits the expression of glucose transporters 1 and 12, facilitating ablation of glycolysis. Sp1 co-operates with p53 in transcriptional repression of HIF1A and SLC2A12 and probably other metabolic genes. Taken together, several pathways triggered by p53 result in robust inhibition of energy production in cells leading to efficient elimination of cancer cells.

HIF1α was ablated by RITA-reactivated p53 under hypoxic and normoxic conditions in MCF7 and HCT 116 cells. This
can be of special interest for tumor therapy of hypoxic tumors because HIF1α can increase the tumor’s resistance to chemotherapeutic agents and radiotherapy (74).

We showed that RITA can downregulate at least some metabolic genes, such as SLC2A12, in HCT116 xenografts in vivo suggesting that glycolytic gene inhibition by RITA-reactivated p53 is not restricted to the in vitro phenomenon. This indicates RITA’s potential as a glycolytic inhibitor in conditions resembling clinical setting and is in line with our previous data on inhibition of specific oncogenes upon RITA treatment in HCT116 but not in HCT116 TP53−/− xenografts (28).

Hexokinase 1 and 2 are frequently overexpressed in tumors along with other glycolytic proteins like Glut1-3, PFK2 and PGM (50). HK plays an important role in immortalizing cancer cells (75). We found that depletion of HK2 by siRNA or inhibition by 2-Deoxyglucose facilitates the induction of apoptosis suggesting that inhibition of glycolytic factors contributes to the robust apoptosis triggered by RITA. Further, it suggests that combination of p53-reactivating drug with inhibitors of glycolysis might be a promising strategy for anti-cancer therapy. While we can not rule out the possibility that RITA and 2-DG activate different pathways which collaborate to induce cell death, our data obtained inSp1- depleted cells suggest that the repression of HIF1A and other metabolic genes plays a role in the growth suppression effect of RITA.

Drug combinations are currently regarded as an efficient way to solve the problem of de novo resistance to cancer therapies, a formidable barrier for successful cure of cancer. Our data indicate that drugs targeting metabolic pathways can be efficiently combined with p53-reactivating compounds.

In summary, our data suggest that reactivation of p53 by small molecules such as RITA, has a high potential for cancer therapy because it simultaneously targets several key enzymes involved in glycolysis.

**Conflict of interest**
The authors declare no conflict of interests.

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FIGURE LEGENDS
Fig. 1. RITA inhibits respiration and induces acidosis in a p53-dependent manner.
HCT116 and HCT116 TP53-/- cells treated with two different concentrations of RITA were analyzed on Bionas® metabolic chip SC 2500 in running medium supplemented with 2% FCS.
A. In HCT116 cells the acidification rate for 1 μM concentration of RITA was reduced to approximately 80%, whereas in control it increased to nearly 500% after 3 days. Treatment with 0.1 μM resulted in the 350% increase of acidification rate. B. In HCT 116 TP53 cells the acidification rate increased to 280% and remained on this level for the last 2 days both in the control and in 1 μM RITA-treated cells, whereas upon 0.1 μM RITA the acidification rate increased to 400%. Measurements were performed in running medium for 4 days. n=2. C. The respiration rates were measured using the same experimental setup. In HCT116 cells treated with 1 μM of RITA the respiration increased up to 220%. In control and in cells treated with 0.1 μM RITA the respiration rate increased to 320%. D. In treated and non-treated HCT116 TP53-/- cells respiration rate increased to 450-500%. Shown are the representative data of two independent experiments.

Fig. 2. Microarray analysis revealed dose- and time-dependent repression of metabolic genes upon RITA treatment.
The microarray data is presented as heat maps made using dChip (DNA chip analyzer). The rows were standardized by subtracting FC (fold change) control and dividing by SD (standard deviation). Vertical columns indicate separate arrays, and horizontal rows indicate genes.
A. Heat map depicting the relative mRNA levels of genes involved in regulation of metabolism in MCF7 cells treated with two concentrations of RITA over indicated periods of time. B. Comparison of changes of metabolic genes expression in MCF7 cells treated with RITA or nutlin3a over indicated periods of time. C. Schematic representation of the ATP-generating pathways indicating the set of metabolic genes altered upon RITA treatment and their regulators. GLUT-glucose transporter, HK-hexokinase, PFKFB-phospho-fructo kinase fructose-bi-phosphate, PFKM-phosphofructokinase muscles, PFKP-phosphofructokinase platelets, PGM-phosphoglycerate kinase, TIGAR- TP53-induced glycolysis and apoptosis regulator, LDH-lactate dehydrogenese, PDK-pyruvate dehydrogenase kinase, PDH-pyruvate dehydrogenase, MCT-monocarboxylate transporter, G6P-glucose-6-phosphate. Adapted from Kroemer and Pouyssegur, (58). Orange – glucose; dark orange – main products of glucose produced during glycolysis and oxidative phosphorylation; cyan-targets of Myc, p53 and HIF1α; purple-p53 targets; green-Myc and HIF1α targets; blue-p53 and HIF1α targets

Fig. 3. p53-dependent ablation of selected metabolic factors upon RITA treatment.
(A, B) Downregulation of SLC2A1 (Glut1), HKII (HK2), PFKFB3, SLC2A12 (Glut12), PDK1, HIF1A 8 hr after treatment with 1 μM RITA in p53-positive cancer cell lines as assessed by qPCR (mean ± SEM, n = 3).
A. Downregulation of selected metabolic genes in colon cancer cell line HCT116 (black bars) and upon inhibition of p53 by small molecule Pifithrin-α (grey bars). B. Downregulation of selected metabolic genes in wild type p53 human breast cancer cells MCF7 (black bars) and upon inhibition of p53 by small molecule Pifithrin-α (grey bars). C. and D. Comparison of changes in mRNA levels of metabolic genes in p53-null cancer cell lines HCT 116 TP53-/- and Saos2 (mean ± SEM, n = 3). E. p53-dependent downregulation of selected metabolic factors on protein level as assessed by immunoblotting.
Fig. 4. RITA represses the expression of SLC2A12 (Glut12) in tumor xenografts in vivo in a p53-dependent manner. qRT-PCR analysis of mRNA levels of SLC2A12 in HCT 116 and HCT116 TP53-/- tumor xenografts treated with RITA for 18 hours. The figure shows the data obtained from five tumor samples treated with vehicle (#1-5) and five RITA-treated tumors (1 mg/kg, #6-10). Primers were designed to be specific for human SLC2A12.

Fig. 5. Contribution of c-Myc and HIF1α inhibition to the repression of metabolic genes upon p53 reactivation under normoxia and hypoxia, respectively. A. C-Myc protein level is downregulated upon 1 µM but not upon 0.1 µM RITA in wild type p53 MCF7 and HCT116 cancer cells, as assessed by Western blot. B. Upper panel, the extent of c-Myc depletion by siRNA was assessed by immunoblotting. Lower panel, inhibition of c-Myc levels by siRNA led to the downregulation of the SLC2A1, HKII and HIF1A mRNA in HCT116 cells, as detected by qPCR (mean ± SEM, n = 3). C. RITA downregulates HIF1α protein levels in a dose- and time-dependent manner under normoxia, as detected by Western blot (short exposure). D. Efficient downregulation of HIF1α by RITA treatment upon its induction by hypoxia mimetic CoCl2 as assessed by immunoblotting (long exposure). E. Western blot analysis revealed the induction of p53 and its target PUMA by 1 and 5µM of RITA in hypoxic conditions. Downregulation of c-Myc and HK2 correlated with p53 induction. HIF1α which is induced by hypoxia, was downregulated by RITA as well. F. qPCR analysis revealed transcriptional repression of SLC2A1, HK2, PFKFB3, SLC2A12 and HIF1α in hypoxia upon treatment with 1µM RITA. Shown is fold change in hypoxia compared to normoxia (mean ± SEM, n = 3).

Fig. 6. Sp1 cooperates with p53 to repress metabolic genes. A. Expression of metabolic genes was compared in cells with depleted Sp1 untreated or treated with RITA for 4 or 8 with that of control transfected cells, untreated or treated with RITA using microarray analysis. Data are presented as heat map. Vertical columns indicate separate arrays, and horizontal rows indicate genes. The rows were standardized by subtracting the mean of the first column. B. Sp1 depletion partially rescued p53-mediated repression of HIF1A, SLC2A12, HKII as assessed by qPCR. * indicates statistically significant differences, calculated using t-Student test. C. Sp1 knockdown rescued MCF7 cells from growth suppression mediated by RITA as assessed by the microscopy analysis of cell morphology.

Fig. 7. Ablation of HK2 contributes to RITA-induced apoptosis. A. HK2 was downregulated by RITA in a dose-dependent manner as detected by Western blot analysis in MCF7 and HCT 116 cells. B. Inhibition of HK2 synergized with 0.1 µM RITA. Microscopy analysis shows the extent of cell death induction by 0.1 and 1 µM of RITA in the presence or absence of HK2 depletion by siRNA. C. Downregulation of HK2 by siRNA synergized with 0.1 µM RITA treatment in apoptosis induction in MCF7 cells. Quantification of cell death induction by RITA treatment in the presence or absence of HK2 depletion by siRNA was performed using trypan blue staining. D. Combination of 0.5 µM RITA with 2-Deoxyglucose with 0.5 µM RITA potentiated growth inhibition of p53-positive HCT116 and MCF7 cells, but not of the HCT116 TP53-/- cells. E. Model depicting the regulatory pathways governing cancer cell metabolism which are affected upon pharmacological activation of p53 by small molecule RITA. For more details, see the text.

Table 1. In vivo p53 binding to metabolic genes, determined by ChIP-seq. Chromosomal coordinates of p53-occupied sites in vicinity to metabolic genes upon 1 µM RITA treatment. Red color indicate the p53-bound fragments occupied also upon 100 µM 5-FU and 10 µM nutlin3a treatments. Higher score indicate better fit to the consensus binding site.

Table 2. Transcriptional factors whose binding sites were found in the promoters of selected metabolic genes.
Transcriptional factors were identified whose specific binding sites were overrepresented in the promoters of the selected set of metabolic genes in comparison with 1000 genes which did not change their expression upon RITA treatment.
Figure 1

ACIDIFICATION RATE

A. HCT 116

RESPIRATION RATE

C. HCT 116

B. HCT 116 TP53–

D. HCT 116 TP53–

control
0.1 µM RITA
1 µM RITA

control
0.1 µM RITA
1 µM RITA

standard acidification rate (%)

standard respiration rate (%)

0 12 24 36 48 60 72 84 96 (h)

0 12 24 36 48 60 72 84 96 (h)

0 12 24 36 48 60 72 84 96 (h)

0 12 24 36 48 60 72 84 96 (h)
Figure 2

A. MCF7

B. MCF7

C.
Figure 3

A. HCT 116, 8 h treatment

- PFTα
- 1 μM RITA
- PFTα + 1 μM RITA

* p<0.05

B. MCF7, 8 h treatment

- PFTα
- 1 μM RITA
- PFTα + 1 μM RITA

* p<0.05

C. HCT 116 TP53⁺, 8 h treatment

- 0.1 μM RITA
- 1 μM RITA

D. Saos2, 8h treatment

- 0.1 μM RITA
- 1 μM RITA

E. MCF7

- RITA [μM]: 0, 1
- HIF1α
- p53
- HK2
- cMyc
- PUMA
- actin

HCT 116

- RITA [μM]: 0, 1
- HIF1α
- p53
- HK2
- cMyc
- PUMA
- actin

HCT 116 p53⁺

- RITA [μM]: 0, 1
- HIF1α
- p53
- HK2
- cMyc
- PUMA
- actin
Figure 4

![Graph showing SLC2A12 expression levels in HCT116 and HCT116 TP53-/- cells treated with vehicle or RITA, with p-values indicated.](Image)
Figure 5

A. MCF7 and HCT 116 treated with 0.1 μM RITA for 0, 4, 8, and 12 hours. cMyc and actin levels are shown.

B. HCT 116 treated with MYC siRNA (0, 10, 20 nM). Log2 fold change in cMyc vs siGFP is shown.

C. MCF7 and HCT 116 treated with 1 μM RITA for 4 and 8 hours. HIF1α and actin levels are shown.

D. MCF7 and HCT 116 treated with RITA 1 μM and CoCl2 (0, 100, 200 μM). HIF1α, p53, and actin levels are shown.

E. Hypoxia treatment of MCF7 and HCT 116 with 1 μM RITA for 0, 1, and 5 hours. HIF1α, p53, HK2, cMyc, PUMA, and actin levels are shown.

F. Log2 fold change in SLC2A1, HK2, SLC2A12, PDK1, and NIF1A in MCF7 treated with hypoxia for 8 hours and 1 μM RITA compared to hypoxic control.
Figure 6

A. MCF7

B. MCF7, 8h treatment

C. MCF7

CDKN1A
PMAIP
BBC3
SLC2A1
PFKFB3
PDK1
HIF1A
HKII
SLC2A12

log2 fold change

* p<0.05
Figure 7

A.  

|        | MCF7 | HCT 116 |
|--------|------|---------|
| **RITA [μM]**| **0** | **0.1** | **1** |
| 6h     |      |         |       |

HK2  
actin

B.  

|        | MCF7 |
|--------|------|
| **RITA [μM]**| **0** | **0.1** | **1** |
| siGFP   |      |         |       |
| siHKII  |      |         |       |

C.  

MCF7  

| **HKII siRNA [pM]**| **0** | **10** | **20** |
|-------------------|------|-------|-------|
| % dead cells      |      |       |       |
| RITA, 0.1 μM      | **-**| **+** | **-** | **+** |
| sRNA              | GFP  | HKII  |       |       |

* expected additive effect siHKII + 0.1 μM RITA

D.  

MCF7  

HIF1A, 0.5 μM:  

|        | MCF7 | HCT 116 | HCT 116 p53-/- |
|--------|------|---------|---------------|
| **no TG**|      |         |               |
| + TG 10 mM |      |         |               |

E.  

RITA  

PS3  

SP1  

cMYC  

HIF1A  

SLC2A12  

SLC2A1  

HKII  

PPFKB3  

PDK1  

ATP production
| Gene Symbol | chr | start | end | Distance to transcriptional start site | Sense of p53 reads | Ratio of p53 reads | p53M1 motif | p53M1M motif | p53M1M score | p53M1 score | p53M1M score |
|-------------|-----|-------|-----|--------------------------------------|-------------------|------------------|-------------|-------------|--------------|-------------|--------------|
| JF8P        | 10  | 3127562| 3127767| 2054                                | 0                 | 4.3              | CATCTTGTTGA TCAAGAGT CACAAGACAGACA            | 64.38       |
| JF8P        | 10  | 3165466| 3165666| 1904                                | 0                 | 10.5             | GGACAGAAGT CACAGAAGCCT                       | 60.63       |
| L36699      | 12  | 4000862| 4001111| 261                                  | 0                 | 22.7             | GACGTGTGTAG AGCACAGTAC                      | 100         |
| L9F1A       | 14  | 61090364| 61090564| 17183                                | 0                 | 3.4              | GACGTGTTGT GACAGTGTGCA                       | 83.62       |
| L9F1A       | 14  | 610901230| 610901280| 715                                | 0                 | 3.6              | AATCCATTCTGC TGCCATTGTGCGGTGCG               | 60.62       |
| R9F1A       | 14  | 61012095| 61012184| 799                                | 0                 | 3.0              | AATCCATTCTGC TGCCATTGTGCGGTGCG               | 60.62       |
| R9F1A       | 14  | 61123081| 61123074| 11015                                | 0                 | 10.5             | TAATCCATTCTGC TGCCATTGTGCGGTGCG              | 96.14       |
| SLGA1       | 1   | 4017893| 4017893| 1158                                | 0                 | 4.0              | AAGCTGTGTTAG AGCACAGTAC                      | 60.78       |
| R8FC        | 12  | 12867184| 12867313| 14509                                | 0                 | 3.9              | AAGCTGTGTTAG AGCACAGTAC                      | 63.39       |
| R8FC        | 12  | 128742733| 128742853| 124                                | 0                 | 2.9              | AAGCTGTGTTAG AGCACAGTAC                      | 82.62       |
| R8FC        | 12  | 128743082| 128743099| 97                                        | 0                 | 3.1              | AATCCATTCTGC TGCCATTGTGCGGTGCG               | 61.53       |
| R8FC        | 12  | 128720852| 128721139| 2846                                | 0                 | 2.6              | AATCCATTCTGC TGCCATTGTGCGGTGCG               | 51.39       |
| R8FC        | 12  | 128765395| 128765614| 2159                                | 0                 | 2.6              | AATCCATTCTGC TGCCATTGTGCGGTGCG               | 53.69       |
| R8FC        | 12  | 128601279| 128601731| 4674                                | 0                 | 2.9              | AATCCATTCTGC TGCCATTGTGCGGTGCG               | 88.51       |
| R8FC        | 12  | 128610155| 128610200| 455                                | 0                 | 2.3              | AATCCATTCTGC TGCCATTGTGCGGTGCG               | 45.86       |
| R8FC        | 12  | 128618268| 128618881| 612                                | 0                 | 2.2              | AATCCATTCTGC TGCCATTGTGCGGTGCG               | 69.13       |
| R8FC        | 12  | 128601096| 128601325| 1242                                | 0                 | 3.2              | AATCCATTCTGC TGCCATTGTGCGGTGCG               | 70.74       |
| SLCA12       | 6   | 134429301| 134429951| 5017                                | 0                 | 10.5             | GGACAGAAGT CACAGAAGCCT                       | 90.8        |

Table 1.


### Table 2.

| Genes | Transcription factor binding site |
|-------|-----------------------------------|
|       | SLC2A1                            | IIKII                                      | MKK3                    |
|       | Pax-6                             | Sp-1, isoform1                             | Pax-3                   |
|       | (5)                               | (12)                                       | (7)                     |
|       | Sp-1, isoform1                    | Pax-3, isoform1                            | Pax-3                   |
|       | (1)                               | (4)                                        | (1)                     |
|       | MAZ                               | MAZ                                        | MAZ                     |
|       | (1)                               | (6)                                        | (2)                     |
|       | HIF1α                             | HIF1α                                      | HIF1α                   |
|       | (7)                               | (1)                                        | (1)                     |
|       | ANR                               | ANR                                        | ANR                     |
|       | (2)                               | (7)                                        | (1)                     |
|       | Egr-1, 2, 3, 4                    | Egr-1, 2, 3, 4                              | Egr-1, 2, 3, 4, 5       |
|       | (7)                               | (7)                                        | (2)                     |
|       | C-Krox, ZBTB78                    | C-Krox, ZBTB78                              | C-Krox, ZBTB78          |
|       | (3)                               | (1)                                        | (1)                     |
|       | SREBP-1                           | SREBP-1                                    |                         |
|       | (2)                               | (1)                                        |                         |
Inhibition of glycolytic enzymes mediated by pharmacologically activated p53: targeting Warburg effect to fight cancer

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