Interaction of HIF and USF Signaling Pathways in Human Genes Flanked by Hypoxia-Response Elements and E-box Palindromes

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Abstract

Rampant activity of the hypoxia-inducible factor (HIF)-1 in cancer is frequently associated with the malignant progression into a harder-to-treat, increasingly aggressive phenotype. Clearly, anti-HIF strategies in cancer cells are of considerable clinical interest. One way to fine-tune, or inhibit, HIF’s transcriptional outflow independently of hydroxylase activities could be through competing transcription factors. A CACGTG-binding activity in human hepatoma cells was previously found to restrict HIF’s access to hypoxia response cis-elements (HRE) in a Daphnia globin gene promoter construct (phb2). The CACGTG factor, and its impact on hypoxia-responsive human genes, was analyzed in this study by genome-wide computational scans as well as gene-specific quantitative PCR, reporter and DNA-binding assays in hepatoma (Hep3B), cervical carcinoma (HeLa), and breast carcinoma (MCF7) cells. Among six basic helix-loop-helix transcription factors known to target CACGTG palindromes, we identified upstream stimulatory factor (USF)-1/2 as predominant phb2 CACGTG constituents in Hep3B, HeLa, and MCF7 cells. Human genes with adjacent or overlapping HRE and CACGTG motifs included with lactate dehydrogenase A (LDHA) and Bcl-2/E1B 19 kDa interacting protein 3 (BNIP3) hypoxia-induced HIF-1 targets. Parallel recruitment of HIF-1α and USF1/2a to the respective promoter chromatin was verified for all cell lines investigated. Mutual complementing (LDHA) or moderating (BNIP3) cross-talk was seen upon overexpression or silencing of HIF-1α and USF1/2a. Distinct (LDHA) or overlapping (BNIP3) promoter-binding sites for HIF-1α and USFs were subsequently characterized. We propose that, depending on abundance or activity of its protein constituents, O2-independent USF signaling can function to fine-tune or interfere with HIF-mediated transcription in cancer cells. Mol Cancer Res; 9(11); 1520–36. ©2011 AACR.

Introduction

Relaying minutes-to-hours of inadequate oxygenation (hypoxia) onto the level of DNA via the hypoxia-inducible transcription factors 1 and 2 (HIF-1 and HIF-2) is a highly conserved signaling event across the animal kingdom (1–3). When exposed to low oxygen partial pressures (pO2), the mammalian HIF-1/2 complexes function as heterodimer of HIF-1α or HIF-2α and HIF-1β subunits (4). Whereas HIF-1β, also known as aryl hydrocarbon receptor nuclear translocator (ARNT), is constitutively present, the activity and abundance of HIF-α subunits are regulated as a function of pO2. In the presence of oxygen, homologs of prolyl hydroxylase domain 1-3 (PHD1-3) dioxygenases catalyze the Fe (II)-dependent hydroxylation of 2 proline residues contained within the oxygen-dependent degradation domain (ODD) domain and the N-terminal transactivation domain (NAD; rear proline only) of HIF-1α and HIF-2α (5–7). Once prolyl hydroxylated, HIF-α subunits are captured by the von Hippel-Lindau tumor suppressor protein and rapidly degraded via the ubiquitin–proteasome pathway (6, 8). A second, O2-requiring posttranslational modification of HIF-1α/-2α targets a single asparagine residue within the subunits’ C-terminal transactivation domain (CAD). It is catalyzed by an asparaginyl hydroxylase called factor inhibiting HIF-1 (FIH-1) to prohibit HIF-α:coactivator interaction and suppress transactivation of genes under high oxygen (9, 10). During hypoxia, both PHD and FIH-1 activities are progressively inhibited, leading to α-subunit accumulation, α/β-subunit dimerization in the nucleus and binding of the heterodimer to hypoxia response elements (HRE) within target genes. Being members of canonical CANNTG E-box...
motifs, HREs consist of a consensus 5'-VNVBRCGTG-3' (V = not T; N = any; B = not A; R = A or G; ref. 11). To date, several hundred potential (12) and more than 70 validated (11) hypoxia-responsive and HRE-flanked gene targets of HIF-1 have been identified. Through this transcriptional outflow, HIF-1 is able to reprogram cellular metabolism, growth, apoptosis, and O2 supply in response to declining pO2 (11).

Nonredundant roles of these hydroxylase systems in the regulation of HIF-1α/−2α activities were only recently unraveled when it became clear that the Michaelis constant (Km) of all 3 PHDs and FIH-1 predicted a distinctly lower oxygen affinity for the former (13). Consequently, PHD1-3 hydroxylases start to experience, relative to FIH-1, inactivation at higher pO2 during progressing hypoxia (14). Differential hydroxylase activities will eventually translate into a differential regulation of HIF-1 targets. By combining transcriptional profiling data (15) with a numerical model of the regulatory dynamics of the FIH-1 and PHD oxygen sensors along a virtual oxygen gradient (16), Pouysségur and colleagues were able to allocate HIF-1 targets into 2 categories: (i) FIH-1−inhibited genes, that is, those induced by progressive hypoxia once the NAD and, subsequently, CAD of HIF-1α/−2α are both released from inhibition (e.g., CA9, PHD3, and LDHA), and (ii) non−FIH-1−inhibited genes, that is, those requiring solely HIF-α NAD activity upon sufficient PHD inhibition while being refractory to any CAD activation (e.g., PGKI and GAPDH; refs. 15, 17). This categorization predicts expression of FIH-1−inhibited genes to be altered during severe hypoxia, whereas moderate degrees of O2 deprivation already affect non−FIH-1−inhibited genes.

Yet, as another and hydroxylase-independent layer of control, HIF’s transcriptional outflow is also prone to be influenced by competing transcription factors. When we previously used reporter constructs of the tripartite globin-2 gene (hb2) promoter (phb2) of the planktonic crustacean Daphnia magna in heterologous transfections of human cancer cells, we noticed a constitutive CACGTG-binding factor which was able to interfere with the HIF-1 transcriptional output as previously reported (19, 20). All primers used for qPCR are listed in Supplementary Table S1.

Antibodies
Mouse monoclonal anti−ATF-1 antibody (25C10G, sc-270) and rabbit polyclonal anti-USF1 (C-20, sc−229) were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-USF1M, anti-USF2F, anti-USF2G, anti-USF2Z, and anti-USF2aO antibodies were kindly provided by Dr. B. Viollet (21). Additional antibody gifts included: (i) rabbit anti-human DEC1 (CW27; ref. 22); (ii) rabbit anti-human MYC (23); (iii) rabbit anti-mouse ARNT (anti-mARNT R-1 IgG; ref. 24); (iv) rabbit anti-human ARNT (anti-hARNT C34; ref. 25); (v) rabbit anti-human USF full-length antibody (USF FL; ref. 26).

Sequence scan for HRE motif and CACGTG palindrome
We used the repeat-masked human genome sequence as provided by the UCSC Genomic Bioinformatics Website (Version hg19, GRCh37) at http://hgdownload.cse.ucsc.edu/goldenPath/hg19/bigZips/chromFaMasked.tar.gz. Gene definitions and transcription start sites were from http://hgdownload.cse.ucsc.edu/goldenPath/hg19/database/refGene.txt.gz (downloaded April 09, 2010). The search for motifs, implemented as R-script, was conducted among the 1,000 base flanking region up−and downstream (= ±1,000 bp) of annotated transcripts. Genes containing both HRE and CACGTG palindrome motifs within ±1,000 bp flanks were considered as HRE/palindrome gene only if the motif−motif distance was 100 bp or less. On the basis of these criteria, we used the GeneGo MetaCore system to identify pathways where HRE/palindrome genes are overrepresented (see Table 1).

Luciferase reporter
With genomic DNA, we amplified the promoter region around the HRE and E-box palindrome motifs via nested PCR (for primers, see Supplementary Table S1).
the 3′-end of any given amplicon is always extended into the first coding exon of the respective gene. In detail, we amplified and cloned the following promoter regions (start/end always relative to translation start ATG codon): (i) human 4EBP1 gene, −518/+403; (ii) human MCIR gene, −880/+9; (iii) human LDHA gene, −2,617/+530; (iv) human TYR gene, −400/+108. Following TOPO cloning of the PCR products into the pCRII-TOPO vector (Invitrogen), the inserted insert was ligated into pGL3-basic luciferase vector (Promega AG) to generate the luciferase reporter constructs. We also obtained the BNIP3/pGL3-basic (−753/+3; ref. 27) and the PHD2/pGL3-basic (−607/+3; ref. 28) luciferase reporter vector as kind gifts. HIF-1α (i.e., pcDNA3.1-hHIF-1-PK tag) as well as USF (pCR3-USF1, pCR3-USF2a, and pCR3-USF2b) expression plasmids were generously supplied by Prof. P. Maxwell (29) and Dr. B. Viollet (30), respectively.

Electrophoretic mobility shift assay and pull-down assay
Isolation of nuclear protein extracts of Hep3B, HeLa and MCF7 cells, and analysis of in vitro protein–DNA interaction by electrophoretic mobility shift assay (EMSA; ref. 18) and pull-down assays (31), was done as previously reported. All oligonucleotide sequences used as probes for either assay are shown in Supplementary Table S1. For EMSA gel supershifts (ss), 1.0 to 1.5 μL of rabbit anti-USF1M, rabbit anti-USF2G, or mouse anti-HIF-1α (mrc3) was added into the reaction (30 minutes, room temperature). Negative supershift controls included 1.5 μL preimmune serum from the same rabbit to be immunized against USF1M or USF2G, as well as 1.0 μL rabbit anti-human immunoglobulin (IgG; code: 309-005-003 Jackson Immuno Research). About pull-down assays, wild-type and mutated phb2 −146 palindrome or −107 HRE oligonucleotides, and wild-type and mutated BNIP3 −251/−246 HRE oligonucleotides, biotinylated at the 5′-end and PAGE purified, were annealed into double-stranded DNA and immobilized on streptavidin-coated magnetic beads (Dynal Biotech) as described (31).

Western blot and coimmunoprecipitation
Proteins were resolved in 10% SDS polyacrylamide gels, transferred onto nitrocellulose membranes (Whatman), and the membranes incubated at 4°C overnight with the following primary antibodies diluted in 5% milk Tris-buffered saline and Tween 20: (i) anti-HIF1α (mrc3; 1:500) or (ii) anti-USF1M or anti-USF2G (1:750). The signal was detected with horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit (1:5,000) and luminol substrate. For coimmunoprecipitation experiments, 150 μg nuclear protein was incubated with 20 μL mouse anti-HIF-1α or 0.75 μg anti-mARNT or 2.5 μL USF antisera and subsequently rotated at 4°C overnight. The next day, 40 μL of protein G beads were added into the mix and incubated at 4°C for another 2.5 hours. The extract/antibody/bead mix was collected by centrifugation, the pellet boiled at 95°C in 1× SDS sample buffer for 10 minutes, and the supernatant analyzed by Western blot.

Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) assays were conducted in human Hep3B, HeLa, and MCF7 cells after a 4-hour exposure to normoxic (air) or hypoxic (1% O2) atmospheres as described (32). In brief, genomic DNA was cross-linked with bound proteins (10 minutes, room temperature) with 1% formaldehyde in 1× PBS and sonicated in a Bioruptor UCD-200 (Diagenode SA) or a Sonifier cell disruptor B15 (Branson) into 500- to 1,000-bp fragments. For immunoprecipitation of the DNA:protein mix, 4.5 μL rabbit polyclonal anti-HIF-1α IgG (ab2185; Abcam) or 10 μL rabbit polyclonal anti-USF1M or anti-USF2G were added into the chromatin solution. Preimmune rabbit antiserum (10 μL) and 2.5 μL rabbit anti-human IgG were used as negative controls. The purified DNA was amplified by PCR using the ChIP primer pairs shown in Supplementary Table S1.

Transient luciferase reporter transfection
Half-confluent Hep3B, HeLa, and MCF7 cells were transfected overnight by the help of the calcium phosphate method with different luciferase reporter constructs and normalization plasmids expressing β-galactosidase. For cotransfections, 15 to 500 ng HIF-1α plasmid and/or 15 to 100 ng USF1, USF2a, or USF2b plasmid were added. In each transfection, pUC18 plasmid was used as filler DNA for a total of 2 to 3 μg DNA. The following day, each batch of transfected cells was split into 2 for parallel 16 hours normoxia and hypoxia exposure. After 16-hour normoxia/hypoxia exposure, cells were lysed and luciferase activity was measured with a commercially available Luciferase Assay System (Promega AG) and a SIRIUS Luminometer (BertHolt Technologies). Luciferase activity was normalized by β-galactosidase activity (β-Galactosidase Enzyme Assay kit; Promega AG) and expressed as “relative luciferase activity” in percent (% RLA) of the total activity of all normoxic and hypoxic reactions of a given assay.

Transient knockdown of HIF-1α, USF1, or USF2a
For transient silencing, the specific siRNA HIF-1α and siRNA USF1 oligonucleotides were selected based on previous publications (33–35). All siRNA sequences (see Supplementary Table S1) were synthesized by Dharmacon Research Inc. SiCONTROL nontargeting siRNA pool #2 was used as scrambled (scr) siRNA control (Dharmacon). Half-confluent Hep3B cells were transfected with a total of 200 nmol/L of siRNAs using Oligofectamine reagent (Invitrogen). In the combined USF1 + USF2a siRNA transfection targeting both USFs, 100 nmol/L of each siRNA were added to the cells.

Statistics
With STATA 10.0 software (Stata 10.0; StataCorp), we compared control versus experimental mean transcript expression levels (Fig. 3) and RLAs for each reporter assay (Fig. 6) within the same oxygen category (either normoxic or hypoxic results; Figs. 3 and 6) or for the hypoxic/normoxic fold inductions (Fig. 3, Table 3). In accordance with prior
Table 1. Top 10 pathways enriched with HRE/CACGTG genes

<table>
<thead>
<tr>
<th>GeneGo pathway name</th>
<th>Pathway description</th>
<th>( P )</th>
<th>Network objects</th>
<th>Enriched network gene symbols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin regulation of translation</td>
<td>Control of cap-dependent mRNA translation— from Insulin receptor (INSR) to PI3K, AKT, mTOR, and eIF4F</td>
<td>3.108e-6</td>
<td>10/42</td>
<td>eEF2K, eEF2B5, eIF4E, 4EBP1, eIF4G1, eIF4H, INSR, MAPK1, PDK1, PRKcz;</td>
</tr>
<tr>
<td>Androgen Receptor (AR) nuclear signaling</td>
<td>Control of cell adhesion, apoptosis, and cholesterol metabolism via AR</td>
<td>6.079e-6</td>
<td>10/45</td>
<td>EGFR, IGF1R, KLK3, MAPK1, RAD9A, SCAP, SMAD3, SD5A1, STAT3, WNT10A;</td>
</tr>
<tr>
<td>Role of IL-8 in angiogenesis</td>
<td>Control of angiogenesis + cell migration via interleukin 8 + VEGFA</td>
<td>1.064e-5</td>
<td>11/58</td>
<td>ACTR3B (¼ ARP3B), CARD11, EGFR, FASN, MBTP1 (¼ S1P), NFκBIE (¼ IκB), PDK1, RELB (NFκB), SCAP, SREBF1, STAT3;</td>
</tr>
<tr>
<td>Receptor-mediated HIF regulation</td>
<td>Control of HIF-1α translation in normoxia— from INSR → mTOR</td>
<td>1.288e-5</td>
<td>9/39</td>
<td>eIF4E, 4EBP1, HIF1A, IGF1R, INSR, IRS4, MAPK1, PDK1, PRKcz;</td>
</tr>
<tr>
<td>Cytoskeleton remodeling</td>
<td>Control of cell adhesion + motility— from extracellular matrix to actin filaments</td>
<td>3.350e-5</td>
<td>14/102</td>
<td>ACTN2, ACTR3B (¼ ARP3B), BCAR1, CFL2, eIF4E, 4EBP1, eIF4G1/3 (eIF4G1, eIF4G3), eIF4H, LIMK1, MAPK1, PLAT (¼ tPA), RPS6KA5 (¼ 56K 90kDa sub., MSK1), SMAD3;</td>
</tr>
<tr>
<td>Ligand-independent activation of ESR1 and ESR2</td>
<td>Control of cellular proliferation + migration via estrogen receptor β</td>
<td>3.636e-5</td>
<td>9/44</td>
<td>DRD1 (¼ dopamine receptor D1, GPCR), EGFR, ESR2 (¼ ER beta), GNAS (¼ Galphα-s), IGF1R, MAPK1 (ERK1, ERK2), PDK1, PRKAR1B;</td>
</tr>
<tr>
<td>Role of SCF complex in cell-cycle regulation</td>
<td>Control of cell-cycle progression via protein ubiquitylation</td>
<td>8.933e-5</td>
<td>7/29</td>
<td>ANAPC (subunit 1/2/4 + FZR1), CDK4, CUL1/RBX1 E3 ligase (CUL1 + RBX1), E2F1, FBX05 (¼ Emi1), PLK1, SMAD3;</td>
</tr>
<tr>
<td>Insulin regulation of fatty acid metabolism</td>
<td>Control of fatty acid homeostasis via insulin/INSR and glucose</td>
<td>1.301 e-4</td>
<td>12/88</td>
<td>FASN, INSR, MAPK1, MBTP1 (¼ S1P), PDE3B, PDK1, PRKAR1B, SCAP, SLC2A4 (¼ GLUT4), SREBF1 (3 SREBP1 transcript variants);</td>
</tr>
<tr>
<td>Apoptosis and survival_BAD phosphorylation</td>
<td>Control of apoptosis and autophagy - from EGFR to mitochondria</td>
<td>1.685e-4</td>
<td>8/42</td>
<td>BAX, EGFR, GNAS (¼ Galphα-s), GNB2 (¼ Gbeta2), IGF1R, MAPK1, PDK1, PPM1A (¼ PP2C), PRKAR1B;</td>
</tr>
</tbody>
</table>

(Continued on the following page)
Table 1. Top 10 pathways enriched with HRE/CACGTG genes (Cont’d)

<table>
<thead>
<tr>
<th>GeneGo pathway name</th>
<th>Pathway description</th>
<th>Enriched network gene symbols</th>
<th>Network objects</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaMKII (sub. delta + gamma), DRD1 (dopamine receptor D1; GPCR), GNAS (Ga-s), IGF1R, MAPK1, PKA(AR1B, RPS6KA5)</td>
<td>Control of amino acid metabolism</td>
<td>CaMKII, DRD1, GNAS, IGF1R, MAPK1, PKA(AR1B, RPS6KA5)</td>
<td>8/44</td>
<td>2.366e-4</td>
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</table>

NOTE: Pathways with the most significant overrepresentation of HRE/CACGTG genes are listed with their GeneGo Name, a brief description and a P value indicating the significance of the overrepresentation of HRE/CACGTG genes. The Network objects column gives the number of network objects of the pathway encoded by HRE/CACGTG genes over the total number of network objects in the pathway. The last column shows the gene symbols of the HRE/CACGTG genes in the pathways. Bold gene symbols denote multiple HRE-CACGTG on July 11, 2017. © 2011 American Association for Cancer Research. mcr.aacrjournals.org Downloaded from candidates can be found in the gene Excel list available upon request. Abbreviation: PI3K, phosphoinositide 3-kinase.

Results

CACGTG palindromic E-boxes often serve as binding sites for several non-HIF basic helix-loop-helix (bHLH) transcription factors, including ARNT (36, 37), MYC (38, 39), USFs (21), STRA13/DEC1 (22), ATF-1, and CREB-1 (40). To identify the factor(s) responsible for the HIF-interfering constitutive activity at the −146 CACGTG element within the promoter of the hb2 gene (phb2) of *Daphnia magna* (18) and map the factor(s) occurrence across different cancer cells, we conducted an EMSA survey using normoxic nuclear extracts from human hepatoma (Hep3B), cervical carcinoma (HeLa), and breast carcinoma cells (MCF7). Because HeLa and MCF7 EMSA screens yielded compatible results, Fig. IA presents Hep3B data only (Fig. 1A). The protein components within the constitutive complex (cc) of the −146 phb2 binding activity were identified using specific antibodies directed against USFs, DEC1, MYC, ARNT, and ATF-1. Of these 5 factors screened by supershifts, only USF1 and USF2 were recognized as main *in vitro* binding factors of the −146 phb2 palindrome (Fig. 1A; lanes 3, 5, 7, 9, and 11) while all other factors either failed (MYC and ATF-1) binding this motif or interacted (DEC1) weakly with it (Fig. 1A, lane 15; 5%–10% of total pool). The preponderance of USFs as protein components in the HIF-interfering complex of *Daphnia’s* hb2 promoter prompted us to subsequently focus on this transcription factor family. Increasing the volume of anti-USF1M (left) and anti-USF2G (right) antiserum in the binding reaction reduced the intensity of the CACGTG complex in a dose-dependent manner (Supplementary Fig. S1A). Binding of USF proteins to the −146 phb2 E-box was clearly oxygen independent (Supplementary Fig. S1A).

We reevaluated our EMSA results through independent pull-down assays of Hep3B, HeLa, and MCF7 nuclear proteins with biotinylated phb2 oligonucleotides bound to streptavidin-coated magnetic beads. In a representative assay with HeLa normoxic and hypoxic nuclear extracts (Fig. 1B and C), wild-type biotinylated oligonucleotides (w-bio), containing the −146 CACGTG phb2 palindrome, were able to pull down 43 kDa USF1 (Fig. 1B), 44 kDa USF2a, and 38 kDa USF2b proteins in an oxygen-independent manner (Fig. 1C). In support of a specific interaction, competition assays (50× comp. lanes) or beads coated with −146 mutant (m-bio) E-box motifs (5’-CAATGT-3’) greatly reduced or abolished the USF pull-down. Similar results
were obtained with extracts from Hep3B and MCF7 cells (not shown). Further pull-down and coimmunoprecipitation analyses verified that (i) USF1/2 (CACGTG preference) and HIF-1 complexes (TACGTG preference) display high-affinity binding to distinct elements within phb2 and (ii) the USF–HIF interference within phb2 is DNA context dependent, because we, and others (41), could not detect any direct physical interaction between USFs and HIF subunits (see Supplementary Fig. S1B–E).

To move beyond the *Daphnia* hb2 promoter as a model for occurring cross-talk among E-box complexes, we adopted *Daphnia* phb2 coordinates to conduct a genome-wide screen and enrichment analysis for human genes that harbor, within 1,000 bases from their transcriptional frame (in up- and downstream direction), both a 5′-VNVBR/CAGTG-3′ HRE consensus motif (11) and a 5′-CACGTG-3′ palindrome with a motif–motif distance of 100 bp or less. According to these criteria, our survey found multiple examples of known HIF targets among the list of HRE/palindrome genes including VEGF factor C (VEGFC), LDHA, phosphoglucomutase 2, enolase 1, transferin (TF), eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), Bel-2/E1B 19 kDa interacting protein 3-like (BNIP3L), and Bel-2-associated X protein (BAX); an Excel file with the detected human HRE/palindrome candidate genes is available upon request). When we looked at the top-scoring GeneGo pathways, whose signaling components showed a highly significant enrichment of HRE/palindrome genes (see Table 1), we noticed several signal transductions where USFs seem to impinge on hypoxia signals in a highly localized manner (e.g., insulin-regulated/cap-dependent mRNA translation, HIF-mediated transcription, and cytoskeletal or cell-cycle control functions). Interestingly, particular focal points of HRE/palindrome gene clusters included the elf4f checkpoint of the cap-dependent translation control, cell surface receptors for insulin and growth factors as well as actin.

Figure 1. EMSA supershifts and pull-down analysis to identify the phb2-binding CACGTG complex in Hep3B cells. A, to identify factor(s) able to bind to the −146 CACGTG motif in phb2, we used the following antibodies in EMSA supershift reactions with Hep3B normoxic nuclear extracts as indicated in the figure underneath each lane. (–), no antibody; PI, preimmune serum; ns, nonspecific; cc, constitutive CACGTG complex; ss, supershifted CACGTG complex. From lanes 4 to 13, EMSA reactions were supplemented by corresponding PI and immune serum from the same rabbit used to generate anti-USF1-directed antibodies (e.g., lanes 4 to 5; PI-1M and anti-USF1M). Results reproduced in n = 3 independent assays. B and C, pull-down analysis with beads (w-bio) coated with −146 phb2 E-box–carrying oligonucleotides (5′-CACGTG-3′) and HeLa normoxic (N) and hypoxic (H) nuclear extracts. Binding specificity was assessed either through beads coated with −146 mutant (m-bio) E-box motifs (5′-CAATGT-3′) or with binding reactions containing 50-fold molar excess of free wild-type oligonucleotide as competitor (50× comp.). Immunoblot of bound factors with α-USF1M antibody (C, left), α-USF2aO antibody (C, right). Staining of ns proteins indicated as loading control. Results reproduced in n = 3 to 4 independent assays.
remodeling genes (see Table 1; Gene symbols). These pathway aggregations of possibly coregulated genes inspired this study to try and provide solid proof-of-principle for a USF-based modulation of, or interference with, the HIF transcriptional outflow for at least some of the known targets listed above. If successful, future work will need to comprehensively assess USF–HIF cross-talk in cancer cells in a physiologic context (see Discussion).

For initial insights on HIF/USF convergence at DNA level, we (i) established those genes with a strong human–mouse–rat (hmr) conservation of the HRE/palindrome motifs within the aligned promoter regions (Table 2; Supplementary Fig. S2) and (ii) examined the mRNA expression of 5 such hmr-conserved HRE/palindrome candidates by quantitative real-time PCR (qRT-PCR) in conjunction with transient, siRNA-based knockdowns of HIF-1α, USF1, and USF2a in Hep3B (see Fig. 2 for knockdown efficacy assessment by Western blot). Transfection with scrambled RNA did not affect steady-state abundance of HIF-1α, USF1, and USF2a proteins [scrambled; compare with nontransfected (non-TF) cells]. In contrast, exposing Hep3B cells to siRNAs specifically directed against HIF-1α (siHIF-1α), USF1 (siUSF1), USF2a (siUSF2a), or the combination of both USFs (siUSF1/2a) resulted in a drastically diminished expression of the respective factor(s). In cells subjected to a USF1 knockdown (siUSF1), effects on the expression of USF2a ranged from unaltered to a slight elevation. Conversely, silencing of USF2a (siUSF2a) was accompanied by a strong reduction of USF1 protein level (Fig. 2, USF1 signal in siUSF2a). Similar to these findings, both USFs were concomitantly lost in USF2 knockout mouse models, suggesting USF2 to be generally required as USF1 transactivator (42).

Following transient knockdown of HIF-1 and USFs in normoxic and hypoxic Hep3B cells, changes in mRNA levels of the HRE/palindrome candidates LDHA, BNIP3, BNIP3L, 4EBP1, and VEGFC were assessed by qRT-PCR (Fig. 3). Transcripts of BNIP3, BNIP3L, LDHA, and VEGFC were all upregulated by 1% O2/16-hour exposure in Hep3B treated with scrambled RNA. The 4- to 5-fold hypoxic induction of the BNIP3 and BNIP3L mRNA levels was entirely (BNIP3) or almost entirely (BNIP3L) driven by HIF-1α (see siHIF-1α data). Silencing USF1 and USF2a expression, however, resulted in moderately (siUSF1) or significantly (siUSF2a) increased fold hypoxic inductions of BNIP3 and BNIP3L genes. The stronger effect on the hypoxic induction of BNIP3 and BNIP3L genes by the siUSF2a treatment could result from its effective double treatment but not impacted by either USF knockdown. Expression of 4EBP1, mildly suppressed in hypoxic hepatoma cells (scr data), was constitutively reduced upon the knockdown of USF factors. The approximate 2-fold hypoxic induction of VEGFC mRNA (scr data) was lost upon USF siRNA treatment due to a strong increase of transcript level in normoxic Hep3B cells. With regard to the vividly O2-responsive BNIP3 and BNIP3L genes, we also noted that the potentiation of the induction in USF-silenced cells subjected to 1% O2 predominantly derived from a reduced normoxic, rather than strengthened hypoxic, gene activation (Fig. 3). This observation highlighted the importance of USF1/2a in maintaining the basal transcription of either gene in oxygenated cells. Because HIF-1 is known to regulate BNIP3 activity with an unusually broad O2 response profile (15, 17), the factor could likely encounter USFs at the BNIP3 promoter even in normoxic cells. In additional qRT-PCR analyses, we therefore assessed if, during episodes of moderate (3% O2) or very mild degrees of O2 scarcity (10% O2), HIF-1

### Table 2. E-box palindromes and HRE sites in promoters of human genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5’–3’</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>TYR</td>
<td>gaaaagtctgCATGTCtccttcca-gccaagaCATGTCataat-aggaaga (atg)</td>
<td>(67)</td>
</tr>
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<td>PHD2</td>
<td>gccggtgtgTACGTCcagacgacgcagcag-gcgcagcgcgcgcc (atg)</td>
<td>(28)</td>
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<tr>
<td>4EBP1</td>
<td>ggggatcCAGGTCgaagc-caaatcctcaggGCGGTGccgcgc-gagacc (atg)</td>
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<td>LDHA</td>
<td>cccgcaCATGTCgacgacccca-CATGTCgcgccgccgc-aat (atg)</td>
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<tr>
<td>MC1R</td>
<td>acgttgtaCAGGTCaggtgtctg-ccccggCATGTCgccgccct-gacagagact (atg)</td>
<td>(61)</td>
</tr>
<tr>
<td>BNIP3</td>
<td>cccgccaCAGGTCccacagcgcaccacc-gcccttgccgcc (atg)</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: HIF-1 and USF coregulated candidate genes: 4EBP1, LDHA, MC1R, and BNIP3; control genes: TYR and PHD2. Translation start site ATG as +1 (in brackets). 5′-flanking region upstream of ATG is given for human TYR, PHD2, 4EBP1, LDHA, MC1R, and BNIP3 genes. HRE and E-box palindromes are capitalized. Human–mouse–rat (hmr) conserved HREs: bold + underlined; variable HREs: bold only; hmr conserved E-boxes: italicized + underlined; variable E-box: italics only. For conservation: see alignments in Supplementary Fig. S2.
and 72 hours. Hypoxia (H, 1% O2) exposure started at time point 42 hours post-siRNA transfection for 6 and 30 hours, respectively. Normoxia (N): air exposure. Scrambled siRNA (scr) and nontransfected (non-TF) cells were used as negative controls. Cells were harvested at 2 post-siRNA transfection time points: 48 and 72 hours hypoxia) up to 72 hours (H/N ratio ≈ 10% O2 [1.6-fold (scr) vs. normoxic [1.0 (scr)] and to a significant extent at 10% O2 [1.6-fold (scr) → 2.2-fold (siUSF1); P = 0.019; Table 3]. This USF1 loss-of-function mediated enhancement resulted from the combination of reduced normoxic [1.0 (scr) → 0.7 (siUSF1)] and, toward milder hypoxia, progressively increasing hypoxic levels of BNIP3 transcripts [with 100% mRNA level (scr) → 84%–95%–105% mRNA level at 1% O2–3% O2–10% O2 (siUSF1)].

Although we were encouraged by these early data suggesting a competitive cross-talk to occur between basally active HIF-1 and USF factors in cells facing a subnormoxic milieu, the remainder of the study focused on providing proof-of-principle evidence for USF-mediated positive or negative functional interactions with HIF-1 at 1% O2. At this level of deoxygenation, HIF-1 activity peaks in many cell lines, hence, its transcriptional control of the bulk of downstream targets is expected to operate with optimal efficacy. Alignments of the homologous regions of BNIP3 promoters had revealed a remarkable hmr conservation around the HRE motif (Supplementary Fig. S2). For this reason, we went on to compare the promoter responses of BNIP3, where the HRE and CACGTG motifs are contained within a single cis-element, with those of MC1R, 4EBP1, and LDHA genes, which all possess distinct HRE and palindrome sites of variable hmr conservation (Table 2; Supplementary Fig. S2). Parallel ChIP experiments confirmed the recruitment of HIF-1α- and constitutive USF1- and USF2a-containing complexes to both LDHA (Fig. 4A) and BNIP3 (Fig. 4B) promoter sequences in intact Hep3B (Fig. 4, left), MCF7 (Fig. 4, right), and HeLa cells (not shown). This coordinate binding of HIF-1 and USFs was seen both at low oxygen (at LDHA + BNIP3 promoter) and in oxygenated nuclei as well (see HIF-1α at BNIP3 promoter; Fig. 4). The latter finding added weight to the notion of HIF-1 controlling BNIP3 transcription even under subnormoxic/normoxic conditions (see previous paragraph).

To further study the convergence of HIF and USF pathways at DNA level, the promoter regions in question were amplified from genomic DNA, cloned, sequence confirmed, and inserted into pGL3 basic luciferase reporter plasmids. The set of luciferase promoter reporters thus obtained included both donated (i.e., BNIP3 and PHD2) and self-generated constructs (TYR, 4EBP1, LDHA, and MC1R),
and covered USF-specific targets (i.e., TYR; ref. 43) or HIF-1–specific targets (i.e., PHD2; ref. 44) plus 4 HRE/palindrome candidates (4EBP1, LDHA, MC1R, and BNIP3; Table 2; Supplementary Fig. S2). We initially examined the hypoxia (1% O2/16 hours) responsiveness of these candidate promoters. Respective reporter transfections of Hep3B, MCF7, and HeLa cells included negative control reactions (i.e., "bVec" = empty pGL3 basic luciferase vector; Fig. 5) to monitor basal and hypoxia nonresponsive luciferase activity. In contrast, the vector; Fig. 5) to monitor basal and hypoxia nonresponsive luciferase activity. In contrast, the vector; Fig. 5) to monitor basal and hypoxia nonresponsive luciferase activity. In contrast, the vector; Fig. 5) to monitor basal and hypoxia nonresponsive luciferase activity. In contrast, the vector; Fig. 5) to monitor basal and hypoxia nonresponsive luciferase activity.

Next, we investigated the possible coregulation of BNIP3 and LDHA reporter by HIF and USF cascades in cotransfections with HIF-1α and USF1, USF2a, or USF2b expression plasmids (Fig. 6). In pilot studies (not shown), we had carefully titrated for each cell line the amount of HIF-1α plasmid (i.e., 15–100 ng) needed for an optimal hypoxic induction of either reporter and of any USF plasmid (i.e., 15–100 ng) needed for an optimal specific activation of either reporter construct (Fig. 6). The activity of the TYR promoter reporter rose constitutively 4- to 7-fold upon USF1-, and up to 20-fold upon USF2a, cotransfection. Overexpression of HIF-1α did not impact the TYR reporter (Fig. 6A). On the contrary, PHD2 reporter activity was significantly increased in normoxic and hypoxic Hep3B upon HIF-1α overexpression. The impact of overexpressed USFs on the PHD2 reporter was either negligible (USF1) or attributed to nonspecific stimulation by the overexpressed factor (USF2a) exerted on the vector backbone. Therefore, both control reporters responded specifically to the overexpression of their respective transcriptional driver(s) (Fig. 6A).

The LDHA promoter was induced by endogenous hypoxia signals almost 2-fold in Hep3B (Fig. 6B), and additionally stimulated upon cotransfection with USF1, and particularly, USF2a and USF2b plasmids. Of note, overexpressed USFs augmented LDHA luciferase activity predominantly under normoxia, thereby reducing the original
hypoxic induction to an almost constitutive expression in Hep3B (Fig. 6B) and MCF7 cells (not shown). The switching from HIF- to USF-driven transactivation modes, and vice versa, was further observed for the endogenous LDHA promoter, particularly in Hep3B (Fig. 4A, left) and HeLa cells (not shown). Here, hypoxia clearly promoted HIF-1 binding and, in parallel, attenuated the occupancy of USF1 and USF2a. Thus, HIF-1 and USF complexes recruited to the LDHA promoter cap the activity of one another to yield a pO2-dependent complementation mode of gene control.

On the basis of these data, transactivation of the LDHA gene by HIF-1 and USFs proceeds from distinct motifs (below) and peaks at varying pO2 (USFs ! aerobic LDHA expression; HIF-1 ! hypoxic LDHA expression). In contrast, both pathways must converge onto a single stretch of DNA within the BNIP3 promoter (Supplementary Fig. S2, and below). The relevant reporter was nearly 3-fold hypoxia induced by endogenous HIF-1α–mediated (Fig. 3) signaling in Hep3B (Fig. 6C) and HeLa cells (Fig. 6D). Cotransfection with USF1 and USF2a, or with USF2b, enhanced BNIP3 promoter activity in both cell lines particularly under normoxia and consequently weakened the reporters’ hypoxic induction. Overexpression of HIF-1α amplified the hypoxic BNIP3 activity robustly in Hep3B (6.6-fold; Fig. 6C) and moderately in HeLa cells (3.2-fold, Fig. 6D). However, this potentiated hypoxia response of BNIP3 by ectopic HIF-1α was significantly impaired through the simultaneous cotransfection with USF1 or USF2a, but not USF2b, in Hep3B and HeLa cells (Fig. 6C and D, see ↓ arrows). Increasing the amount of USF1/2a plasmids further (15 → 100 ng) converted the hypoxic transactivation of the reporter into an increasingly constitutive response, especially in Hep3B cells (Fig. 6C). These data suggest that

Table 3. BNIP3 mRNA fold inductions in mildly deoxygenated Hep3B transfected with siRNAs

<table>
<thead>
<tr>
<th>O2</th>
<th>scr</th>
<th>siHIF-1α</th>
<th>siUSF1</th>
<th>siUSF2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>3%</td>
<td>3.325 ± 0.754</td>
<td>1.078 ± 0.148</td>
<td>4.583 ± 0.472</td>
<td>4.222 ± 0.907</td>
</tr>
<tr>
<td>P (siX vs scr)</td>
<td>—</td>
<td>P = 0.0072</td>
<td>P = 0.0704</td>
<td>ns</td>
</tr>
<tr>
<td>10%</td>
<td>1.622 ± 0.177</td>
<td>1.056 ± 0.038</td>
<td>2.145 ± 0.159</td>
<td>1.560 ± 0.313</td>
</tr>
<tr>
<td>P (siX vs scr)</td>
<td>—</td>
<td>P = 0.0056</td>
<td>P = 0.0189</td>
<td>ns</td>
</tr>
</tbody>
</table>

NOTE: Hep3B transfections with scrambled (scr) control RNA or HIF-1α, USF1 or USF2a siRNA followed the identical protocol used for the quantitative expression analyses in Fig. 3. Bnip3 mRNA levels were quantified by qRT-PCR and normalized by L28 expression. Fold inductions of Bnip3 transcript levels in mildly deoxygenated (H: 3% or 10% O2; 16 hours) versus normoxic (N: air; 16 hours) Hep3B are given as means (± SD) of 3 independent experiments. P values for H/N-fold changes between siRNA-treated experimental groups (i.e., siX = siHIF-1α or siUSF1 or siUSF2a) and the scr control group of the same O2 category (i.e., comparison done only within 3% or 10% O2 category) are indicated.

Figure 4. ChIP determination of in vivo HIF-1 and USF binding to LDHA and BNIP3 promoters. ChIP assay was carried out in Hep3B (left) and MCF7 (right) cells with indicated antibodies or preimmune serum and nonspecific antibody IgG as negative controls. Purified DNA was subjected to PCR with LDHA primer (A, 159-bp amplicon) or BNIP3 primer (B, 314-bp amplicon). N, air; H, 1% O2 4 hours. PI-2G preimmune serum for anti-USF2G; neg. PCR with H2O.
HIF-1α and USF1 or USF2a compete dose dependently with each other over the control of the BNIP3 site. The functionality of the computed HIF-1 and USF1/2 sites in the LDHA and BNIP3 promoter was assessed by EMSA screens with Hep3B, HeLa, and MCF7 normoxic and hypoxic nuclear extracts. Representative results are shown for LDHA (MCF7 nuclear extracts, Fig. 7A) and BNIP3 (Hep3B nuclear extracts, Fig. 7B). The wild-type CACGTG-motif in region I (Supplementary Fig. S2) of the LDHA promoter was weakly bound by a hypoxia-regulated complex containing HIF-1α [see supershift (ss)—lane 4; Fig. 7A] and avidly bound by the cc factors USF1 and USF2a (see ss in lanes 6, 7, 9, and 10; Fig. 7A, reg.I wt). Another radiolabeled oligonucleotide, spanning the wild-type region II and III (Supplementary Fig. S2; reg. II/III ww) was tightly bound both by HIF-1α and USF1/2 (Fig. 7A, bottom; detection of HIF-1α ss: lane 4; USF2G ss: 9 + 10). When using a reg. II/III double site oligonucleotide carrying a mutation in region II and an unaltered wild-type sequence in region III (reg. II/III mw), only the hypoxia-inducible complex, supershifted by anti–HIF-1α, was detected in conjunction with a complete loss of the constitutive binding activity by USFs (Fig. 7A: reg. II/III mw). The reverse sequence alteration in region III but not II (reg. II/III ww) left the oligonucleotide attachment by the constitutive USF complex undisturbed, but erased any interaction with HIF-1. Thus, the LDHA region II acts as exclusive, high-affinity site for USFs, whereas LDHA region III attracts HIF-1 to this promoter in deoxygenated nuclei.

In contrast to this segregated binding of HIF-1 and USFs in the LDHA promoter, either complex interacted with the −259/−236 DNA of the BNIP3 promoter containing the HRE at −251/−246 (Supplementary Fig. S2; Fig. 7B). Specific supershifts were able to positively identify HIF-1α (ss: lane 4) as constituent of a hypoxic binding activity (lane 2), and USF1 (ss: lanes 6 + 7) and USF2 (ss: lanes 9 + 10) as participants of a constitutive complex (lanes 1 + 2; Fig. 7B). We further elaborated whether the HIF–USF interplay at the −251/−246 core element of BNIP3 is governed by differential affinities of the respective factors. To that end, we conducted additional pull-down analyses using magnetic beads coated with w-bio BNIP3 HRE probes (Supplementary Table S1) and assessed binding specificity and avidity in reactions containing 10× or 50× excess of wt competitor (comp.) oligonucleotides or beads coated with mutant (m-bio) probes (Supplementary Fig. S3). Excess probe (10× and 50×) eliminated the initial binding activity (set to 100% in each case) to a mean (n = 3–4 independent assays) residual activity of: (i) approximately 8.8% (10×) and approximately 5.4% (50×) for hypoxic HIF-1; (ii) approximately 1.3% and approximately 0.4% for normoxic or hypoxic USF1; and (iii) approximately 2.6% and approximately 1.4% for normoxic or hypoxic USF2, respectively. The fact that 10×/50× excess probe sufficiently eliminated almost all of the USF–bead interaction showed the, relative to hypoxic HIF-1, much weaker in vitro affinity by which USF1 and USF2a constitutively bind to the BNIP3 HRE (Supplementary Fig. S3).

Figure 5. Endogenous response of PHD2, 4EBP1, LDHA, MC1R, and BNIP3 luciferase reporter. Hep3B (A), MCF7 (B), and HeLa (C) cells were transfected with 2.0 μg of 4EBP1, LDHA, and MC1R or 1.5 μg of PHD2 or BNIP3 luciferase reporter plasmid in a total of 3.0 μg plasmid. After 16-hour hypoxic (1% O2; H, black bars) or normoxic (air; N, white bars) exposure, RLA was determined and normalized with ß-galactosidase (null bVec PHD2; 4EBP1, LDHA, and MC1R or 1.5 μg of PHD2 or BNIP3 luciferase reporter plasmid) was used as negative controls. Mean H/N-fold inductions of each reporter are indicated above the respective pair of columns.
Discussion

One way to fine-tune, or inhibit, HIF’s transcriptional outflow independently of hydroxylase activities could be through competing transcription factors. We reported earlier (18) that binding of a Hep3B factor to CACGTG motifs was able to counteract the HIF-driven induction of the phb2 reporter from HREs at adjacent positions. Evidently, palindrome factors can engage in positive or negative cross-talk with nearby HIF/HRE complexes (45–47).

En route toward a more physiologic understanding of hypoxic signaling, we thought to analyze gene control mechanisms not just as a function of the stability/activity of HIF-1 per se. Rather, the dynamic interplay between transcriptional complexes that governs the hierarchy by which HIF-1 and related factors gain access to DNA and regulate expression was considered.

This study, thus, aimed to identify the phb2 CACGTG-binding entity in human cancer cells and investigate the factors interplay with HIF-1 in the control of selected examples of cotargeted genes.

Both, EMSA supershifts and oligonucleotide pull-down assays consistently identified USF1 and USF2a/2b as the main phb2 CACGTG complex in nuclear extracts from Hep3B and HeLa (Fig. 1) or MCF7 cell lines. Our

Figure 6. Regulation of BNIP3 and LDHA luciferase activity by overexpressed HIF-1α and USFs. A, Hep3B transfections of USF and HIF-1 control reporter with either 1.5 μg TYR plasmid or 0.5 μg PHD2 plasmid. B, Hep3B transfections with 0.3 μg LDHA reporter plasmid. Hep3B (C) or HeLa (D) transfections with 0.3 μg BNIP3 reporter plasmid. For reporter regulation by overexpressed HIF-1α and USFs, nanogram (ng) amounts of HIF-1α and USF expression plasmids used are indicated underneath the respective cotransfection. RLA is given as in Fig. 5 (mean ± SD; RLA; n = 3 independent experiments). N and H conditions and presentation of mean H/N-fold inductions as in Fig. 3. Statistics done in regard to same O2 category (i.e., N or H): (i) endo/exo test, endogenous controls (reporter alone; no overexpressed HIF/USF) versus HIF/USF cotransfection (reporter with exogenous HIF-1α and/or USF1/2a); (ii) hif/combi test, reporter + exogenous HIF1α versus reporter + exogenous (HIF1α + USF1/2a). Symbols for P < 0.05 for (i) nonparametric Wilcoxon rank-sum tests (endo/exo, #; hif/combi, ¶); (ii) ANOVA/Sidak tests (endo/exo, +; hif/combi, +). See Materials and Methods for more details.
pull-down assays also documented the preferential in vitro docking of HIF-1 to the asymmetric −107 phb2 HRE and of USFs to the symmetrical −146 phb2 palindrome motif (Supplementary Fig. S1B and C, 50× comp. lanes). Thus, the single base substitution within the hexameric core of either motif (i.e., −107 HRE: 5′-TACGTG-3′; −146 palindrome: 5′-CACGTG-3′), and presumably additional changes in neighboring nucleotides, are key in conferring the vastly differing affinities of HIF and USF transcription factors to these motifs. This observation fits well with the general perception that CACGTG-palindromes tend to attract non-HIF bHLH factors (25, 36, 37, 47), and, consequentially, are notably underrepresented amongst functional HIF elements (48–50). Our coimmunoprecipi-
tations further demonstrated the interactive precipitation of HIF-1α by ARNT proteins and vice versa but failed to reveal any physical contact between USF2a and either subunit of HIF-1 [HIF-1α and ARNT; see Fig. S1D, S1E and ref. 41]. Thus, the constitutive USF1/2α are the main factors that indirectly interfere with the HIF/HRE-driven induction of phb2 globin gene by binding to the phb2 CACGTG palindrome in human cancer cells (18, 31).

Upstream stimulatory factors belong to the bHLH-leucine zipper family of transcription factors (21, 51, 52). They can mutually influence each other’s expression, both in positive [USF2 transactivates USF1 gene (42); USF2a knockdown yields diminished USF1 levels; Fig. 2] and negative ways [USF1 represses USF2 gene; USF1<sup>−/−</sup> mice...
show elevated USF2 levels (42)]. USFs have been implicated in conferring the UV-induced tanning response in melanocytes and in acting as antiproliferative agents in cells transformed by overexpressed MYC or activated RAS signaling (53). Following a marked depletion of intracellular calcium during the differentiation of erythroid progenitor and erythroleukemia cells, endogenous USFs start to accumulate and transactivate several adult marker genes (e.g., β globin) that ultimately drive the cells into maturity (54). Beyond MYC, other palindrome complexes can also tailor, or interfere with, HIF’s transcriptional read-out. To date, 3 human genes have been examined as HIF/USF coregulated targets, that is, the genes encoding plasminogen activator inhibitor-1 (PAI-1; refs. 55–57), the catalytic subunit telomerase reverse transcriptase (TERT) of the telomerase complex (58–60), and the glycolytic enzyme 1-type pyruvate kinase (L-PK; ref. 41).

Guided by phb2 coordinates of Daphnia, we conducted a genome-wide computational survey for HIF/USF-coregulated human genes that were flanked by closely adjacent or overlapping CACGTG palindrome and HRE motifs. Among those, we found LDHA and BNIP3 to be expressed and hypoxia induced at transcript level in human Hep3B cells (Fig. 3). This induction was entirely dependent (BNIP3) or aided (LDHA), by HIF-1α in Hep3B cells. The O2-responsive control of BNIP3 via HIF-1α ranged, in Hep3B’s at least, from harsh (1% O2) to moderate (3% O2) to mild (10% O2) degrees of deoxygenation. Because the promoter of either gene recruited both HIF-1 and USFs in hypoxic Hep3B and MCF7 cells and hypoxia induced at transcript level in human Hep3B cells (Fig. 3). This induction was entirely dependent (BNIP3) or aided (LDHA), by HIF-1α in Hep3B cells. The O2-responsive control of BNIP3 via HIF-1α ranged, in Hep3B’s at least, from harsh (1% O2) to moderate (3% O2) to mild (10% O2) degrees of deoxygenation. Because the promoter of either gene recruited both HIF-1 and USFs in hypoxic Hep3B and MCF7 cells in vitro (Fig. 4), control of LDHA and BNIP3 expression was considered suitable to examine HIF/USF cross-talk at DNA level in greater detail. Previous studies had already validated human LDHA and BNIP3 genes as hypoxia-inducible HIF-1 targets in HeLa and MCF7 cells, respectively (48, 61).

Luciferase reporter of the LDHA (~2-fold) and BNIP3 (3.5- to 7-fold) promoter yielded a robust upregulation by hypoxic (1% O2/16 hours) conditions across Hep3B, HeLa, and MCF7 cells (Fig. 5). In cotransfection assays in Hep3B cells, overexpressed USFs were found to upregulate the LDHA reporter particularly in normoxic conditions (Fig. 6B). The role of USFs in transactivating LDHA in oxygenated cells implies the factors as candidate drivers of aerobic glycolysis in cancer cells (Warburg effect). A previous study had already described rat LDHA as MYC target and further noticed the weak upregulation of the gene by USFs under normoxia via binding of both E-box sites, regions I and II, within the rat LDHA promoter (62). The human LDHA promoter was, during low pO2, predominantly bound by HIF-1, which, in Hep3B and HeLa but not MCF7 cells, evidently served to displace LDHA-attached USFs (Fig. 4A).

Subsequent EMSA gel supershift assays revealed region I of the 5’ flank of the LDHA gene as weak HIF-1 and strong USF1/2a site. The region II palindrome and region III asymmetric E-box of LDHA, however, functioned as USF1/2 (reg. II) and HIF-1 (reg. III) binding sites, respectively (Fig. 7A). To rationalize these data, we surmise that, in Hep3B and HeLa cells at least, the sites in regions I to III do not seem to be segregated. Upon changes in pO2, they rather act as adaptable platform for the dominant transcription factor entity. Thus, in hypoxic Hep3B and HeLa cells, HIF-1 might variably expand its LDHA occupancy from its holdout at region III onto regions I and II as well and, by doing so, displaces bound USFs from these regions in intact cells. At the same time, the presence of distinct, nonoverlapping sites in the LDHA promoter forms the very foundation for the complementing control of this gene by HIF-1 and USF pathways (Figs. 6A and 7A). This complementation allows the HIF-driven expression of LDHA during hypoxia to eventually switch to a USF (and MYC)-mediated control under high pO2 which, in turn, ensures production of this glycolytic enzyme in response to a broader O2 spectrum and additional microenvironmental stimuli of solid malignancies (i.e., acidic milieu).

Cotransfection of Hep3B and HeLa cells with HIF-1, USF1, and USF2a revealed the dose-dependent interference with the HIF-1-mediated BNIP3 induction at 1% O2 by USFs (see arrows, Fig. 6C and D). Conversely, the transient loss of USF1 + USF2a functions (siUSF2a treatment, Fig. 2) or of USF1 activity alone (siUSF1 treatment), resulted in Hep3B cells in a significantly augmented induction of BNIP3 (and BNIP5) genes at harsh (1% O2) and mild (10% O2) degrees of deoxygenation, respectively (Fig. 3, Table 3). These data support the notion of an increasing competition of HIF’s BNIP3 control by endogenous USF1 in mildly O2-deprived cells (see also below). The HRE at position −251/−246 in the promoter of the human BNIP3 gene was first identified by Kothari and colleagues to function as a direct functional binding site for HIF-1 under hypoxia (61). Our EMSA screen with a single site oligonucleotide (−259/−236; Supplementary Table S1) characterized this HRE-containing sequence as being cotargeted by HIF-1 and USFs in hypoxic (1% O2/16 hours) cells (Fig. 7B). Additional pull-down assays revealed hypoxic (1% O2/16 hours) HIF-1 complexes to dock, in vitro, much more tightly to the BNIP3 HRE than do USF1 and USF2a factors (Supplementary Fig. S3). Because pull-downs use, EMSA-like, nuclear extracts with free, DNA-dissociated α-subunits, they cannot provide insights on the binding of normoxic HIF-1 to the αi-element in question. Nonetheless, we extrapolate a markedly inferior affinity of USFs to the BNIP3 HRE when compared with hypoxic HIF-1 complexes under in vivo conditions as well.

Although USF expression manipulations affected BNIP3 gene/reporter activity particularly in normoxia [i.e., note BNIP3 inductions at 1% O2 in (i) Fig. 3, USF1/2a knockdown: 4.8-fold (scr) → 6.6- to 7.6-fold knockdown; (ii) Fig. 6C and D; USF1/2a overexpression: 2.8-fold (endogenous) → 1.5- to 1.8-fold (overexpression)], several additional observations implied the USF/HIF convergence onto BNIP3 to follow far more intricate rules than a LDHA-like segregation between normoxic/USF and hypoxic/HIF occupations would suggest. First, ChIP analysis of all 3 cell lines (Hep3B, MCF7, and HeLa) showed measurable amounts of HIF-1 during normoxia, and of USF1/2 complexes during normoxia and hypoxia, attached to the BNIP3 promoter chromatin (Fig. 4 and data not shown). Second, the USF1/2
broadened O2 sensitivity. In contrast, the single BNIP3
allow switching between USF/normoxic and HIF-1/hypoxic
(63).
proin
onset of cellular senescence in aerobic murine embryonic
documented HIF-1 to be strictly necessary in delaying the
exception is a study by Welford and colleagues which
mildly deoxygenated or completely aerobic cells. A noted
when it comes to events emanating from HIF signaling in
the hypoxia-inducible factor during low pO2 (Fig. 7B),
dimers, tethered to the same −251/−246 core sequence as
the hypoxia-inducible factor during low pO2 (Fig. 7B),
showed virtually no signs of displacement by incoming
HIF-1 (Fig. 4). Such a persisting attachment of USF factors
during hypoxic periods of HIF-1 occupancy could be
achieved by the presence of secondary docking sites within
the BNIP3 promoter. Indeed, we noted hnrm conserved
CACGCC motifs dubbed E1 and E2, separated by 3-
nucleotide spacers on either side of the −251/−246 BNIP3
HRE (i.e., 5′-CACGC GCCAGTG ACGCAGCA-3′; E1/E2 = capital; spacers = small; HRE = capital/bold; see Table 2; Supplementary Fig. S2). Subsequent EMSA experiments with triple site E1-HRE-E2 oligonucleotide probes in www (all 3 sites intact) and mwm (HRE intact, E1 + E2 mutated) configuration revealed for hypoxic
extracts (HIF-1 docked to HRE) a 1.3- to 2.3-fold stronger
binding of USF complexes to www versus mwm sequences
(not shown). Thus, intact E1/E2 motifs (and several other
CACGCN promoter sites) can temporarily provide alterna-
tive USF sites at the BNIP3 HRE. When HIF-1 approaches
the BNIP3 HRE during low pO2 in vivo, USFs may be able to
sidestep displacement by sliding onto E1 and E2.

The fact that we detected HIF-1α tethered to the BNIP3
promoter in normoxia only in vivo (ChIP), but not by in
vitro (EMSA, pull-down) measures, may highlight the pro-
tective effect of chromatin and/or the required HIF-1
heterodimer under conditions where free α-subunits are
all but depleted due to ongoing hydroxylation and degrada-
tion. Because none of the usual proinflammatory agents (e.g.,
cytokines, growth factors, and reactive oxygen species),
known to spark a strong nonhypoxic HIF-1α accumulation
and induced expression of HIF-1 targets, were added to our
cultures the select interaction of HIF-1 with the BNIP3
promoter inoxic cells seems to occur independently of
changes in cytokine/growth factor/ROS concentrations.
About the question whether DNA-bound HIF-1 in oxic
cells maintains transcriptional functionality, we found that,
in Hep3B cells subjected to 16-hour periods of 3% and 10%
O2, HIF-1α still manages to drive the induction of BNIP3
(Table 3). Overall, however, our knowledge is meager at best
when it comes to events emanating from HIF signaling in
mildly deoxygenated or completely aerobic cells. A noted
exception is a study by Welford and colleagues which
documented HIF-1 to be strictly necessary in delaying the
onset of cellular senescence in aerobic murine embryonic
fibroblasts, in part via the transcriptional control of the
proinflammatory macrophage migration inhibitory factor
(63).

Taken together, the distinct sites of the LDHA promoter
allow switching between USF/normoxic and HIF-1/hypoxic
states, in favor of a complementing expression profile with a
broadened O2 sensitivity. In contrast, the single BNIP3
sequence around the −251/−246 CACCTG motif seems to
be the platform for a pO2-dependent, conditional compe-
tition between USF1/2a and HIF-1 [i.e., (i) normoxic/sub-
normoxic state: USF → HIF-1; (ii) hypoxic (1% O2) state:
HIF-1 only; with ↔ = competition]. If so, this conditional
competition would argue for different DNA affinities and/or
transactivation competences of normoxic and hypoxic HIF-
1 complexes, respectively. Inadequate α:β subunit inter-
actions, however, are unlikely be involved in debilitating
affinity or transcriptional capacity of normoxic HIFs (rela-
tive to species at 1% O2). Recent fluorescence resonance
energy transfer measurements revealed, at least for proteol-
ysis-saturating levels of overexpressed HIF-2α and ARNT,
identical α:β distances in normoxic and hypoxic HIF-2
heterodimers (64). Although the assembly of HIF in oxy-
genated and severely hypoxic nuclei appears to match one
another, the α-subunits in such high pO2, HRE-tethered
dimers should show hydroxylation at ODD-prolyl and/or
CAD-asparaginyl residues.

The recent categorization of HIF-1 targets into PHD-
driven by HIF-1α NAD activity) and PHD/FIH-depen-
dent cohorts (driven by HIF-1α NAD + CAD activity) has
yielded some surprising new insights about the O2 profiles of
LDHA and BNIP3 genes (15–17). Here, BNIP3 expression
was characterized by extremely low FIH-1 sensitivity scores,
indicating full responsiveness of the gene by marginal drops
of pO2 and transcriptional gene induction (rather than
inhibition) by active FIH-1. In line with this concept, we
find BNIP3 to be induced via HIF-1α in hepatoma cells at
1%, 3%, and 10% O2 (Fig. 3, Table 3). Although possible
functions of BNIP3, in addition to the factors proapoptotic/
proautophagic dichotomy (65, 66), need to be clarified for
mildly versus profoundly hypoxic cancer cells, the binding of
HIF-1 to the BNIP3 promoter under a wide range of pO2,
and to the LDHA sites selectively during hypoxia, agrees
with such nonredundant impacts of PHDs and FIH-1 on the
activity of these HIF targets. In USF1−silenced Hep3B,
BNIP3 mRNA expression levels were, relative to controls
transfected with scrambled RNA, reduced in normoxia and
steadily increasing in cells experiencing ever milder degrees
of hypoxia. This mix of effects yielded, as result of the USF1
knockdown, a progressive potentiation of the BNIP3 induc-
tion from harsh (1% O2) to moderately (5% O2) to mildly
(10% O2) deoxygenated Hep3Bs (Fig. 3, Table 3). We
therefore surmise that endogenous USFs primarily cap the
activity of the HIF-1/HRE complex at BNIP3 during
normoxia-mild hypoxia; that is, interfere with HIF-1 com-
plexes whose α-subunits remain either fully (i.e., at O2)/
NAD + CAD) or partially (i.e., at CAD) hydroxylated. The
HIF-1/HRE complex under strictly hypoxic (1% O2) con-
tions, that is, with complete dehydroxylation of α-sub-
units at NAD and CAD regions, is, however, due to HIF's
superior DNA affinity dominant over endogenous USFs.
Now, upstream stimulatory factors will only through over-
expression still be able to shift the binding equilibrium to the
BNIP3 HRE in their favor and guard the site against HIF-1.
Thus, USF1 and USF2a are best viewed as pO2-dependent
conditional, not compulsory, HIF-interfering factors. Their
delimiting impact on hypoxic signaling likely occurs most
effectively toward HIF’s temporal or O2 limits (i.e., during
anoxia or reoxygenation), or in response to a strong phys-
ologic activation of the USF pathway [i.e., (i) UV-induced
USF1 phosphorylation in cells of melanocytic origin (53);
(ii) Ca2+ depletion–based protection from proteolysis in
differentiating erythroid progenitor/erythroleukemia cells [54]). Future work should tap into genome-wide implications of coactivated cross-talk between endogenous USFs and HIF-1 in appropriate models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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