

Acetylation mediated by the p300/CBP-associated factor determines cellular energy metabolic pathways in cancer

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Abstract. Normal cells produce energy either through OXPHOS in the presence of oxygen or glycolysis in its absence. Cancer cells produce energy preferably through glycolysis even in the presence of oxygen, thereby, acquiring survival and proliferative advantages. Oncogenes and tumour suppressors control these metabolic pathways by regulating the expression of their target genes involved in these processes. During hypoxia, HIF-1 favours high glycolytic flux by upregulating glycolytic enzymes. Conversely, p53 inhibits glycolysis and increases OXPHOS expression through *TIGAR* and *SCO2* gene expression, respectively. We hypothesise that the p300/CBP-associated factor (PCAF) as a common co-factor shared between p53 and HIF-1 plays an important role in the regulation of energy production by modulating *SCO2* and *TIGAR* gene expression mediated by these two transcription factors. The possible involvement of HIF-1 in the regulation of *SCO2* and *TIGAR* gene expression was investigated in cells with different p53 status in normoxia- and hypoxia-mimicking conditions. Putative hypoxia response elements (HREs) were identified in the regulatory region of *SCO2* and *TIGAR* gene promoters. Chromatin immunoprecipitation experiments suggested that HIF-1 was recruited to the putative HREs present in the *SCO2* and *TIGAR* promoters in a cell type-dependent manner. Transcriptional assays endorsed the notion that PCAF may be involved in the determination of the *SCO2* and *TIGAR* cellular levels, thereby, regulating cellular energy metabolism, a view

supported by assays measuring lactic acid production and oxygen consumption in cells ectopically expressing PCAF. The present study identified HIF-1 as a potential regulator of *SCO2* and *TIGAR* gene expression. Furthermore, evidence to suggest that PCAF is involved in the regulation of cellular energy production pathways in hypoxia-mimicking conditions is presented. This effect of PCAF is exerted by orchestrating differential recruitment of HIF-1 α and p53 to the promoter of *TIGAR* and/or *SCO2* genes, thereby, tailoring physiological needs and environmental conditions to *SCO2* and *TIGAR* gene expression.

Introduction

It is well established that unlike normal tissues cancer cells switch the pathway of energy production from oxidative phosphorylation (OXPHOS) to aerobic glycolysis under normoxic conditions (Warburg effect) even though the efficiency of ATP production through OXPHOS is much higher than that of glycolysis (1,2). The high demand of rapid energy release in fast proliferating cancer cells is covered more efficiently by glycolysis, since this pathway can be regulated effectively without requiring the replenishment of suitable metabolites as is the case with the aerobic respiration which is a slower process. In addition, glycolysis confers the ability to cancer cells to evade apoptosis, resist immune responses and use the glycolytic intermediate products as substrates for anabolic reactions, explaining the preference of these cells to produce energy through glycolysis (3-5).

Cellular energy needs are covered by the coordination of the gene expression of a network of metabolic genes carried out by oncogene and tumour suppressor pathways coupling environmental conditions to cellular physiology. For example, in conditions of low oxygen concentration, which is a common event in the tumour microenvironment, HIF-1, the major orchestrator of transcription under these conditions, induces gene expression of glycolytic enzymes and reduces mitochondrial activity switching the energy production pathway from oxidative phosphorylation to glycolysis thus facilitating the cellular adaptation to hypoxia (6-8).

The p53 tumour suppressor on the other hand, displays diverse effects on energy metabolism by fine tuning the gene expression of proteins involved in both OXPHOS and

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glycolysis (9,10). Recently synthesis of cytochrome *c* oxidase 2 (*SCO2*) and TP53-induced glycolysis and apoptosis regulator (*TIGAR*), two genes involved in oxidative phosphorylation and glycolysis respectively, have been documented as p53 transcriptional targets (11,12). *SCO2* is involved in the assembly of the cytochrome *c* oxidase complex (complex IV subunit 2) in the mitochondrial respiratory electron transport chain and the delivery of copper to this complex (13). Mutations in *SCO2* gene severely impair cytochrome *c* oxidase assembly accompanied by cellular copper deficiency which result in various mitochondrial diseases such as encephalomyopathy and hypertrophic cardiomyopathy (14). By inducing *SCO2* gene expression, p53 enhances mitochondrial respiration (9,10) while in its absence glycolysis prevails (11). *TIGAR* inhibits glycolysis as it displays homology to the phosphatase domain of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/FBPase2) enzyme which restrains glycolysis by dephosphorylating fructose-2,6-bisphosphate (F2,6BP). The kinase domain of the PFK2/FBPase2 induces the phosphorylation of fructose 6-phosphate (F6P) thereby increasing the intracellular levels of the F2,6BP, whereas the phosphatase domain of this bi-functional enzyme dephosphorylates F2,6BP to F6P. F2,6BP is an allosteric activator of PFK-1 therefore its levels play a crucial role in the determination of the glycolytic rate, the blockade of the Warburg effect and the redirection of glycolysis to the pentose phosphate pathway (PPP) (3,15,16). Induction of *TIGAR* gene expression is an alternative way by which p53 determines the cellular fate by depleting the intracellular NADPH hence mediating antioxidant defence (3,12).

We have previously demonstrated the role of the p300/CBP associated factor (PCAF) as common coregulator of the function of p53 and HIF-1 α in hypoxia indicating that the acetylation function of PCAF is crucial in determining cell cycle arrest and survival or apoptosis in hypoxic conditions in a manner depending on its intrinsic HAT activity (17). PCAF mediated acetylation and Sirt-1 dependent deacetylation of HIF-1 α have been more recently shown to modulate cellular responses in hypoxia (18). Taking into account the fact that both PCAF and p300 interact with Sirt-1 (19,20) and Sirt-1 is a well-known cellular sensor of reduced glucose and NAD⁺ availability (21), we hypothesised that PCAF as common regulator of p53 and HIF-1 α , might coordinate the pathways for cellular energy production regulating the physiologically distinct pathways of OXPHOS and glycolysis.

It is known that p53 and HIF-1 α play important roles in the determination of sensitivity/resistance of cancer cells to chemotherapy or radiotherapy (22). In this respect, p53 degradation, or HIF-1 α stabilisation and fine tuning of their transcription target selectivity contribute to the response to radiation damage and affect the therapeutic efficacy of agents that regulate these properties. It is therefore of crucial importance to study the factors that can distinguish between the beneficial and detrimental properties of both these transcription factors in cancer therapy in order to improve the efficacy of various therapeutic approaches. We present herein evidence that the HAT activity of PCAF beyond its role in the control of cell proliferation and survival/apoptosis in hypoxic conditions (17) is also implicated in the modulation of metabolic pathways, such as OXPHOS or glycolysis regulating energy and oxidative stress homeostasis in cancer cells. This function of

PCAF is in accord with the recently presented view suggesting the existence of common regulatory systems shared between cell cycle progression and metabolic pathways (23).

Materials and methods

Cell lines, cell culture and constructs. U2OS and MCF7 (p53 wt) and MDA-MB-231 (p53 mutant) and SaOS2 (p53^{-/-}) cells were cultured in DMEM (Sigma-Aldrich, UK) supplemented with 10% v/v heat inactivated fetal calf serum (Gibco, UK) and 1% of penicillin and streptomycin 10,000 U/ml (Lonza, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Wherever mentioned, cells were treated with 250 μ M desferrioxamine (DSFX) (Sigma-Aldrich) for 16 h.

The PCDNA3-Flag-TIGAR expression construct was a generous gift from Professor K. Vousden (Beatson Institute, Glasgow, UK) the PCDNA3-*SCO2* expression construct was provided by Professor P. Hwang (NIH, Bethesda, MD, USA) and the pCiFlag-PCAF(wt) and pCiFlag-PCAF(Δ HAT) were obtained from Dr I. Talianidis (Athens, Greece) (24). Human *SCO2* and *TIGAR* luciferase reporters containing the consensus HREs and p53 binding sites were constructed by amplifying the -287 to -1712 fragment of the *SCO2* and the +400 to -508 region of the *TIGAR* (counted from the translation initiation codon) (primer sequences are shown in Table I) and inserting them in the pGL3 promoter luciferase vector (Promega, USA). The calcium phosphate method (25) and the polyfect transfection system (Qiagen, UK) were used to transfect cells. Luciferase reporter assays were carried out as described previously (25).

Immunoblotting and antibodies. Cells were harvested in 240 mM TNN buffer (50 mM Tris-HCl pH 7.4, 240 mM NaCl, 5 mM EDTA and 0.5% NP-40) and equal amounts of protein were loaded and resolved by SDS-PAGE and western blotting. After incubating with primary and secondary antibodies, the blots were developed with ECL substrate according to the manufacturer's instructions (Pierce, Thermo Scientific, USA). The following antibodies were used for western blotting: β -actin (Abcam, UK), *SCO2* (ProSci Inc., USA), *TIGAR*-IN1 (ProSci Inc.), HIF-1 α (H1 α 67; Calbiochem, EMD Chemicals, USA), p53 (DO1; Santa Cruz Biotechnology, USA), PCAF (E-8; Santa Cruz Biotechnology), anti-Flag (M2; Sigma-Aldrich) and PFKFB3 (Abgent, USA).

Quantitative RT-PCR. Quantitative RT-PCR analysis was carried out as described previously (17). Briefly, total RNA was extracted from cells using RNeasy plus mini kit (Qiagen, USA) following the manufacturer's instructions. The RNA was then reverse transcribed to cDNA and used for qPCR analysis using SYBR Green fluorescent probe. Analysis was performed using the Opticon Monitor (Bio-Rad Laboratories, USA) or Realplex (Eppendorf, UK) software. The primer sequences used in qPCR reaction are provided in Table II.

Chromatin immunoprecipitation. Chromatin immunoprecipitation analysis was performed in U2OS cells as described previously (17). Briefly, chromatin was cross-linked using 1% formaldehyde and the protein DNA complex was sonicated to produce ~500 bp DNA fragments which were then immuno-

Table I. Luciferase primers.

Gene name	Forward primer	Reverse primer
TIGAR	5'-CTCGAGGGGTGGGTGGGTCTAAGTCT-3'	5'-GAGCTCGGACGAGCAATTCTGCAAAC-3'
SCO2	5'-ACCGTGGAGCTGGTCC-3'	5'-CAGCAAGGTGAACCTCT-3'

Table II. qPCR primers.

Gene	Orientation	Primer sequence	Product size (bp)
TIGAR	Forward	5'-ATGGAATTTTGGAGAGAA-3'	84
	Reverse	5'-CCATGGCCCTCAGCTCAC-3'	
SCO2	Forward	5'-TCACTCACTGCCCTGACATC-3'	149
	Reverse	5'-CGGTCAGACCCAACAGTCTT-3'	
Rpl19	Forward	5'-ATGTATCACAGCCTGTACCTG-3'	122
	Reverse	5'-TTCTTGGTCTCTTCCTCCTTG-3'	

Table III. ChIP primers.

	Forward	Reverse
TIGAR		
HRE 1	5'-GTGTTGGAATCTCGGCTCAC-3'	5'-CAAGGCAGGTCAGGAGAATC-3'
HRE 2	5'-CCGTGTTAGCCAAAATGGTC-3'	5'-CACATTTGGCCTTCTGAACA-3'
HRE 3 and E2F1	5'-CTATAGAAGGGTGCCTCCTT-3'	5'-TGACTCCTTCCCATACCTA-3'
HRE 4	5'-ACACGGTGAAACCCTGTCTC-3'	5'-AGTGCAGTGGTGTGATCTCG-3'
HRE 5	5'-CACAGTCTGTTGGTCGCTG-3'	5'-GATTCCTTCCCTCGATAGCC-3'
HRE 6	5'-AGGAATCCTACCGCGGACT-3'	5'-CTACCTCCCCACACCACT-3'
SCO2		
HRE 1	5'-TGGTGCTGCACGAGCTCGG-3'	5'-CTCACCACGGCGCAGCCTC-3'
HRE 2	5'-CTCTGCAGGGACCCCCTGGC-3'	5'-GCGGTCGGAGAGTACGAGCG-3'
HRE 3	5'-CAGGAGGCGCTCGTACTCT-3'	5'-GACAGGCTCTCAGCGCGTGC-3'
HRE 4	5'-CATGCGCAGTCCGGGGAC-3'	5'-ACGAGAGGAAGCGCCGACCT-3'
HRE 5 and p53	5'-GCCAGAGAGTTACCCACCTCCTTTTAA-3'	5'-CTGTACCCGCACCCTGCCC-3'
HRE6	5'-GTGTGGTTGCCCAGGTGTGGA-3'	5'-GGCTGCCCCTGCGACTTGAG-3'

precipitated with HIF-1 α -ChIP grade (AB2185; Abcam) or an irrelevant antibody (HRP conjugated anti-rabbit). The reverse cross-linked DNA fragments were then amplified in PCR reactions with specific primers (Table III) flanking different HREs within the *TIGAR* and *SCO2* promoter and analyzed using Opticon Monitor software (Bio-Rad Laboratories) or 2% agarose gel electrophoresis.

Measurement of oxygen consumption. A Clark-type oxygen electrode system (Rank Brothers, Cambridge, UK) was used to measure the ability of the cells to uptake oxygen. The Clarke type electrode uses polarising voltage to create a current or flow of electrons between one silver and one platinum electrode. The

output of the electrode is connected to a voltage adaptor and the output of this adaptor is connected to a Pico Log recorder (Pico Technology Ltd., UK). As the oxygen concentration in the incubation chamber changes, the current flowing between the two metal electrodes changes in proportion of the oxygen concentration in the incubation chamber. These changes in the current are converted to changes in the voltage which was recorded in the Pico Log recorder. Cells (2×10^5) were collected in 2-ml medium loaded into the incubation chamber maintained at 37°C and stirred continuously using a magnetic stirrer. The value of the voltage indicating the amount of oxygen available in the incubation chamber at each time-point in DSFX-treated cells was divided with the respective value

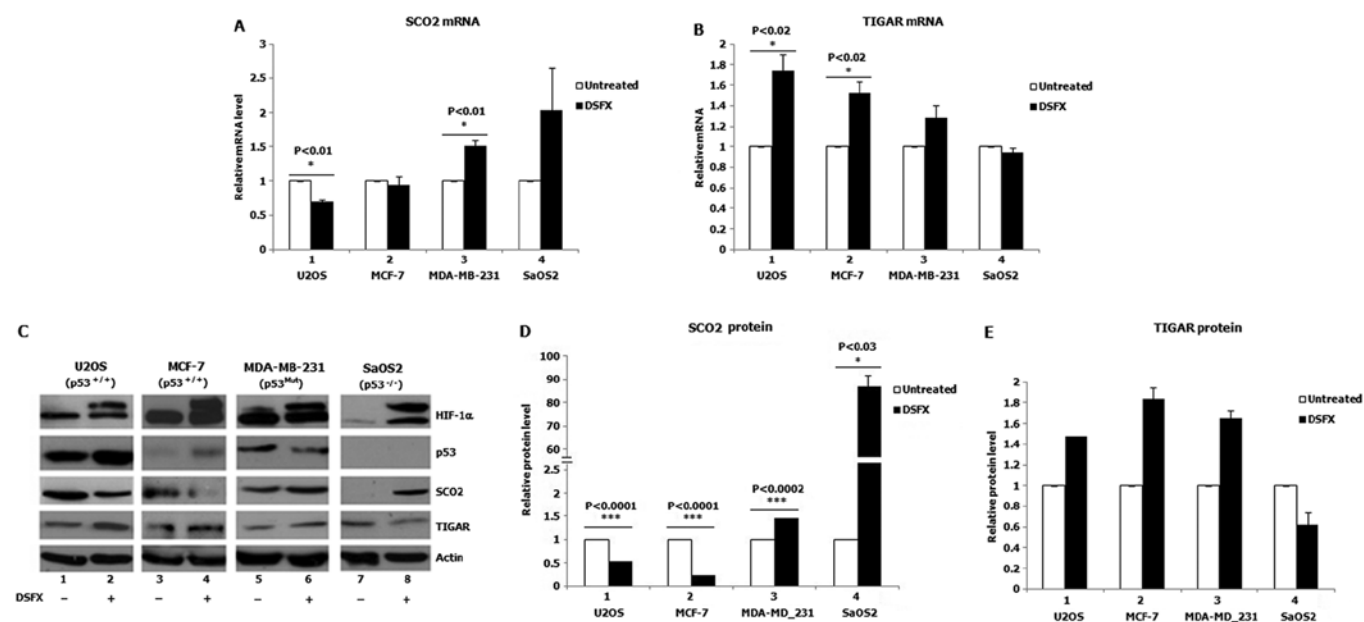


Figure 1. Regulation of *SCO2* and *TIGAR* gene expression in hypoxia mimicking conditions. *SCO2* (A) and *TIGAR* (B) mRNA and protein (C) levels were followed by qRT-PCR and western blotting, respectively, in non-treated or DSFX-treated U2OS, MCF-7, MDA-MB-231 and SaOS2 cells. Rpl19 was used as an internal control in mRNA assays and actin as loading control in western blotting. The mRNA levels of *SCO2* and *TIGAR* in non-treated cells was normalised to 1 (white bars). Densitometric analysis of *SCO2* and *TIGAR* protein levels is shown in (D) and (E) respectively. Asterisks indicate statistical significance of the results.

obtained for the amount of oxygen available in the chamber in untreated cells. The inverted numbers attained from this calculation showing the oxygen consumption in treated and non-treated cells were plotted as a slope. Oxygen consumption was measured from 1 to 600 sec.

Measurement of lactic acid production. Cells were grown in 6-well plates, transiently transfected and treated as described in the text and figure legends. Lactate levels were quantified using Lactate Reagent kit (Trinity Biotech, Ireland) following the manufacturer's instructions. Lactate production rates were expressed as nmol/min per milligram protein.

Results

***SCO2* and *TIGAR* gene expression in hypoxia mimicking conditions.** To investigate whether gene expression of the p53 transcription targets *SCO2* and *TIGAR* was differentially affected in hypoxia mimicking conditions, we followed *SCO2* and *TIGAR* mRNA and protein levels in p53wt (U2OS and MCF7), p53 mutated (MDA-MB-231), or p53-deficient (SaOS2) cells treated with the hypoxia mimicking agent DSFX. Significantly decreased *SCO2* mRNA levels were observed in DSFX-treated U2OS cells (p53wt) compared to those in the non-treated cells (Fig. 1A, compare black bar 1 to white bar 1). In contrast, increased *SCO2* mRNA levels were monitored in DSFX-treated MDA-MB-231 cells (mutated p53) and SaOS2 cells (p53 null) compared to those in non-treated cells (Fig. 1A, compare black bars 3 and 4 to white bars 3 and 4). Similarly lower *SCO2* protein levels were detected in DSFX-treated versus non-treated U2OS and MCF-7 cells (Fig. 1C, compare lanes 2 to 1 and 4 to 3; Fig. 1D, compare black bars 1 and 2 to white bars 1 and 2) and higher in DSFX-treated as opposed to

non-treated MDA-MB-231 and SaOS2 cells (Fig. 1C, compare lanes 6 to 5 and 8 to 7; Fig. 1D, compare black bars 3 and 4 to white bars 3 and 4).

In contrast to *SCO2*, increased *TIGAR* mRNA and protein levels were recorded in DSFX-treated U2OS and MCF-7 compared to non-treated cells (Fig. 1B, compare black bars 1 and 2 to white bars 1 and 2; Fig. 1C, compare lanes 2 to 1 and 4 to 3; Fig. 1E, compare black bars 1 and 2 to white bars 1 and 2). Although *TIGAR* mRNA levels did not change, downregulation of *TIGAR* protein was observed in DSFX-treated SaOS2 cells compared to non-treated cells (Fig. 1C, compare lanes 8 to 7; Fig. 1E, compare black bar 4 to white bar 4). Higher *TIGAR* mRNA and protein levels were documented in DSFX-treated MDA-MB-231 cells compared to those in non-treated cells (Fig. 1B, compare black bar 3 to white bar 3; Fig. 1C, compare lanes 6 to 5; Fig. 1E, compare black bar 3 to white bar 3). Taken together results presented in Fig. 1 are suggestive of a differential regulation of the *SCO2* and *TIGAR* cellular levels in hypoxia mimicking conditions in a manner dependent on the presence of p53.

Putative HREs are present in the regulatory region of the *SCO2* and *TIGAR* promoters. To gain a better understanding of the transcriptional events regulating *SCO2* and *TIGAR* gene expression in DSFX-treated cells and in particular to test whether HIF-1 was involved in these events, the upstream regulatory region of the *SCO2* and *TIGAR* promoters were submitted to bioinformatics analysis to investigate whether hypoxia responsive elements (HREs) were present in these regions. Putative HRE sites were identified within the regulatory region of both *SCO2* and *TIGAR* promoters (Fig. 2).

The presence of binding sites for several transcription factors in addition to the known p53 binding sites (11,16)

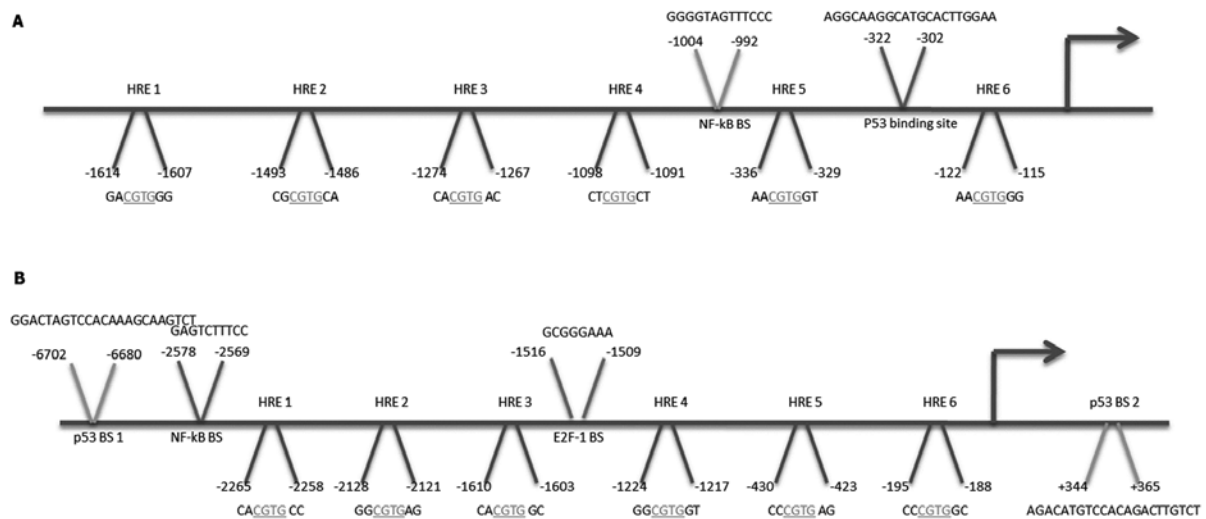


Figure 2. Schematic diagram indicating the HRE and p53 binding sites within the upstream regulatory region of the SCO2 (A) and TIGAR (B) promoters.

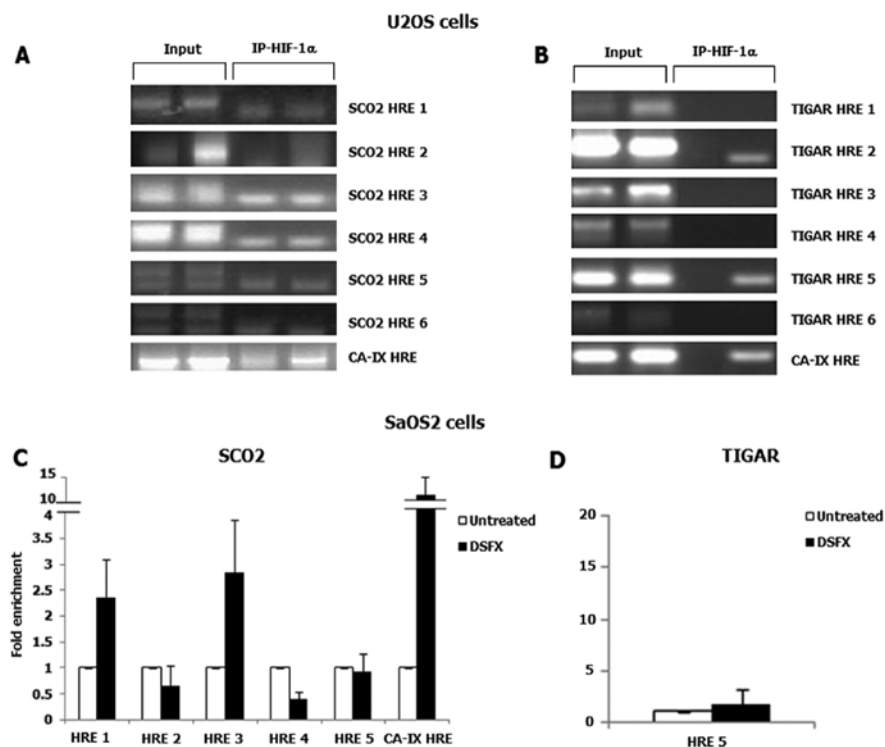


Figure 3. HIF-1 α is recruited to the promoters of SCO2 and TIGAR in a cell type-dependent manner. HIF-1 α bound to chromatin was immunoprecipitated from cellular extracts of non-treated or DSFX-treated U2OS (A and B) and SaOS2 (C and D) cells. The immunoprecipitated chromatin was amplified using specific primers flanking various regions containing the putative binding sites for HIF-1 in SCO2 (A and C) and TIGAR (B and D) promoter regions. Rabbit IgG was used as a negative control and 10% input as loading and positive control. One representative out of three independent experiments is shown in (A) and (B). The average of two independent experiments performed in duplicates is shown in (C) and the average of three experiments performed in duplicates in (D).

within the upstream regulatory region of *SCO2* and *TIGAR* promoters implies that gene expression of these genes is under a complex control of multiple transcription factors. ChIP assays were carried out to assess the recruitment of HIF-1 to the putative binding sites identified in *SCO2* and *TIGAR* promoters. HIF-1 complexes with chromatin were immunoprecipitated using HIF-1 α specific ChIP grade antibody. Rabbit IgG was used as a negative control and 10% input as loading control. The known HIF-1 transcription target gene

CA-IX was used as a positive control (Fig. 3A-C). In U2OS cells HIF-1 α recruitment was not detected in any of the putative HREs identified within the *SCO2* promoter (Fig. 3A), whereas only the HRE 5 within the *TIGAR* promoter was found to be occupied by HIF-1 α in DSFX-treated U2OS cells (Fig. 3B, compare lanes 3 with 4; the band appearing in the TIGAR HRE 2 panel is not specific). However, in SaOS2 cells, HIF-1 α was found to be recruited in the putative HRE 1 and HRE 3 sites within the *SCO2* promoter (Fig. 3C).

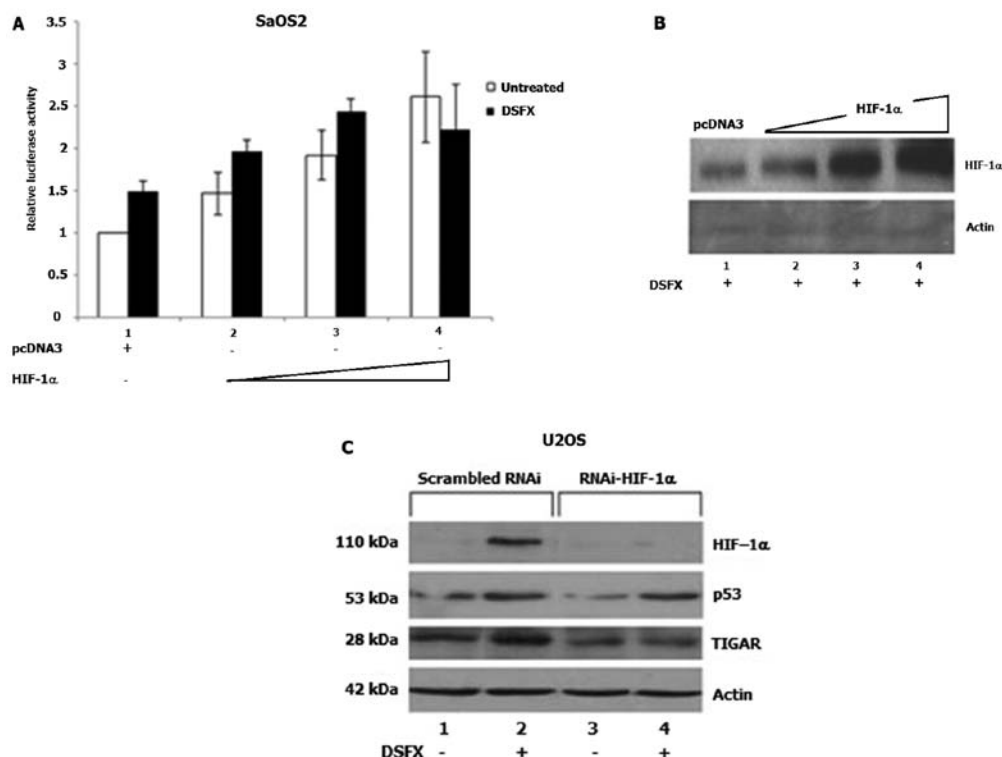


Figure 4. SCO2 and TIGAR are possible HIF-1 α transcription targets. (A) SCO2-HRE1-5-p53-Luc reporter assay in SaOS2 cells overexpressing increasing amounts of HIF-1 α were left untreated (white bars) or treated with DSFX (black bars) and subjected to luciferase reporter assays. (B) Cells transfected as described in (A) were submitted to western blotting and probed for HIF-1 α . Actin was used as a loading control. (C) TIGAR protein levels in U2OS cells transfected with either scrambled RNAi (tracks 1 and 2) or RNAi against HIF-1 α (tracks 3 and 4). The cells were left untreated (lanes 1 and 3) or treated with DSFX (lanes 2 and 4). Actin was used as a loading control.

Furthermore, HIF-1 α was not detected bound to the HRE 5 of the *TIGAR* promoter in the Saos2 cells (Fig. 3D).

To further investigate the involvement of HIF-1 in the transcriptional regulation of *SCO2* gene expression, the fragment of the *SCO2* promoter containing the putative HREs 1-5 (Fig. 2A) was cloned in the pGL3-Luc reporter vector as described in Materials and methods. The constructed SCO2-HRE1-5-p53bs-Luc was used to carry out luciferase reporter assays in DSFX-treated U2OS and SaOS2 cells ectopically expressing increasing amounts of HIF-1 α . Results shown in Fig. 4A indicated that increasing amounts of HIF-1 α (Fig. 4B) induced SCO2-HRE1-5-p53bs-Luc expression in SaOS2 cells signifying the potential involvement of HIF-1 α in the regulation of *SCO2* gene expression.

Possible involvement of HIF-1 α in the regulation of *TIGAR* gene expression was evidenced in U2OS cells transfected with RNAi against HIF-1 α and monitoring TIGAR protein levels (Fig. 4C). Increased TIGAR protein levels were observed in DSFX-treated U2OS cells transfected with scrambled RNAi compared to those detected in non-treated cells (Fig. 4C, compare lanes 2 to 1), whereas reduced TIGAR protein levels were recorded in U2OS cells transfected with RNAi-HIF (Fig. 4C, compare lanes 3 and 4 to lanes 1 and 2, respectively).

The HAT activity of PCAF is potentially involved in the determination of SCO2 and TIGAR cellular levels. We have previously reported that PCAF HAT activity plays important role in the determination of both p53 and HIF-1 α transcription target selectivity in hypoxia mimicking conditions thus coor-

inating cell cycle arrest or apoptosis under these conditions (17). It is also known that lysine acetylases (such as p300 and PCAF) and deacetylases (such as Sirt-1) act antagonistically to promote or suppress the transcriptional activity of many transcription factors including p53 and HIF-1, thereby regulating important cellular functions (19,20).

Since *SCO2* and *TIGAR* were identified as possible common transcription targets of p53 (11,16) and HIF-1 α (Figs. 1-4) we were intrigued to examine whether the HAT activity of PCAF played coordinative role in selectively targeting p53 or HIF-1 to the *SCO2* and *TIGAR* promoter in response to diverse environmental stimuli. For this purpose, *SCO2* protein levels were followed in non-treated and DSFX-treated U2OS and SaOS2 cells transfected with either, Flag-PCAFwt or Flag-PCAF Δ HAT expression vectors (Fig. 5). Reduced *SCO2* protein levels were detected in DSFX-treated U2OS cells overexpressing Flag-PCAFwt compared to non-treated cells (Fig. 5A, compare lanes 2 to 1) while increased *SCO2* protein levels were observed in DSFX-treated U2OS overexpressing Flag-PCAF Δ HAT compared to the non-treated cells (Fig. 5A, compare lanes 4 to 3). In Flag-PCAF Δ HAT overexpressing SaOS2 cells, reduced *SCO2* protein levels were identified in DSFX-treated cells compared to the non-treated cells (Fig. 5D, compare lanes 4 to 3). No change in *SCO2* protein levels was observed in Flag-PCAFwt overexpressing SaOS2 cells treated or not treated with DSFX (Fig. 5D, compare lanes 2 to 1).

Flag-PCAFwt overexpression in DSFX-treated U2OS cells downregulated luciferase expression driven by the SCO2-HRE1-5-p53bs-Luc reporter (Fig. 5B, compare bars

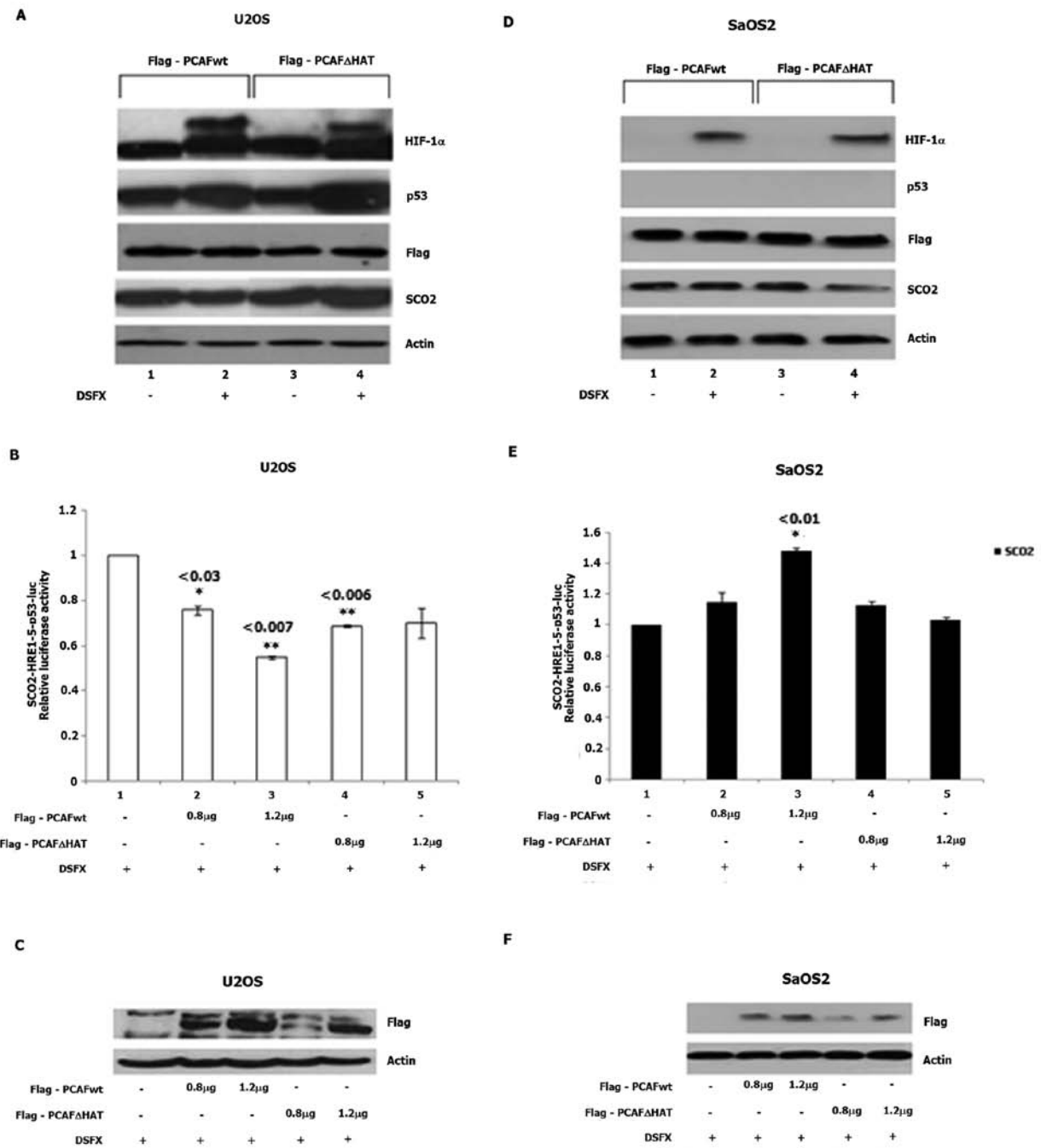


Figure 5. PCAF regulates SCO2 gene expression. SCO2 protein levels were followed in non-treated or DSFX-treated U2OS (A) and SaOS2 (B) cells transfected with either Flag-PCAFwt or Flag-PCAFΔHAT. The exogenously expressed proteins were detected using a specific antibody against Flag. Actin was used as a loading control. DSFX-treated U2OS (C) and SaOS2 (D) cells were transfected with increasing amounts of Flag-PCAFwt and Flag-PCAFΔHAT along with SCO2-HRE1-5-p53-Luc reporter and subjected to luciferase reporter assays. The protein levels of the transfected Flag-PCAFwt and Flag-PCAFΔHAT in U2OS (E) and SaOS2 (F) cells are shown below the reporter assay diagrams. One representative out of three independent experiments is shown in (A), (B), (E) and (F) and the average of three experiments performed in duplicates in (C) and (D).

3 to 1). Increasing amounts of Flag-PCAFwt transfected in SaOS2 cells resulted in 1.5-fold increase in SCO2-HRE1-5-p53bs-Luc activity compared to that exhibited in the SaOS2 cells transfected with the empty vector (Fig. 5E, compare bars 1 to 3). No difference in the SCO2-HRE1-5-p53bs-Luc reporter activity was observed in U2OS and SaOS2 cells treated with DSFX and ectopically expressing increasing amounts of Flag-PCAFΔHAT (Fig. 5B and E, bars 4 and 5). Increasing expression of Flag-PCAFwt and Flag-PCAFΔHAT

transfected in U2OS and SaOS2 cells is shown in Fig. 5C and F, respectively.

To test whether PCAF plays a role in the regulation of TIGAR gene expression, we followed TIGAR mRNA levels in DSFX-treated U2OS and SaOS2 cells transfected with Flag-PCAFwt or Flag-PCAFΔHAT expression vectors. A statistically significant reduction of *TIGAR* mRNA levels was observed in U2OS cells ectopically expressing Flag-PCAFwt (Fig. 6A, compare bars 2 to 1), whereas *TIGAR* mRNA levels

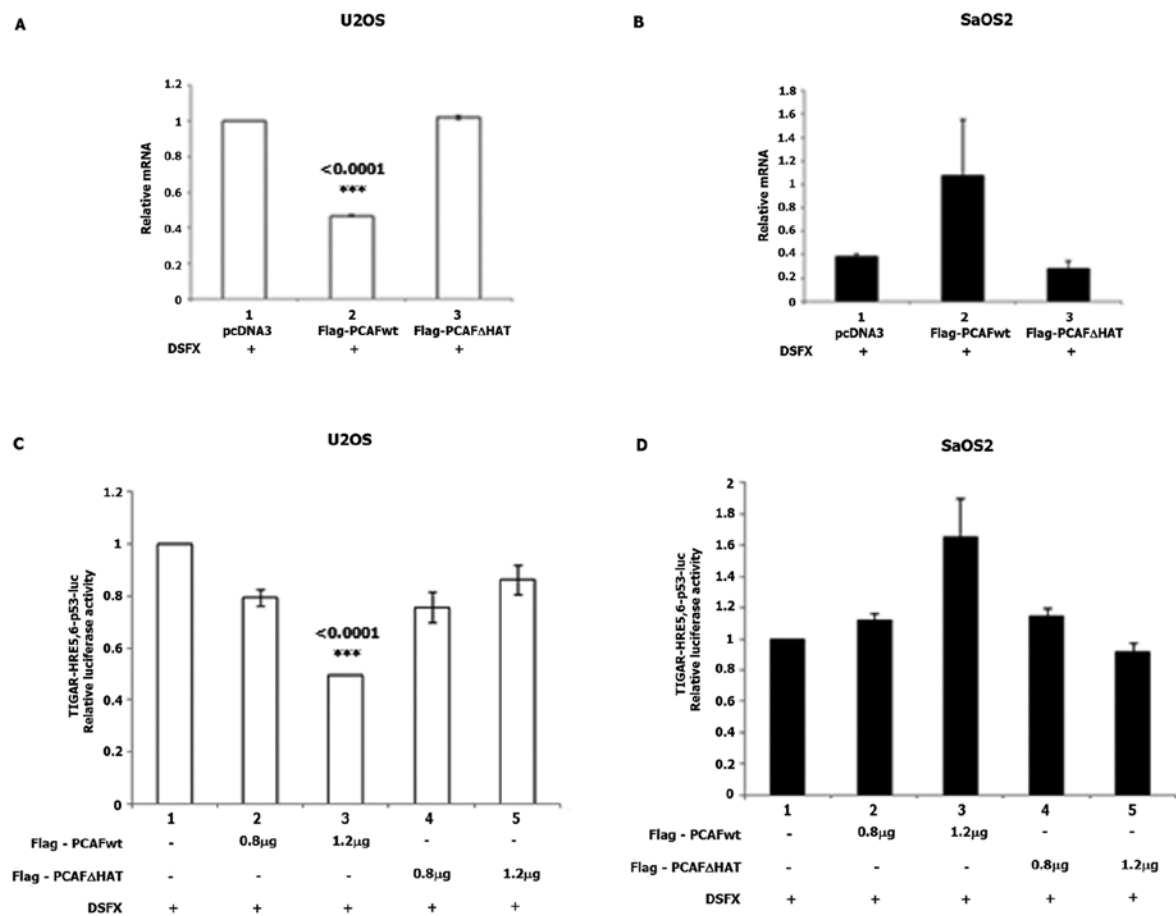


Figure 6. PCAF regulates *TIGAR* gene expression. DSFX-treated U2OS (A) and SaOS2 (B) cells were transfected with empty vector PCDNA3 or Flag-PCAFwt or Flag-PCAFΔHAT and the *TIGAR* mRNA levels were followed with qRT-PCR. DSFX-treated U2OS (C) and SaOS2 (D) cells were transfected with increasing amounts of Flag-PCAFwt and Flag-PCAFΔHAT along with TIGAR-HRE5,6-p53-Luc reporter and subjected to luciferase reporter assay. The average of three independent experiments performed in duplicates are shown in (A-D).

were not affected in Flag-PCAFΔHAT-transfected cells (Fig. 6A compare bars 3 to 1). Upregulated *TIGAR* mRNA levels were detected in SaOS2 cells expressing Flag-PCAFwt (Fig. 6B, compare bars 2 to 1) and no effect of Flag-PCAFΔHAT (Fig. 6B, compare bars 3 to 1).

Flag-PCAFwt overexpression in DSFX-treated U2OS cells repressed the luciferase activity of the TIGAR-HRE5,6-p53bs-Luc reporter (Fig. 6C, compare bars 3 to 1). In DSFX-treated SaOS2 cells overexpression of Flag-PCAFwt induced the luciferase activity of the TIGAR-HRE5,6-p53bs-Luc reporter (Fig. 6D, compare bars 3 to 1). Flag-PCAFΔHAT overexpression did not affect the luciferase activity of the TIGAR-HRE5,6-p53bs-Luc reporter in either U2OS or SaOS2 cells (Fig. 6C and D respectively, bars 4 and 5). The increasing amounts of Flag-PCAFwt and Flag-PCAFΔHAT transfected in U2OS and SaOS2 cells are shown in Fig. 5C and F, respectively. Taken together the results shown in Figs. 5 and 6 support the notion that the HAT activity of PCAF regulates *SCO2* and *TIGAR* cellular levels by fine tuning p53 and HIF-1α transcriptional activities and the interplay between these transcription factors (12,16,17).

To investigate whether PCAF played a role in the regulation of the expression of the HIF-1α transcriptional target gene *PFKFB3* the protein levels of this glycolytic regulator were followed in U2OS and SaOS2 cells overexpressing either Flag-

PCAFwt or Flag-PCAFΔHAT in the presence or absence of DSFX. Reduced PFKFB3 protein levels were observed in the Flag-PCAFΔHAT compared to Flag-PCAFwt overexpressing and DSFX-treated U2OS cells (Fig. 7A, compare lanes 4 to 2 and Fig. 7C, compare black bars 2 to 1). In SaOS2 cells the HAT activity of PCAF exhibited the opposite effect to that observed in U2OS on the PFKFB3 protein levels (Fig. 7B, compare lanes 4 to 2 and Fig. 7D, compare black bars 2 to 1).

Functional significance of the HAT activity of PCAF in the regulation of cellular energy metabolism. The functional significance of the regulation of *SCO2* and *TIGAR* gene expression mediated by the acetyltransferase activity of PCAF was investigated by assessing the lactate levels produced by cells transfected with either Flag-PCAFwt or Flag-PCAFΔHAT. The lactate efflux observed in DSFX-treated cells was normalised to that produced by non-treated cells. Increased lactate production by DSFX-treated U2OS cells overexpressing Flag-PCAFwt was observed compared to that estimated in these cells expressing Flag-PCAFΔHAT under the same conditions (Fig. 8A, compare white bars 2 with 3). The opposite was the case in SaOS2 cells where overexpression of Flag-PCAFwt led to reduced lactate efflux in DSFX-treated versus non-treated cells whereas transfection of Flag-PCAFΔHAT resulted in increased production of lactate in hypoxia mimicking conditions (Fig. 8A, compare

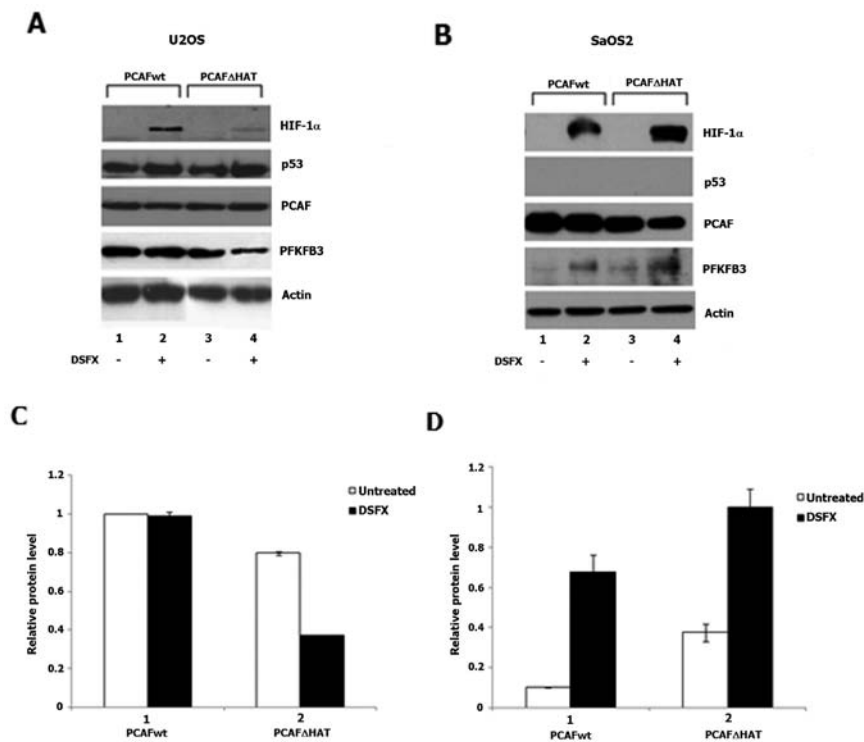


Figure 7. PCAF regulates PFKFB3 protein levels. U2OS (A) and SaOS2 (B) cells transfected with Flag-PCAFwt or Flag-PCAFΔHAT were left untreated or treated with DSFX and subjected to western blot analysis. Densitometric analysis of PFKFB3 protein levels in U2OS is shown in (C) and in SaOS2 cells in (D).

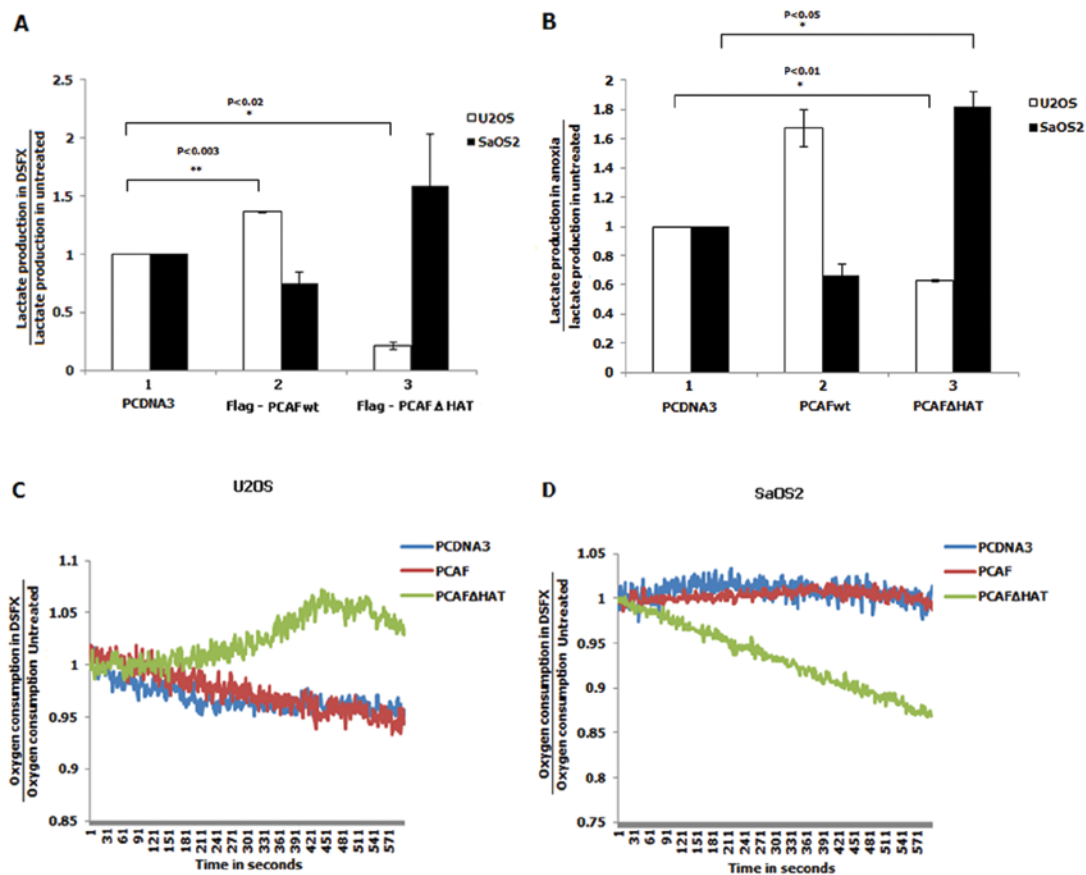


Figure 8. Lactate production and oxygen consumption are differentially regulated by the HAT activity of PCAF in U2OS and SaOS2 cells. Lactate production was assessed in DSFX-treated cells (A) or cells incubated in the anoxic chamber (B). U2OS (white bars) and SaOS2 (black bars) cells were transfected with either PCDNA3 (1) or PCAFwt (2) or PCAF-ΔHAT (3) and lactate production was measured as described in Materials and methods. Oxygen consumption was performed in U2OS (C) and SaOS2 (D) cells transfected with PCDNA3 (blue), or PCAFwt (red) or PCAFΔHAT (green). Asterisks indicate statistical significance of the results.

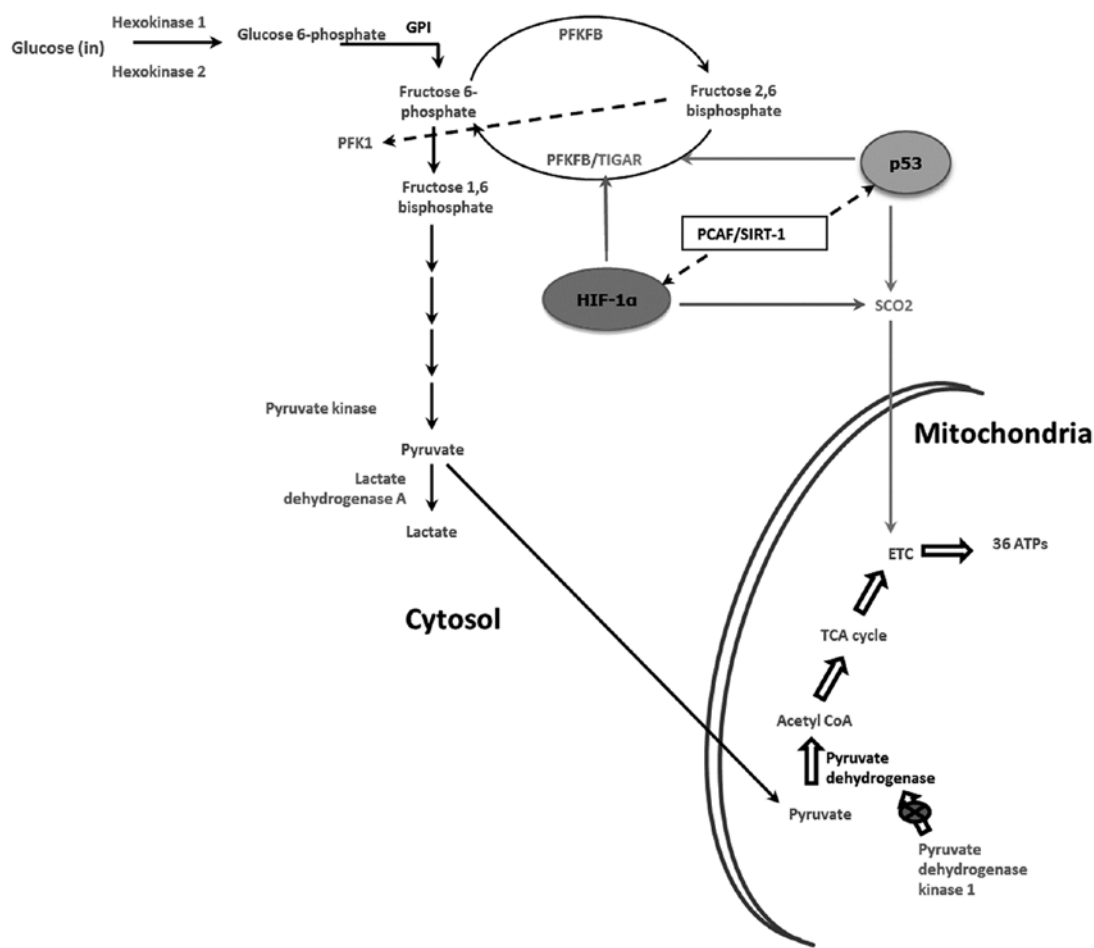


Figure 9. Schematic diagram illustrating the potential regulation of cellular energy metabolism mediated by PCAF. The balance between PCAF and Sirt-1 activity fine tunes the HIF-1 and p53 transcriptional activity and target selectivity regulating TIGAR and SCO2 gene expression thereby orchestrating glycolysis and OXPHOS pathways of cellular energy production under diverse environmental conditions.

black bars 2 with 3). Lactate production measured in U2OS and SaOS2 cells incubated in an anoxic chamber presented similar results with those obtained from DSFX-treated cells (compare Fig. 8A with B). These findings indicated that the HAT activity of PCAF increased glycolysis in hypoxic U2OS cells and reduced it in SaOS2 cells corresponding with downregulated *TIGAR* gene expression in DSFX-treated and Flag-PCAFwt expressing U2OS cells and upregulated *TIGAR* gene expression in SaOS2 cells under these conditions (Fig. 6). Thus the differential lactate production in U2OS and SaOS2 cells could be mechanistically explained at least in part by the opposite effect of PCAF on the *TIGAR* gene expression in the two cell lines (Fig. 6).

Inability to produce energy through OXPHOS in cancer cells is compensated by increased glycolysis (26). Oxygen uptake is an indicator of the ability of the cells to produce energy through the OXPHOS pathway (11). The role of the HAT activity of PCAF in the regulation of OXPHOS was studied by quantifying the ability of Flag-PCAFwt, Flag-PCAFΔHAT or PCDNA3 empty vector transfected in U2OS or SaOS2 cells to uptake oxygen (Fig. 8C and D, red, green and blue lines respectively). The oxygen consumption in DSFX-treated and non-treated cells was calculated as described in Materials and methods. While the lactate production of DSFX-treated U2OS cells transfected with Flag-PCAFΔHAT was decreased

compared to these cells transfected with Flag-PCAFwt under the same conditions (Fig. 8A, compare white bars 3 with 2), the oxygen consumption was found to be increased in the same conditions (Fig. 8C, compare green line with red and blue lines). In contrast to U2OS cells, DSFX-treated SaOS2 cells overexpressing Flag-PCAFΔHAT consumed less oxygen compared to cells transfected with Flag-PCAFwt (Fig. 8D, compare green line with red and blue lines). Taken together results shown in Fig. 8 indicate that the HAT activity of PCAF plays an important role in determining the pathway of cellular energy production in hypoxia mimicking conditions.

Discussion

Proliferation status and physiological conditions are some of the factors determining the cellular energy demands. Among the transcription factors that orchestrate the pathways of cellular energy metabolism to harmonize cell cycle progression and energy metabolism with environmental physiological conditions is the tumour suppressor p53 (27). Inactivation of p53 results in substantial OXPHOS deficiency (11) and increased dependence on glycolysis accompanied by elevated lactate production (28). The p53 mediated switch from OXPHOS to glycolysis is in part implemented by the transcriptional regulation of the expression of a number of genes involved in both

OXPHOS and glycolysis by this transcription factor (11). For example, p53 acting in a cell type-dependent mode stimulates the expression of genes essential for the completion of the OXPHOS pathway such as *SCO2* (11) and inhibits glycolysis by repressing the glucose transporters GLUT-1 and GLUT-4 (29).

In cancer tissues the deprivation of cellular oxygen within hypoxic regions contributes to the shift to glycolysis and eventually tumour cell growth. In response to low oxygen concentration the gene expression of a variety of metabolic enzymes such as GLUT and the regulatory bifunctional PFKFB3 enzyme is directed by the HIF family of transcription factors (30,31). Dramatic increase of the ratio of kinase:phosphatase activity of the HIF-1 α transcription target PFKFB3 in hypoxic conditions results in increased F2,6BP concentration in hypoxic solid tumours (31,32). The homologue to the bisphosphatase domain of the PFK2/FBPase2 gene *TIGAR* has been identified as a p53 transcriptional target (16) suggesting that the reduction of the phosphatase activity of FBPase2 in hypoxia and increased glycolysis could be exaggerated as a result of active repression of *TIGAR* gene expression under these conditions. In addition, HIF-1 α mediated downregulation of mitochondrial oxygen consumption and preservation of the Crabtree effect might be the result of differential modulation of *SCO2* gene expression in hypoxia (6).

In order to identify p53 and HIF-1 α common transcription target genes involved in energy production pathways we tested the possibility the gene expression of the known p53 targets *SCO2* and *TIGAR* was under HIF-1 α control. We followed the mRNA and protein levels of these genes in hypoxia mimicking conditions, which revealed that both *SCO2* and *TIGAR* cellular levels were differentially regulated in normoxia compared to hypoxia mimicking conditions (Fig. 1). To find out whether HIF-1 α was one of the factors involved in the regulation of *SCO2* and *TIGAR* mRNA and protein levels we searched for the existence of potential HRE sites within the regulatory region of the promoters of both these genes (Fig. 2) and tested the activity of these sites employing luciferase reporter and chromatin immunoprecipitation assays, which supported the notion that *SCO2* and *TIGAR* were under HIF-1 transcriptional control (Figs. 3 and 4).

PCAF has been shown to acetylate both HIF-1 α and p53 and coordinate their selective recruitment to pro-survival or apoptotic genes such as p21 and BID thereby regulating cell cycle arrest and apoptosis in hypoxic conditions (17). Given the fact that cell cycle regulation and metabolism share common regulatory pathways (23) and PCAF is a co-factor regulating cell growth effects mediated by both p53 and HIF-1 α (17), we expanded our studies to investigate whether PCAF was involved in the fine tuning of p53 and HIF-1 α mediated effects on metabolism. Luciferase reporter assays pointed out that the HAT activity of PCAF was involved in the HIF-1 α mediated regulation of the expression of *SCO2* (Fig. 5) and *TIGAR* (Fig. 6) gene expression indicating a role of PCAF mediated acetylation as a major regulator for the determination of the pathway of cellular energy production in hypoxia mimicking conditions. Furthermore, downregulation of PFKFB3 in U2OS cells overexpressing Flag-PCAF Δ HAT in hypoxia mimicking conditions and upregulation of this enzyme in SaOS2 under the same conditions (Fig. 7) implied that the HAT activity of

PCAF regulated both *TIGAR* (Fig. 6) and PFKFB3 (Fig. 7) cellular levels. Given the fact that *TIGAR* shares structural and functional similarity with the phosphatase domain of PFKFB3, this result suggested that *TIGAR* might compensate for the lack of phosphatase activity of PFKFB3 in hypoxia mimicking conditions.

The interplay of the two main bioenergetic pathways OXPHOS and glycolysis mediated by the HAT activity of PCAF was investigated by analysing lactate production and oxygen consumption in cells overexpressing Flag-PCAFwt or Flag-PCAF Δ HAT variants (Fig. 8). Results shown in Fig. 8A and B suggested that Flag-PCAFwt overexpressing U2OS cells depend more on glycolysis in hypoxia mimicking and anoxic conditions than untransfected or Flag-PCAF Δ HAT expressing cells under the same conditions (Fig. 8A and B). In contrast, in hypoxia mimicking or anoxic conditions SaOS2 cells overexpressing Flag-PCAFwt exhibited higher lactate level production than those transfected with Flag-PCAF Δ HAT (Fig. 8A and B). Increased ability of Flag-PCAF Δ HAT overexpressing U2OS cells to uptake oxygen in hypoxia mimicking conditions (Fig. 8C) could be indicative of elevated capacity of these cells to produce energy through OXPHOS. In addition, the increased lactate production observed in U2OS cells overexpressing Flag-PCAF Δ HAT comply with the reduced oxygen consumption observed in these cells (Fig. 8A, B and D). Reduced oxygen uptake could be an indication of reduced oxidative phosphorylation, but the increased oxygen consumption may or may not indicate increased OXPHOS (33).

Collectively, the results presented provide evidence to support a role for the HAT activity of PCAF, at least in part, in determining the pathway of cellular energy production in U2OS and SaOS2 cells by fine tuning the crosstalk between p53 and HIF-1 α (Fig. 9).

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