HINCUTs in cancer: hypoxia-induced noncoding ultraconserved transcripts

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Recent data have linked hypoxia, a classic feature of the tumor microenvironment, to the function of specific microRNAs (miRNAs); however, whether hypoxia affects other types of noncoding transcripts is currently unknown. Starting from a genome-wide expression profiling, we demonstrate for the first time a functional link between oxygen deprivation and the modulation of long noncoding transcripts from ultraconserved regions, termed transcribed-ultraconserved regions (T-UCRs). Interestingly, several hypoxia-upregulated T-UCRs, henceforth named ‘hypoxia-induced noncoding ultraconserved transcripts’ (HINCUTs), are also overexpressed in clinical samples from colon cancer patients. We show that these T-UCRs are predominantly nuclear and that the hypoxia-inducible factor (HIF) is at least partly responsible for the induction of several members of this group. One specific HINCUT, uc.475 (or HINCUT-1) is part of a retained intron of the host protein-coding gene, O-linked N-acetylglucosamine transferase, which is overexpressed in epithelial cancer types. Consistent with the hypothesis that T-UCRs have important function in tumor formation, HINCUT-1 supports cell proliferation specifically under hypoxic conditions and may be critical for optimal O-GlcNAcylaton of proteins when oxygen tension is limiting. Our data gives a first glimpse of a novel functional hypoxic network comprising protein-coding transcripts and noncoding RNAs (ncRNAs) from the T-UCRs category.

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Hypoxia is one of the classic features of the neoplastic microenvironment and is causally associated with resistance to conventional anticancer therapy. Under hypoxic conditions, cells undergo a complex adaptive process involving genes regulating angiogenesis, cell invasion, metabolism and survival pathways. Novel insights into the pathways regulated by low oxygen concentration have shown that in addition to protein-encoding genes, the noncoding transcriptome is also affected by hypoxia. We and others previously reported that hypoxia regulates specific microRNAs (miRNAs), which in turn regulate signals of relevance for tumor biology,2–5 including angiogenesis, cell cycle, DNA repair and energy metabolism.5,6 During the past few years, it has become clear that long noncoding RNAs (ncRNAs) (>200 bp in length) also participate in complex genetic events in eukaryotes.7 One intriguing family of long ncRNAs8 is transcribed from regions that exhibit extremely high conservation between the orthologous regions of human, rat and mouse genomes,9,10 hence the term ‘transcribed-ultraconserved regions’ (T-UCRs) or ultraconserved genes.8 Their striking evolutionary retention strongly suggests profound biological roles in a wide variety of physiologic responses. Recent studies have identified alterations in T-UCR-expression patterns that are associated with specific tumor phenotypes, pointing towards a mechanistic involvement of T-UCRs in cancer development. In particular,

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Abbreviations: miRNA, microRNA; ncRNA, noncoding RNA; T-UCR, transcribed-ultraconserved gene; PCR, polymerase chain reaction; RT-qPCR, quantitative reverse transcription PCR; DMOG, dimethyl oxide glycin; DMSO, dimethyl sulfoxide; CA9, carbonic anhydrate IX; HIF, hypoxia-inducible factor; CRC, colorectal carcinoma; Cq, quantification cycle; OGT, O-linked N-acetylglucosamine (GlcNAc) transferase; HINCUTs, hypoxia-induced noncoding ultraconserved transcripts; VHL, Von Hippel–Lindau tumor suppressor; WT, wild type; FACS, fluorescence activated cell sorting

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alterations in T-UCR expression have been described in adult chronic lymphocytic leukemia, colorectal cancer (CRC), hepatocellular carcinomas and neuroblastomas. In the human genome, T-UCRs are often found to overlap with exons of genes involved in RNA splicing or are located within host gene introns or in close proximity to genes that are involved in transcription and development regulation.

Whereas ultraconserved sequences appear to act as tissue-specific regulators of gene expression during development, very little, if anything, is currently understood about the mechanisms underlying the cancer-associated profile alterations of the T-UCR. To address this limitation, we hypothesized that decreased oxygenation contributes to the deregulation of specific T-UCRs in cancer, pursuing a similar rationale to our original work on hypoxia-regulated miRNAs. We initiated the search by exposing several cancer cell lines to low oxygen followed by quantification of changes in T-UCR expression versus normoxic controls using a genome-wide microarray profiling. Subsequently, we interrogated the contribution of hypoxia-inducible factor (HIF) activity to the upregulation of a set of hypoxia-induced noncoding ultraconserved transcripts (HINCUTs). Finally, the leading candidate of this group was functionally dissected in detail.

Results

T-UCR-expression changes under hypoxic conditions in malignant cells. We re-interrogated our previous microarray data (Array Expression accession: E-TABM-184) obtained from CRC samples (78 CRCs compared with 21 normal colonic mucosas) to identify T-UCRs overexpressed in CRC that could be potentially induced by hypoxia (for a detailed workflow, see Figure 1a and Supplementary Figure 1). The same type of microarrays was used for the discovery of hypoxia-induced miRNAs. We identified 61 T-UCRs that had a significantly (P-value < 0.05 Welch t-test with Benjamini–Hochberg correction) altered expression in CRC when compared with normal colon tissue (59 overexpressed and two with lower expression). Twenty-seven T-UCRs were upregulated compared with normal colonic mucosa at a more stringent P-value < 0.005, and further analyzed (Supplementary Table 1).

In order to validate the microarray data, we exposed two cancer cell lines (HCT-116 CRC and MCF-7 breast cancer cells) to 1% oxygen or 500 μM DMOG (dimethylxylglycine) – a widely used hypoxia mimetic – for 24 and 48 h and measured T-UCR expressions using quantitative reverse transcriptase PCR (RT-qPCR). Comparisons were performed with normoxic controls (21% oxygen) or dimethyl sulfoxide (0.5% DMSO). We identified a set of T-UCRs (uc.63, uc.73, uc.134 and uc.475) that was induced more than two-fold (P-value < 0.05 by Student’s t-test) after 48 h both of hypoxia and DMOG exposures (Figure 1b and Table 1). All of these transcripts were predominantly present in the nucleus, as identified by nuclear and cytoplasmic fractionation experiments (Supplementary Figure 2). Robust activation of hypoxia signaling was confirmed by HIF-1α (or HIF-2α) protein overexpression (Figures 1b and c), carbonic anhydrase IX (CA9) (data not shown) and miR-210 (Supplementary Figure 3) gene expression levels, which are both well-established coding and noncoding HIF targets, respectively. Henceforth, these T-UCRs will be referred to as HINCUTs; their functional dissection under low-oxygen conditions is described below.

Evidence for HIF as HINCUT’s regulator. The effect of DMOG on the expression of HINCUTs suggests a central role for HIFs as an upstream regulator. To further address the role of HIF, we used the renal cell carcinoma (RCC) cell line 786-O, which exhibits homozygous inactivation of the von Hippel–Lindau tumor suppressor gene (VHL), the well-established E3 ubiquitin ligase for all HIF family members. 786-O cells exhibit constitutive activation of the HIF pathway. We hypothesized that HIF-2α-induced T-UCR expression would have a similar pattern. To this end, when we compared normoxic 786-O cells expressing an empty vector with cells expressing wild-type VHL to restore the defects, and consistent with being bona fide HIF-2α targets, all HINCUTs were upregulated at least 1.5-fold in the VHL-deficient cell line (VHL−) compared with the VHL-restored (VHL+) cells (Figure 1c).

Time course experiments in hypoxic models revealed that uc.475 was the most consistently induced (Supplementary Figure 4). As no T-UCR promoter has been characterized to date, we performed an in silico search for HIF-binding sites within the 15-kb genomic region surrounding HINCUTs. For uc.63, uc.73, uc.134 and uc.475, we found multiple putative HIF-binding sites per UCR (Supplementary Table 2). Then T-UCRs with yet unknown functional role and more than one binding sites identified were also included in these studies. In the uc.63 promoter region, we identified the following three candidate HIF-binding sites: Q3_uc.63A (63A.1), Q3_uc.63A (63A.2) and Q3_uc.63A (63A.3), where for uc.475 the following three candidate sites were found: Q3_uc.475 5'-GCTGCAATGCAGGCGCGG-3' (475.1) and two in close proximity with each other, Q5_uc.475 5'-CAGCTGCGCCGCG-3' and Q6_uc.475 5'-CAGCTGCGCCGCGC-3' (475.2) (Figure 2a).

To further substantiate the role of HIF in the regulation of uc.475 and uc.63, we performed chromatin-immuno-precipitation assay (ChIP) of HIF-1α in MCF-7 cells under normoxic conditions or following treatment with the hypoxia mimic, DMOG. We confirmed direct recruitment of endogenous HIF-1α at the promoters of uc.63 (data not shown) and uc.475, which was more evident during hypoxic conditions (Figure 2a; genomic regions from The University of California, Santa Cruz (UCSC) Genome Browser (http://genome.ucsc. edu)NCBI36/hg18). For each ChIP reaction, we used the promoter of CA9, a well-characterized HIF target, as the positive control and hemoglobin beta (HBB) as the negative control.

We further confirmed the transcriptional activation of uc.475 by hypoxia and HIF-1α using promoter fragments subcloned into the pGL4-10 (luc2) basic expression vector. These constructs were co-expressed with the pRL-TK (Renilla) control vector and constitutively active HIF-1α constructs (pcDNA 3.1-HIF-1α P/A (proline/alanine mutant) and pcDNA 3.1-HIF-1α 9M (proline/alanine/asparagine mutant) in two CRC cell lines, HCT-116 and HT-29 cells (Figures 2b and c).
We found that hypoxia and the active HIF-1αs induced an increase (up to five-fold) in luciferase activity (Figure 2b), which was correlated with the uc.475 expression in these cells (Supplementary Figure 5). In contrast, luciferase activity of the mutant clones that lack HIF-1α-binding sites (PT1 and PT2) was significantly reduced (Figure 2c) (P-value < 0.005), suggesting that the two predicted binding sites are functional HIF-recognition sites that mediate hypoxia effects on uc.475 transcription.

**Biological effects of uc.475 downregulation.** To determine the biological significance of HINCUTs, we performed knockdown experiments for uc.63 and uc.475 using custom-designed siRNAs and found that these effected cell proliferation using the general strategy described initially for uc.73.8 siRNA-specific oligonucleotides for HINCUTs were designed after evaluating by strand-specific RT-qPCR if the sense or antisense transcript was transcribed at higher levels (Supplementary Figures 5 and 6). No major biological effect
on the dynamics of COLO-205 culture was identified for uc.63 (Supplementary Figure 7) and henceforth, we focused our efforts on uc.475. Notably, the primary uc.475 transcript has the same orientation as its host gene, O-linked N-acetylglucosamine (GlcNAc) transferase (OGT) (Supplementary Figure 6). The effect of uc.475 downregulation on cell survival was tested in HT-29 cells under hypoxic and normoxic conditions. These cells were chosen based on their high endogenous uc.475 expression level (Supplementary Figure 5). We employed a custom-designed pool of siRNAs to knockdown uc.475; each of the three siRNAs tested to be efficient reduced the basal expression of uc.475 by more than 65% as confirmed by RT-qPCR (Supplementary Figures 7 and 8 and Supplementary Table 3).

Under normoxic conditions, downregulation of uc.475 had no significant impact on the total cell number versus control (siRNA-NEG) cells for up to 120 h (Figure 3a, left). In contrast, under hypoxic conditions (1% O₂), uc.475 knockdown significantly decreased cell proliferation (Figure 3a, right). This effect was not the result of increased apoptosis, as poly ADP-ribose polymerase, a critical component of programmed cell death, remained uncleaved (data not shown). Instead, through the analysis of the cell cycle, using fluorescence-activated cell sorting (FACS), we observed that uc.475 knockdown under hypoxic condition enriched the G2/M fraction more than two-fold at 72 and 96 h (from 10.3 and 8.2% with the negative control to 21.7 and 18.8% with siRNA, respectively), suggesting a G2/M blockade. No similar effect was observed after treatment with siRNA against another intron of OGT (Supplementary Table 3 and Supplementary Figure 8) under normoxia and hypoxia, and only minimal effects on cell cycle progression were observed (Supplementary Figure 9b). To exclude the possibility that siRNA against uc.475 downregulates pre-mRNA of OGT and leads to a phenotypic effect, we designed another siRNA targeting another intron of OGT. We selected intron 12 to knockdown the pre-mRNA, as this siRNA exhibited the highest efficiency among several siRNAs we have designed. As a result, although OGT protein expression was moderately downregulated by OGT-intron 12 knockdown, more significant reduction was observed with uc.475 knockdown (Figure 3c), and no proliferation inhibition or altered cell cycle distribution was observed after the knockdown of OGT-intron 12 (Supplementary Figure 11). These results indicate that the alteration of OGT expression and proliferation inhibition by uc.475 knockdown is a distinct effect of the OGT pre-mRNA knockdown.

OGT locus: a mix of OGT and HINCUT-1 transcripts. As mentioned previously, and in line with earlier reports, the uc.475 sequence (396 bp in length) is found inside the fourth intron of the OGT gene (UCSC Genome Browser; GRCh37/hg19, Figure 4a). OGT has two known isoforms (OGT1 of 5497 bp and OGT2 of 5467 bp) because of a variant splice site, which is known to encode proteins of 1046 or 1036 amino acids (116 and 103 kDa), respectively, and that catalyze polypeptide glycosylation by the addition of N-acetylglucosamine (O-GlcNAc) to serine and threonine residues (O-GlcNAcylation) on many nuclear and cytosolic proteins. Recently, this modification was proposed to be a new link between glucose metabolism and cancer growth and invasion in breast, colon and lung cancer types. In addition, OGT was found to be highly expressed in prostate cancer compared with the normal epithelium. Taking into account the link between OGT and cancer, we hypothesized that this, at least in part, could be explained by the associated uc.475 transcript with a potential role as the regulator of its host gene expression during hypoxia.

By using a multiple-primer amplifications approach, we cloned a transcript of 392 bp that included the uc.475 region but excluded the flanking OGT exons (for details see Supplementary Figure 12). In contrast, amplification levels were much lower when primers in the ‘flanking’ OGT exons (Exons 4 and 5) and intron region containing uc.475 were used in combination; the OGT and uc.475 transcripts were 2–10 times more abundant than the amplicons obtained with the mixed combinations of primers between flanking sequences of uc.475 and exons of OGT (Figure 4b). These data support two possibilities: (a) the existence of an OGT-independent transcript that contains the fourth intron or (b) the existence of a primary transcript that contains both OGT and uc.475 but is further spliced into two transcripts, one noncoding (the intronic uc.475) and one coding (the OGT isoforms).

### Table 1 Genomic information for the five HINCUTs identified in the present study

<table>
<thead>
<tr>
<th>T-UCR</th>
<th>Genomic location</th>
<th>Symbol of the host gene</th>
<th>Gene location</th>
<th>Host gene orientation*</th>
<th>Length (nt)</th>
<th>HCT-116 HYP/ NORM (48 h)</th>
<th>MCF-7 DMOG/ DMSO (48 h)</th>
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<tr>
<td>uc.475+</td>
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<td>RSRC1 (Arginine/serine-rich colliled-coil 1)</td>
<td>Intron 7</td>
<td>S</td>
<td>211</td>
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Abbreviations: DMOG, dimethyloxalylglycine; DMSO, dimethyl sulfoxide; HINCUT, Hypoxia-induced noncoding ultraconserved transcripts.

*Host gene orientation is reported versus HINCUT orientation considered as reference.
To further substantiate the tight link between OGT and uc.475, we performed experiments under stress conditions for which OGT function is relevant. As OGT-mediated O-GlcNAcylation is proportional to substrate (glucose) availability, we performed experiments under glucose-deprivation conditions (OGD experiments) – two tightly associated characteristics of the tumor microenvironment. In HT-29 cells, the expression levels of uc.475

Figure 2  Confirmation of a direct association between HIF and T-UCR using ChIP and luciferase assay. (a) Direct recruitment of HIF on T-UCR-expression induction in hypoxia-simulated conditions by DMOG in the MCF-7 breast cancer cell line. We confirmed a direct connection between HIF and T-UCR (uc.475) using ChIP method with HIF-1α antibodies or an IgG control. To enrich genomic fragments that contain hypoxia-response elements (HIF-interacting sites), we amplified the regions represented on the genome graphic using specific primers for pointed locations. The ChIP figures are inverted for a better view of expression induction. NC, negative control; HBB, hemoglobin globin beta, which is supposed to be transcriptional inactive; and carbonic anhydrase IX (CA9), which was used as a positive control in the ChIP experiment. (b) The direct effect of HIF on the uc.475 promoter reporter construct was confirmed by using luciferase assay and was shown as an effect of exogenous pcDNA 3.1-HIF-1α vector constructs (HIF-1α P/A and HIF-1α 3M) on cloned promoter activity. A stronger effect was detected by using luciferase assay normalized using empty pcDNA 3.1 vector. The error bars represent the standard deviation from two independent experiments, each performed in triplicate and measured in duplicate with a luciferase assay. (c) Luciferase activity in HCT-116 and HT-29 cells was measured after transfection with truncated promoter reporter constructs (PT1 and PT2) with a complete deletion of the HIF-1α-binding sites that were present in the originally designed uc.475 promoter reporter construct (WT). Filled circles show the position of the HIF-1α-predicted binding site. ***P-value <0.001 compared with luciferase activity of an empty vector; WT, wild type; PT1, truncated promoter 1; PT2, truncated promoter 2.
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and OGT mRNA significantly increased during 12 and 24 h of normoxia in glucose-free media. After 48-h exposure under these conditions, the expression of both decreased dramatically to the same or lower level observed under hypoxia in the presence or absence of glucose (Figure 4c). These data show that the responses of uc.475 and OGT mRNA expression to oxygen and glucose deprivation are similar.

In parallel, we attempted to visualize the sizes of the transcripts from this locus by using Northern blot analysis. This would allow us to determine whether uc.475 exists as an independent transcript, or only part of a hybrid transcript with OGT, or as both. We used RNA isolated from MCF-7 cells after exposure to hypoxia or normoxia for 24 h. Using a probe specific to uc.475, we identified two transcripts (~8 and ~9.5 kb) with higher expression under hypoxia in MCF-7 cells, whereas the OGT-coding region transcripts (4.2 kb long) had barely detectable signal (Figure 5, left). Hybridization with an OGT-specific probe detected two transcripts (1.7 and 4.2 kb) with similar intensity under both normoxia and hypoxia conditions (Figure 5, right). Therefore, we believe that uc.475 is the part of a larger OGT primary transcript, which retains intron 4 and which is expressed in a hypoxia-dependent manner.

When we screened for potential open reading frames (ORF) in the ‘retained intron’, we detected no ORF > 100 bp. The 9-kb transcript was not predicted to encode for either of the two OGT isoforms, as the predicted ORF corresponds to 665 amino acids, potentially representing the smaller 78-kDa version of the OGT reported recently19,20,27 (Supplementary Figure 13). In addition, we have searched for miRNAs and other putative ncRNAs in the uc.475 region using the UCSC database, in which current integrated RNA deep-sequencing experiment data (RNA-seq and miRNA-seq) are available, and found no annotated miRNAs or other ncRNAs to be located within the uc.475 region.

**Uc.475 inhibition has an impact on OGT expression and function.** We first investigated whether OGT expression at RNA and protein levels was affected by uc.475 knockdown in HT-29 cells. Expression of OGT mRNA levels was decreased by knocking down uc.475 (Figure 6a), as well as OGT protein levels, which was more evident in hypoxia (Supplementary Figure 10). Silencing OGT with siRNAs (siRNA-OGT) resulted in decreased levels of uc.475 and OGT mRNAs (Figure 6b) and OGT protein expression (Figure 6c). Interestingly, and contrary to the mRNA, the OGT protein level does not increase in hypoxia, as evidenced by measurements performed in three different cell lines – HCT-116 colon cancer, MCF-7 breast cancer and U-87 glioblastoma cells (Supplementary Figure 14).

To provide functional support for the impact of uc.475 downregulation on OGT function, we quantitated the O-GlcNAcylation rate following siRNA-mediated knockdown of the transcript containing the ultraconserved sequence (Supplementary Figure 15). This was performed in HT-29 cells treated with PUGNAc (1,5-hydroximolactone), an inhibitor of the N-acetylhexosaminidases (OGA or MGEA5), in order to prevent the removal of O-GlcNAc from proteins.28 Downregulation of uc.475 led to a significant decrease in the global O-GlcNAcylation rate, an effect similar to treatment with OGT-siRNA (Figure 6d), whereas the inhibition of OGT protein and global O-GlcNAcylation was relatively modest after treatment with siRNA against OGT-intron 12, which targets OGT pre-mRNA.

**The enhancer-like function of the DNA region containing uc.475 ncRNA.** It has been reported that ultraconserved regions can act as developmental enhancers, so we determined whether, in addition to having a transcriptional activity, the uc.475 could also have an enhancer function.29 We used sequence-transcription stimulation by an adjacent heterologous promoter region as previously described.30 The 2.7-kb long region (~1 kb upstream and +1.3 kb downstream of uc.475) (Supplementary Figure 12) was subcloned into the pGL3-TK vector (Figure 7a). This cloned insert revealed 2-3 fold luciferase activity enhancement, under both normoxia and hypoxia, in U-87 cells (Figure 7b). These findings demonstrate that the uc.475 DNA region also has an enhancer activity both under hypoxic and normoxic conditions.

**Discussion**

We aimed to investigate whether hypoxia induces the expression of noncoding ultraconserved transcripts in a similar manner to the conventional hypoxia-regulated genes or more recently studied miRNAs. To this end, we employed a strategy that integrates tumor data with a microarray-based screen.2 We identified in multiple cell types the following hypoxia-induced, noncoding ultraconserved transcripts: uc.63, uc.73, uc.106, uc.134, and uc.475, and termed these HINCUTs. Similar to the earlier work on miRNAs,2 we are providing evidence that HIF has a critical role for their hypoxic regulation, based on induction with chemical inactivators of the HIF prolyl hydroxylases, effect of the VHL tumor-suppressor inactivation and effect of exogenous HIF-1α on luciferase reporters containing fragments from the candidate promoters of HINCUTs. We identified HIF-1α-binding sites upstream of uc.475. This region in fact overlaps the OGT gene promoter region; therefore, the transcriptional control of uc.475 is not considered to be completely distinct from OGT transcription. Our in silico prediction detects no putative binding site for HIF-1α very close to OGT intron 4, where uc.475 is located. Recent reports have demonstrated that many intronic miRNAs possess their own promoter, which is independent of the host gene transcription.31

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**Figure 3** Uc.475 biological significance under normoxia and hypoxia. (a) The effect of siRNA-targeting uc.475 on cell proliferation was determined under normoxic (left) and hypoxic (right) conditions by using a pool of three siRNAs against uc.475 (siRNA-uc.475). Three independent experiments were performed, and as control scrambled siRNA sequence, siRNA-NEG, was used. (b) FACS analysis was performed 72 and 96 h post transfection with either siRNA-uc.475 or siRNA-NEG in normoxia (left) and hypoxia (right). (c) Western blot analysis to detect differences of uc.475 host gene (OGT) protein expression levels. Hypoxic conditions were confirmed by CA9 induction. Protein-specific bands were normalized to beta-actin. siRNA against OGT pre-mRNA was designed to target OGT-intron 12. *P*-value < 0.05, **P*-value < 0.01, ***P*-value < 0.001.
Figure 4 Transcripts produced at the uc.475 genomic locus in an environment deprived of oxygen and/or glucose. (a) The genomic region of OGT from the UCSC Genomic Browser, GRCh37/hg19 with the known transcripts of OGT isoforms 1 and 2. The rounded rectangle represents region containing uc.475. Northern blot-probe locations are indicated. The intron 4 region where uc.475 is located is zoomed in for better view of region of conservation in mammals. (b) RT-qPCR data showing that the uc.475 noncoding transcript and the coding OGT transcript are expressed at similar levels, whereas the ‘hybrid’ amplicons between the intron and the two adjacent exons are barely expressed. (c) Glucose- and oxygen-deprivation effects on mRNA expression level of uc.475 and OGT. CA9 mRNA expression was analyzed as a hypoxia-induced gene.
Further investigation is required to reveal whether there is an additional transcriptional factor-binding site closer to the uc.475 region.

Our data suggest that uc.475 is part of a primary (potentially retained intron) transcript that also includes the coding sequences of its ‘host gene’ OGT. The role of OGT is to catalyze the addition of O-GlcNAc on proteins, which are involved in transcription, cellular signaling pathways and processes, and the cell cycle.27,32 OGT was also recently reported to be overexpressed in breast cancer and primary and metastatic prostate cancer,22,24 further supporting OGT as a therapeutic target in solid malignancies.

Our results may expand the understanding of the complexity of the OGT genomic locus, as it reveals the presence of a noncoding ultraconserved sequence, part of a retained intron, at the core of a hybrid nuclear transcript. We provide evidence that the OGT locus generates multiple coding and noncoding transcripts. The retained intron that contains uc.475 does not possess a coding ORF but does include a second start codon (ATG) or it could be part of a long 5'-untranslated region (3.27 kb).21

We also noticed a contrast between the hypoxia-inducible nature of OGT/uc.475 at RNA and protein levels. Indeed, measurements in multiple cell lines indicate that OGT is either unchanged (Supplementary Figure 14) or even decreased during oxygen deprivation (data not shown). This is likely part of the wide translational suppression consistently reported under hypoxia. Higher transcript levels may serve to compensate this decrease, and it is entirely conceivable that in the absence of mRNA induction the level of O-GlcNAcylation would be compromised below the level compatible with survival.

What is therefore the role of uc.475-containing noncoding region in the context of OGT-hypoxic regulation? The luciferase experiments (Figure 7) suggest that on one hand this sequence may serve as an enhancer, which could cooperate with HIF for elevated transcription. The predominantly nuclear localization of the hypoxia-inducible hybrid transcript may point also to a role in RNA stability. To date, retained introns within several genes were found to enhance almost every step of gene expression and also affects mRNA stability. However, some studies showed that intron retention has little or no effects on mRNA half-life,33–35 whereas others confirmed that it is important for mRNA stability and has an effect on prolonged mRNA half-life.36–38 In addition, cells under hypoxia were even found to inhibit nonsense-mediated RNA decay (NMD) mechanisms to enable the expression of genes critical for cell adaptation and response to a cellular stress.39 Moreover, many studies found that constitutively spliced introns are required for optimal gene expression,40,41 and one possible way through which introns can affect transcription is by acting as repositories for transcriptional regulatory elements such as enhancers and repressors.41

Although further mechanistic studies as well as an understanding of the specific roles of the many transcripts at the OGT locus are needed, this is the first study that purposely investigates T-UCRs as a part of the hypoxic response and extends the knowledge of the interplay between coding and noncoding transcripts under stress conditions.

Materials and Methods

The complete version is included in the Supplementary Material.

Cell cultures and growth conditions. Colon cancer (HCT-116, HT-29 and COLO-205), breast cancer (MCF-7), bladder cancer (UC-2) and glioblastoma (U-87) cell lines were obtained from ATCC (Manassas, VA, USA). RCC cell lines were obtained from Dr. Maria Czyzyk-Krzeska (University of Cincinnati, Cincinnati, OH, USA). HCT-116 and HT-29 were maintained in McCoy’s 5A (enriched with L-glutamine and sodium bicarbonate; Thermo Scientific, Logan, UT, USA); UC-2 was maintained in minimum essential Eagle medium (MEM; Celtgro Meditech, Manassas, VA, USA); MCF-7 was cultured in Dulbecco’s modified Eagle’s
Figure 6  Impacts on OGT expression and function after uc.475 inhibition and O-GlcNAcylation change after knocking down uc.475 or OGT. (a) RT-qPCR expression levels of OGT and uc.475 after uc.475 knockdown in HT-29 cells under normoxia and hypoxia. Primers were designed to detect both OGT1 and OGT2 isoforms. Error bars indicate standard deviation. UCR or uc, ultraconserved gene; OGT, O-linked N-acetylglucosamine (GlcNAc) transferase; siRNA, small interfering RNA; HT-29, colon cancer cell line; NOR, normoxia; HYP, hypoxia; PPIA, reference gene cyclophilin A; * P-value <0.05, *** P-value <0.001. (b) RT-qPCR confirmation of OGT gene knockdown using siRNA against OGT. Primers specific for uc.475 and each of the OGT isoforms were used. (c) Western blot performed to detect differences of uc.475 host gene (OGT) protein expression levels after OGT silencing. Hypoxic conditions were confirmed by CA9 induction. Protein-specific bands were normalized to beta-actin as a reference protein. (d) O-GlcNAc protein levels were detected in samples exposed to hypoxic conditions for 72 h or simultaneously transfected with siRNA-targeting either uc.475, OGT pre-mRNA or OGT. All levels were compared with sample transfected with siRNA-NEG. Different concentrations of PUGNAc were added in cell media (40 and 80 μM) to prevent O-GlcNAc from being removed from the proteins. siRNA against OGT pre-mRNA was designed to target OGT-intron 12.
high-glucose medium (DMEM; Cellgro Mediatech); U-87 was cultured in special DMEM supplemented with F12 50/50 mix (Cellgro Mediatech); COLO-205 was maintained in RPMI 1640 (Cellgro Mediatech); and 786-O VEC (VHL −) and 786-O WT (VHL +) were cultured in DMEM: Ham’s F12 supplemented with G418 (200 μg/ml). All cell lines were Mycoplasma-free, which was confirmed by using the MycoAlert mycoplasma detection kit (Lonza, Rockland, ME, USA).

Before being used, cell lines were validated by STR DNA fingerprinting using the AmpFISTR Identifier kit, according to the manufacturer’s instructions (Applied Biosystems, cat. no. 4322288). The STR profiles were compared with known ATCC STR Identifier kits, according to the manufacturer’s instructions (Applied Biosystems). All cell lines used were negative for O-GlcNAc-modified proteins, which was confirmed by washing with phosphate-buffered saline (PBS) and then harvested for RNA and protein extractions to determine siRNA knockdown efficiency and to detect changes in O-GlcNAc, respectively. Primary and mouse-specific secondary antibodies for detecting O-GlcNAc were reported by Caldwell et al.22

**Hydroxyurea experiments.** Hypoxic experiments were performed in an InVivo200 hypoxia workstation (Ruskinn, Inc., Cincinnati, OH, USA), where oxygen concentration was maintained at 1 or 0.2% (in 5% CO2 and 95% N2) for 24, 48, 72 and 96 h. RNA and proteins were collected for further analysis using RT-qPCR and western blot, respectively.

Chemical induction of HIF-1α expression under normoxic conditions was performed using DMOG (Frontier Scientific, Logan, UT, USA). DMOG was added to the growth medium to a final concentration of 0.5% DMSO vehicle versus 0.001% DMSO vehicle as control.

RNA extractions, RT-qPCR, real-time PCR controls and reference genes are detailed in Supplementary Material.

**Data analysis for RT-qPCR.** Data analysis was performed using the Bio-Rad CFX Manager Software (Bio-Rad, Hercules, CA, USA). Only samples with a single clear peak in melting temperature and no expression in nontargeted control duplicates. Relative expression, as the fold change or difference, was calculated using the 2^(-ΔΔCt) formula, in which ΔCt represents the difference in Ct values between the target gene and the mean Ct of the reference gene. Each sample, amplified using RT-qPCR, was tested in duplicate. Standard deviation (S.D.) of each target gene was calculated.

Western blot analysis, siRNA transfection and FACS are detailed in Supplementary Material.

**HIF-1α-binding site predictions.** We scanned the putative promoter sequence (−10 kb upstream and +10 kb downstream of the 5’ end of the annotated T-UCR) for predicted HIF-1α-binding sites generated using the MATCH program and the VS$HIF1_Q3 (5′-ggkACGTGggn-3′), VS$HIF1_Q5 (5′-nglACGTGgng-3′) and VS$HIF1_Q8 (5′-nRCGTGgng-3′) position weight matrices from the TRANSFAC database, version 9.1 (http://www.gene-regulation.com). The position weight matrices describe the position preferences of different nucleotides in the HIF-binding site.22

CHIP and the promoter reporter assays are detailed in Supplementary Material.

**Detection of global O-GlcNAc cellular-protein modifications.** To detect O-GlcNAc-modified proteins, we chemically (PUGNAc as a 1,5-hydroximolactone is an inhibitor of various N-acetylhexosaminidases) prevented the removal of O-GlcNAc modifications by the glycosidase MGEA5 (mismannose-expressed antigen 5 (hyaluronidase)).22 PUGNAc treatment was initiated 24 h after siRNA transfection. Fresh complete media containing PUGNAc was added to final concentrations of 0, 40 and 80 μM, respectively, and in duplicate wells. The stock PUGNAc solution (SIGMA; cat. no. 132489-69-1) was prepared following the manufacturer’s instructions and stored at −20°C. After 72 h under normoxic or hypoxic condition, cells were washed with phosphate-buffered saline (PBS) and then harvested for RNA and protein extractions to determine siRNA knockdown efficiency and to detect changes in O-GlcNAc, respectively. Primary and mouse-specific secondary antibodies for detecting O-GlcNAc were reported by Caldwell et al.22

**ORF detection.** OGT isoforms 1 and 2, and the retained intron sequence (intron 4) of the OGT gene were all tested for ORF using the NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

**Cell fractionation.** Cells (HCT-116, 2 × 106) were pelleted, and cytoplasmic and nuclear fractions were obtained using the PARIS kit following the manufacturer’s protocol (Ambion RNA, Life Technologies, Carlsbad, CA, USA). Oxygen and Glucose deprivation assays are described in Supplementary Materials.

**ncRNA array data analyses.** Microarray chips were developed with a total of 481 human UCR sequences as in http://www.soe.ucsc.edu/~jill/ultra.html. For each UCR, two 40-mer probes were designed, one corresponding to the sense genomic sequence (named ‘+’) and the other to the complementary sequence.
Conflict of Interest

The authors declare no conflict of interest.

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Authors Contributions

Study concept and design: Calin, Ivan and Kunej; development of methodology: Calin, Ivan, Kunej and Davuluri; acquisition of data: Ferdin, Wu, Nisholda, Shah, Devín, Ling, Shimizu, Kumar, Cortez and Schmittgen; analysis and interpretation of data: Ferdin, Nisholda, Voorhoeve, Regnato, Negrini, Davuluri, Kunej, Ivan and Calin; statistical analysis: Ferracin, Bi, Yang, Czerkies, Zang and Davuluri; administrative, technical or material support: Shimizu, Schmittgen and Regnato; drafting of the manuscript: Ferdin, Nisholda, Ivan, Kunej and Calin. All authors critically reviewed the manuscript and approved the final version.


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