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# HIF-2α expression is suppressed in SCLC cells, which survive at moderate and severe hypoxia when HIF-1α is repressed

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## Running title

Hypoxic adaptation in SCLC

## Key words

Small cell lung carcinoma (SCLC), hypoxia, hypoxia-inducible factor, HIF-1α, HIF-2α, non-small cell lung carcinoma (NSCLC)

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## Abstract

Small cell lung carcinoma (SCLC) is extremely aggressive and frequently widely metastasized at an early stage of the disease. Since tumor hypoxia is related to aggressive tumor behavior and the hypoxic adaptation of SCLC is poorly documented, we stained SCLC tumors arranged in a tissue microarray for HIF-1α and HIF-2α proteins. We found an overall lack (34/35) of HIF-2α protein expression, which was confirmed in large tumor sections. HIF-1α protein was strongly expressed in most tumors (20/31), frequently adjacent to necrotic regions. In line with tumor data, cultured SCLC but not non-small cell lung carcinoma cells, showed no or extremely low levels of HIF-2α mRNA and no HIF-2α protein at hypoxia. HIF-1α was stabilized after 4 h at hypoxia and the accumulation increased up to 96 h. SCLC cells survived well and showed net proliferation and low cell death at modest (1% oxygen) and severe hypoxia (0.1% oxygen). HIF-1α repression did virtually not influence cell death or viability despite reduced levels of hypoxia-inducible genes, such as *BNIP3* and *BNIP3L*. At 1% oxygen no increased autophagy (LC3B-II activation) or NF-κB signaling were detected, while unfolded protein response was activated at severe hypoxia. Our data indicate that HIFs are not exclusively required for SCLC cell survival at modest or severe hypoxia and that additional, yet uncharacterized hypoxia-driven adaptation pathways may become activated.

## Introduction

Lung cancer is the most common cancer form worldwide and approximately 1.3 million people die yearly of the disease (GLOBOCAN 2008: Cancer Incidence and Mortality Worldwide, *http://globocan.iarc.fr,* last accessed November 30, 2011). The majority of lung tumors are classified as non-small cell lung carcinoma (NSCLC) whereas 15-18% is diagnosed as small cell lung carcinoma (SCLC). SCLC is very aggressive, characterized by high proliferation rate and early onset of the metastatic process, with 60-70% of patients presenting with clinically generalized disease. Despite good initial response to chemotherapy, the vast majority of SCLCs relapse. The 5-year survival is 20% in patients with limited disease and only a few percent at disseminated disease [1].

Regions of hypoxia (~1% oxygen or lower) are common features of solid tumors and are consequences of an increasing number of oxygen consuming, dividing tumor cells and incomplete tumor vascularization [2] [3]. Tumor cells adapt to low oxygen pressure by changing the transcription of genes involved in a variety of processes, such as angiogenesis, metabolism, cell survival and metastasis. This adaptive response is primarily driven by the heterodimeric transcription factors hypoxia-inducible factor (HIF)-1 and HIF-2 [4] [5]. Under hypoxic conditions, the HIF-α subunits become stabilized and bind to the dimerization partner aryl hydrocarbon receptor nuclear translocator (ARNT/HIF-1β), which is constitutively expressed. The HIF heterodimers together with cofactors such as CBP/p300 bind to hypoxia-response elements (HRE) of target genes and initiate transcription [5]. HIF-1 and HIF-2 may have slightly different target gene preferences, and there is a cell context dependence that is poorly understood. Although genes involved in glycolysis [6] and genes like *BNIP3* have been reported to predominantly be induced by HIF-1 [7], recent data suggest a time-dependent regulation of HIF target genes in addition to factor-specific regulation. In lung

adenocarcinoma, neuroblastoma, and breast carcinoma cells HIF-1α accumulates transiently and primarily mediates an acute hypoxic response, whereas HIF-2α continues to accumulate and mediates a prolonged response to hypoxia [8] [9] [10] [11]. Thus, the majority of genes regulated by HIF-2α are regulated by HIF-1α as well but with different kinetics [8].

In rodents, the HIF-α transcription factors show different distributions in normal tissue; HIF-1α mRNA is widely expressed [12] [13], whereas high HIF-2α mRNA levels are mainly limited to distinct cell populations of organs such as the lung, heart, liver, brain, and kidney [14]. Despite that the alveolar epithelium is exposed to high oxygen pressure, about 100 mm Hg (~13% oxygen) [15], the lung has one of the highest HIF-2α mRNA levels [14]. During development of fetal lung the oxygen level in the surrounding environment is generally low [16] [17] and HIF-1α and HIF-2α are important factors for the maturation of the lung as shown by gene targeting [18] [19]. While HIF-1α mRNA levels are stable and comparatively low, the mRNA levels of HIF-2α increase during maturation of the lung in both human and mouse and stay high in the adult lung [20] [18] [21].

HIF-α protein expression is associated with aggressive tumor behavior and poor outcome in several tumor forms [22] [23]. HIF-1α protein is expressed in NSCLC, though there are contradicting results whether it is associated with poor outcome or not [24] [25] [26] [27] [28] [29] [30]. HIF-2α expression is less frequent in NSCLC, but has been related to poor prognosis [26]. In SCLC, HIF-1α expression is associated with poor overall survival [31], whereas correlations between HIF-2α expression and clinical parameters have not been reported.

The importance of both HIFs for proper lung development and the comparatively high expression of HIF-2α in the developing lung led us to investigate the expression of HIFs in

SCLC tissue and the adaptive response of SCLC cells to hypoxia. We show here that SCLC cells in tumor tissue and in culture virtually lack expression of HIF-2α mRNA and protein. Despite this shortage, SCLC cells adapt well to hypoxia, and they survive and propagate even at severe hypoxia. Furthermore, the expression kinetics of HIF-1α and HIF-2α in lung carcinoma cell lines markedly differed in SCLC and NSCLC cells, indicating that the mechanisms by which these lung cancer cells adapt to hypoxia differ. The facts that HIF-2α protein is abundant and needed during normal lung development and that HIF-2α mRNA is high in normal lung may indicate that deregulation of HIF-2α expression is an abnormality associated with SCLC tumorigenesis**.**

## Materials and Methods

### Cell Culture

The human SCLC cells U-1690, U-1906, U-2020, U-1285, and U-1568 (a large-cell morphological variant of SCLC) were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO). The human NSCLC cells U-1810 (large cell lung carcinoma), U-1752 (squamous cell lung carcinoma) and H-125 (lung adenocarcinoma) were cultured in F-10 medium (Gibco, Paisly, UK), and A549 (lung adenocarcinoma, ATCC, Manassas, VA) in F-12 Kaighn´s modification (Thermo Scientific HyClone, South Logan, UT). Immortalized human alveolar epithelial cells, P2G and P2GH [32] [33] were grown in DCCM-1 medium (Biological Industries ltd, Kibbutz Beit Haemek, Israel) supplemented with 2 mM L-glutamine (Sigma-Aldrich) and the human SK-N-BE(2)c neuroblastoma cells as described [34]. All media were supplemented with 10% FCS (EuroClone Ltd., Paignton, UK), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). Cells exposed to 5%, 1% and 0.1% oxygen, were cultured in humidified oxygen-regulated chambers, H35 Hypoxystation (Don Whitley Scientific, West Yorkshire, UK).

**Tissue Microarray, Immunochemistry and Generation of Anti-HIF-2α Antibodies**

Paraffin-embedded human tumor specimens from 44 SCLC and NSCLC patients (ethics approval no. 2004/762 and 2008/702, Lund University, Sweden) were histologically evaluated by a lung cancer pathologist (NM). Tumor specimens were assembled in a tissue microarray by two representative tissue cores per donor block using an automated arraying device (ATA-27; Beecher Instruments, Inc. Sun Prairie, WI). After antigen retrieval, immunoreactivities were detected using the Envision Flex kit (DAKO A/S, Glostrup, Denmark) and DAKO Autostainer Plus. Antibodies used were anti-HIF-1α (1:50, mouse monoclonal, BD Biosciences, San José, CA) and anti-HIF-2α antibody (1:200, mouse monoclonal, Novus Biologicals, Littleton, CO). Staining specificity was checked as described [11]. All immunohistochemical stainings were evaluated by a pathologist (MJ).

For immunocytochemical analyses, U-1690 and SK-N-BE(2)c cells were pelleted, fixed in 4% paraformaldehyde, and embedded in paraffin. Cells were stained with an anti-HIF-2α antibody (1:1 000, rabbit polyclonal) produced by immunizing rabbits with a peptide corresponding to amino acids 852-870 of human HIF-2α (with a cysteine residue added to its amino terminus; CGSSTLLQGGDLLRALDQAT) coupled to keyhole limpet hemocyanin by use of m-maleimidobenzoyl-N-hydroxysuccinimideester. Antibodies were affinity purified by sequentially passing the antiserum over a column of immobilized peptide corresponding to amino acids 808-826 of human HIF-1α (with a cysteine residue added to its amino terminus; CGSRNLLQGEELLRALDQVN), followed by adsorption to a column with immobilized HIF-2α peptide (essentially as described by [35]).

**Quantitative Real-time PCR**

RNA preparation, subsequent cDNA synthesis and quantitative real-time PCR (Q-PCR) reactions were performed as described [36]. The comparative Ct method was used for relative quantification of expression levels [37] and each reaction was done in triplicate. *HPRT1, TBP* and *UBC* were used as reference genes and selected based on most stable expression at hypoxia according to geNorm. Primers were designed using Primer Express (Applied Biosystems, Foster City, CA); sequences are listed in Table 1.

**Western Blot Analyses**

Whole-cell RIPA buffer protein lysates were separated by SDS-PAGE and blotted onto Hybond-C-Extra nitrocellulosa membranes (Amersham Bioscience, Little Chalfont, UK). After blocking, the following primary antibodies were used: anti-HIF-1α antibody (1:650, mouse monoclonal, Abcam, Cambridge, UK), anti-HIF-1α antibody (1:500, rabbit polyclonal, Millipore, Temecula, CA), anti-HIF-2α antibody (1:750, rabbit polyclonal, Abcam), anti-HIF-2α antibody (1:200, rabbit polyclonal, see *Tissue Microarray, Immunochemistry and Generation of Anti-HIF-2α Antibodie*s), anti-actin antibody (1:2 000, mouse monoclonal, MP Biomedicals, Illkirch, France) and anti-SDHA antibody (1:2 000, mouse monoclonal, Abcam). For autophagy analysis, harvested cells were directly boiled in sample buffer, proteins were separated by SDS-PAGE and blotted onto Immobilon-P Transfer membrane (Millipore). After blocking, the anti-LC3B antibody (1:1 000, rabbit monoclonal, Cell Signaling Technology, Danvers, MA) was used. Immunoreactivity was detected by HRP-conjugated secondary antibodies and EZ-ECL chemiluminescence detection kit (Biological Industries ltd). Data from immunoblots were quantified using Fiji Image J software (http://pacific.mpicbg.de/ wiki/index.php/Main_Page).

**Cell Viability, CellTrace CFSE Cell Proliferation and Cell Cycle Analyses**

For cell viability analyses, approximately 200 trypan blue (Sigma-Aldrich) stained cells per

triplicate cultures were counted by light microscopy. Proliferative capacity was analyzed by flow cytometry (BD Biosciences) in non-synchronized U-1690 cells labeled with 5 μM CellTrace carboxyfluorescein diacetate, succinimidyl ester (CFSE) solution (Invitrogen, Eugene, OR). Cell cycle progression was analyzed with propidium iodide in non-synchronized U-1690 cells. After fixation in 70% ethanol, cell pellets were labeled with Vindelöv solution (3.5 μM Tris-HCl pH 7.6, 50 μg/ml propidium iodide, 20 ug/ml RNase, 10 mM NaCl and 0.1% v/v NP40). DNA content was analyzed by flow cytometry and gating of the populations was performed using the FlowJo software (version 6.4.7, Tree Star, Inc. Ashland, OR).

**siRNA Transfections and Reporter Assay**

SCLC cells were cultured in antibiotic-free medium and adherent cells were transfected/treated with siRNA against HIF-1α, BNIP3L or an unspecific siRNA (Applied Biosystems) at concentrations of 25-50 nM, or Lipofectamine 2000 Reagent alone (Invitrogen, Carlsbad, CA). To improve knockdown efficiency the transfection procedure was repeated the following day. For cell viability experiments, cells were reseeded the day after last transfection. The next day, the cells were cultured for another 72 h before analyses.

For NF-κB luciferase activation assay (pNFκB-Luc vector, BD Biosciences) transfections were made with FuGene HD transfection reagent (Roche, Mannheim, Germany). Transfection efficiency was assayed using constant amount of pRL-TK Renilla vector (Promega, Madison, WI). Luciferase and Renilla activities were measured the day after transfection after 1-4 h of cell culture at normoxia or hypoxia using the Dual-Luciferase Assay System (Promega). TPA (Sigma) stimulation (100 ng/ml) over night was used as positive control. The experiment was repeated twice and done in triplicate.

### Statistical Analyses

Data are reported as means +/- SD. Group comparisons were made using a 2-tailed unpaired Student's *t*-test or by ANOVA followed by Duncan's multiple range test. Statistical significance was set at levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

## Results

### HIF-2α is virtually absent in human SCLC specimens

We analyzed the expression of HIF-1α and HIF-2α proteins in lung cancer sections; 35 SCLC and 9 NSCLC specimens arranged in a tissue microarray and a limited number of large tumor sections. A similar fraction (~65%) of the SCLC and NSCLC tumors contained tumor cells staining positive for HIF-1α and the expression was mostly nuclear and present in cells surrounding necrotic zones (Figure 1A-C). The percentage of positive cells and the intensity of HIF-1α staining varied among the cases. The frequency of HIF-2α positivity differed markedly between SCLC and NSCLC specimens. HIF-2α protein was detected in only 1 of 35 (~3%) analyzed SCLC specimens. In the positive tumor case, only a small number of tumor cells were positive with a cytoplasmic immunoreactivity. In line with previous observations [26], 5 of 9 (~56%) NSCLC specimens showed HIF-2α positivity and the staining was mainly cytoplasmic (Figure 1A-C). The HIF-2α immunoreactivity ranged from being present in only a small number of tumor cells to nearly all tumor cells. Specific immunohistochemical staining of HIF-1α and HIF-2α was verified in cultured normoxic and hypoxic tumor cells (Figure 1D). The specificity of the HIF-2α immunostaining was further corroborated by HIF-2α positive infiltrating immune cells serving as internal controls, as demonstrated in a SCLC section (arrowheads in Figure 1B, upper right panel). Altogether, these results reveal that HIF-2α protein is differentially expressed in lung cancers with almost no detectable HIF-2α protein in SCLC.

**No HIF-2α but sustained HIF-1α protein expression in hypoxic SCLC cells**

In line with our immunohistochemical data on primary tumors, HIF-2α mRNA levels in four SCLC cell lines were extremely low compared to those in NSCLC cells, at both normoxia and hypoxia (Figure 2). In contrast, there was a robust expression of *HIF1A* at 21% and 1% oxygen in all analyzed lung cancer cell lines (Figure 2). In agreement with mRNA expression data, HIF-2α protein was not detected in SCLC cells during the entire culture period studied (up to 96 h, Figure 3A) or in sections of paraffin embedded U-1690 cells cultured for 72 h at 21% and 1% oxygen (Figure 3B). In all SCLC cell lines tested, the HIF-1α protein was expressed in hypoxic cells after 4 h, and this accumulation remained and increased over time (Figure 3A). In contrast, in NSCLC cells HIF-1α protein was expressed at the highest levels after 4 and 24 h at 1% oxygen and then the accumulation markedly declined (Figure 3A). The HIF-2α protein was induced in NSCLC cells after 4 h of exposure to hypoxia and HIF-2α remained high up to 96 h. Hypoxic SK-N-BE(2)c neuroblastoma cells were used as positive controls for HIF-1α and HIF-2α protein after 4 and 72 h of culture, respectively (Figure 3A-B), since HIF-1α protein primarily mediates an acute response and HIF-2α a more prolonged response to hypoxia in neuroblastoma cells [8]. Thus, the presence and kinetics of the HIF proteins in NSCLC cells follows the previously observed patterns seen in tumor cells of other derivations.

As *HIF2A* is highly expressed in developing lung we investigated HIF protein expression in the non-tumorigenic immortalized human alveolar epithelial P2G and P2GH cell lines. HIF-2α protein was readily detected in P2G and P2GH cells also at normoxia, while HIF-1α protein was robustly, but almost exclusively expressed in hypoxic cells (Figure 4). Interestingly, in the well-characterized A549 lung adenocarcinoma cells, HIF-1α expression

pattern at 1% oxygen were similar to those of the P2G and P2GH cells, with HIF-1α protein expression at 4 hours and this accumulation sustained up to 96 h. Though, in A549 cells HIF-2α was induced after 4 h of exposure to hypoxia and markedly up-regulated after 72 h (Figure 4).

The U-1568 cell line is a morphological variant of SCLC cells with slightly larger cells [38] representing the intermediate form of SCLC sometimes diagnosed in the clinic. Interestingly, these cells showed intermediate HIF mRNA and protein expression levels between that of SCLC and NSCLC cells, e.g. HIF-1α protein was expressed at 1% oxygen after 4 h, but this stabilization was slightly reduced over time whereas HIF-2α was induced after 4 h exposure to hypoxia and the protein levels were moderately up-regulated over time (see Supplemental Figure S1A-B at http://ajp.amjpathol.org).

To summarize, we found that HIF-2α is not expressed in SCLC cells *in vivo* or *in vitro* and thus, that HIF-1α and HIF-2α proteins in SCLC are differentially expressed and regulated in comparison to all other investigated lung tissue-derived cells (NSCLC cells, a large-cell morphological variant of SCLC cells, and immortalized alveolar epithelial cells).

**SCLC cells have high capacity to adapt to hypoxia**

Despite that SCLC cells essentially lack HIF-2α, SCLC cells tolerated hypoxic conditions very well. To investigate in more detail the survival capability of SCLC cells, we chose oxygen levels to mimic a physiological end capillary oxygen pressure of approximately 5% as well as low oxygen pressures (1 and 0.1%) reflecting oxygenation stages occurring in solid tumors [39]. The SCLC cells survived well at all investigated oxygen levels, although the number of viable cells was notably lower at 0.1% oxygen (Figure 5A-C). However, also at

0.1% oxygen there was a pronounced net growth over 72 h with almost a doubling of U-1690 cells and about 8 to 7 times increase in number of U-1906 and U-2020 cells. The proportion of dead cells was somewhat increased with lower oxygen tensions but never exceeded 17% in the SCLC cultures. The low amount of dead cells was verified by TUNEL staining (data not shown). The proportion of dead cells was not higher at earlier time points (24 and 48 h, data not shown). As shown in Figure 5D, the NSCLC U-1752 cells, showed a similar capacity as SCLC cells to survive at lower oxygen levels although with a higher proportion of dead cells at all investigated oxygen levels (with a highest level of 23% at 0.1% oxygen).

The reduced number of SCLC cells at hypoxia could be explained by a lower rate of proliferation (Figure 5E-G). As demonstrated by CFSE staining in non-synchronized hypoxic cells, there was a slower rate of cell divisions as indicated by a smaller shift in CFSE intensity (Figure 5E). A larger fraction of undivided cells was also shown (Figure 5F) and this result was supported by cell cycle distribution analyses indicating a somewhat delayed cell cycle progression (Figure 5G). Altogether these results indicate that SCLC cells survive well during hypoxia with continuous, but slightly reduced cell propagation rate also at severe hypoxic conditions, albeit accompanied by a small increase in cell death.

**Well-established hypoxia-inducible genes are modestly up-regulated in hypoxic SCLC cells**

To further evaluate how the lack of HIF-2α in SCLC cells influenced their adaptation to hypoxia, mRNA levels of known hypoxia-inducible genes were examined. Overall, a modest and inconsistent induction of the investigated genes was observed in SCLC as compared to NSCLC and neuroblastoma cells (Figure 6). Of the studied genes, only *BNIP3*, involved in apoptosis and autophagy, and *HK2*, involved in glycolysis, was pronouncedly up-regulated in

all tested SCLC cell lines. The well-characterized hypoxia-inducible gene *VEGF* was expressed at comparably low levels and was not consistently induced at hypoxia. Also genes predominantly driven by HIF-1α in other cell systems [40] [41] [42] like *GLUT1/SLC2A1*, *GLUT3*/S*LC2A3* and *CA9* were at most modestly up-regulated by hypoxia (Figure 6). *OCT4* as well as *SERPINB9,* the latter mainly regulated by HIF-2α in neuroblastoma cells, demonstrated only minor changes in the expression levels at hypoxia (Figure 6). Altogether, these results suggest that a classical HIF-dependent response to hypoxia is reduced in SCLC cells. Most of the analyzed genes, like *BNIP3*, *VEGF, GLUT1, GLUT3, HK2* and *CA9*, were up-regulated in NSCLC cells after 72 h at 1% oxygen, which might suggest that HIF-2α, as the predominantly expressed HIF-α at later time points, also is important for the hypoxia-regulated expression of these genes. The most distinct response to hypoxia was seen in the adenocarcinoma cells H-125 and A549, which was comparable to that seen in neuroblastoma cells.

**SCLC cells still survive at hypoxia when HIF-1α expression is reduced**

The role of HIFs at hypoxic adaptation in SCLC cells was examined by knocking down HIF-1α expression using siRNA. A specific reduction of HIF-1α mRNA (~60%) was observed at 21% and 1% oxygen after 72 h and this decrease was even more evident (73%) at the protein level (Figure 7A). In the knockdown cells, the induced expression of both *BNIP3* and *CA9* at 1% oxygen was significantly diminished (see Supplemental Figure S2A at http://ajp.amjpathol.org), confirming that these genes are regulated predominantly by HIF-1α at sustained exposure to hypoxia. The same trend was observed for *GLUT1* and *VEGF* expression (see Supplemental Figure S2A at http://ajp.amjpathol.org), however, the effect appeared modest in light of the efficient knockdown of the HIF-1α protein. HIF-2α mRNA level was slightly increased in HIF-1α siRNA-treated cells but was still considerably lower

than in NSCLC cells (see Supplemental Figure S2B at http://ajp.amjpathol.org) and did not result in detectable levels of protein in hypoxic cells (Figure 7A). To exclude a possible involvement of HIF-2α-dependent gene regulation at hypoxia, the low basal HIF-2α mRNA expression was further reduced by siRNA but there was no effect on the expression levels of the investigated target genes (data not shown).

The importance of HIF-1α for SCLC cell viability was evaluated in HIF-1α knocked down SCLC cells at severe hypoxia for 72 h. Interestingly, despite an oxygen level as low as 0.1%, the viability of HIF-1α knocked down U-1690 and U-1906 cells was still high and the cells continued to divide to a large extent (Figure 7B). In U-1906 cells, in which the HIF-1α knockdown was most efficient (87 ± 3% knockdown in three independent experiments, Figure 7C), there was a seven times net increase of viable cells, although the total number of viable cells was lower compared to control-siRNA-transfected cells. In addition, the amount of dead cells was not significantly changed (7-10% dead cells in control-siRNA-treated and si-HIF-1α-treated cells) (Figure 7B). Despite efficient knockdown of HIF-1α, HIF-2α protein was still not detectable (Figure 7C).

From the experiments above we conclude that the transcription of a set of well-known hypoxia-driven genes in SCLC is modestly and some times inconsistently induced at hypoxia and only partly driven by HIF-1α and not by HIF-2α. The observations suggest alternative adaptation mechanisms to hypoxia in SCLC cells. The autophagy marker LC3B did not convert from LC3B-I to LC3B-II in hypoxic SCLC cells (see Supplemental Figure S3A at http://ajp.amjpathol.org), suggesting that increased activation of an autophagy survival program is not important for survival of SCLC cells at hypoxia. In line with these results, the HIF-driven genes *BNIP3* and *BNIP3L*, which are associated with both autophagy and cell

death, were significantly down-regulated in HIF-1α knocked down cells (see Supplemental Figure S3B at http://ajp.amjpathol.org), but this did not change the total cell number or resulted in more dead cells even at severe hypoxia (Figure 7B). Also, knockdown of BNIP3L it self did not influence hypoxic SCLC cell viability (see Supplemental Figure S3C at http://ajp.amjpathol.org). Likewise, the activity of the oxygen-sensitive transcription factor NF-κB was not significantly increased in hypoxic (1% oxygen) compared to normoxic SCLC cells (see Supplemental Figure S4 at http://ajp.amjpathol.org). On the other hand, at severe hypoxia the unfolded protein response (UPR) was activated as shown by significantly increased splicing of *XBP1* mRNA compared to moderate hypoxia (Figure 7D). This activation was sustained upon HIF-1α knockdown (Figure 7E) indicating, as expected from the literature [43], a HIF-independent UPR-induction at low oxygen level (Figure 7C). In summary, our data strongly suggest that SCLC cell survival at modest or severe hypoxia is not entirely an effect of HIF-driven gene expression.

## Discussion

SCLC differs from NSCLC according to pathological and clinical characteristics. Here we present data further underscoring the biological differences between SCLC and NSCLC. Our results demonstrate an overall lack of HIF-2α expression in SCLC cells and an atypical temporal regulation of HIF-1α protein levels during prolonged hypoxia. The response to hypoxia in SCLC cells stands in contrast to that in NSCLC cells, which stabilize both HIFs with HIF-1α stabilization primarily during an acute and HIF-2α stabilization during a sustained phase of hypoxia. The HIF-1α expression pattern we observed in SCLC specimens was in line with a previous investigation [31], as was the overall staining result of HIF-1α and HIF-2α proteins in NSCLC specimens [26]. Thus, SCLC cells revealed different HIF expression patterns as compared to NSCLC cells and immortalized alveolar epithelial cells

evaluated here, as well as in previously examined tumor cells of other derivations [8] [9] [11]. Our data demonstrate that no HIF-2α protein was detectable in hypoxic SCLC cells, however, *HIF2A* seems to be functional in SCLC cells since a small increase at the mRNA, but not at the protein level, was observed upon knockdown of HIF-1α. A similar repression of *HIF2A* expression by HIF-1α has been observed in other cell systems [10] [11].

HIF-2α is highly expressed in the developing lung and is required for proper lung development. The observation that HIF-2α expression is uniformly down-regulated in SCLC cells, opens up for speculations regarding a role of silenced HIF-2α during SCLC tumorigenesis. However, to establish a link between HIF-2α down-regulation and SCLC is not trivial, as the specific cell population giving rise to SCLC has not been definitely defined. One hypothesis is that SCLC and NSCLC originate from a common pulmonary stem cell, a model supported by the fact that SCLC can display a mixture of SCLC and NSCLC cells [44]. Another hypothesis is that SCLC and other neuroendocrine lung tumors originate from rare pulmonary neuroendocrine cells, primarily located in the upper airways [44], and this is suggested by recent data using a mouse SCLC model [45]. A distinct origin of SCLC and NSCLC is also supported by the fact that a multipotent epithelial stem/progenitor cell exists in normal mouse lung that can generate cells of both airway and alveolar epithelial lineages but not neuroendocrine cells [46]. The heterogeneity seen in SCLC might instead be explained by the effect of mutations in pulmonary neuroendocrine cells giving rise to both non- and neuroendocrine tumor cells [47]. The presence/absence and putative role of HIF-2α in pulmonary neuroendocrine cells and in SCLC initiation and progression remains to be determined.

It is reasonable to assume that the prolonged accumulation of HIF-1α is a mechanism for SCLC cells to compensate for the absence of HIF-2α in a hypoxic stress situation. Indeed, a HIF-1α-dependent induction of known hypoxia-inducible genes both at short and at longer time points was observed. Thus, the exclusive expression and utilization of HIF-1α in SCLC may implicate that HIF-1α is crucial for the viability and growth capacity of these cells at low oxygen pressures. However, when directly tested by knocking down HIF-1α, the SCLC cells survived very well at hypoxia and the number of dead cells was unchanged. These observations suggest that other mechanisms than those driven by HIF-1 may be crucial for survival of SCLC cells at modest to severe hypoxia. We could not demonstrate increased NF-κB activity or autophagic response in hypoxic SCLC cells and therefore we conclude that neither NF-κB activation nor autophagy is of major importance for the survival of hypoxic SCLC cells. The UPR pathway involving XBP1 was activated at 0.1% oxygen presumably contributing to the HIF-1α-independent SCLC cell survival observed at severe hypoxia.

Hypoxic areas are common features in SCLC tumors and a correlation between HIF-1α protein expression and poor clinical outcome in SCLC has been demonstrated [31]. Thus, the expression of HIF-1α at prolonged hypoxia, as demonstrated here, might contribute to the aggressive properties, as shown for HIF-2α in other tumor forms [11, 23]. However, our results suggest that HIF-1α is not exclusively required for the viability of SCLC cells, but HIF-1α may be crucial for other properties linked to aggressiveness like for instance migration and invasion, properties not investigated in this study. Since the lung is a tissue where oxygen levels fluctuate, it is reasonable to assume that it is important for cells in such environments to be less sensitive to changes in oxygen levels. Therefore complementary adaptive mechanisms to hypoxia might be a survival advantage for cells of the lung as well as lung-derived tumor cells. At present we do not fully understand how SCLC cells adapt to hypoxia and further

characterization of the adaptation process in these cells might unravel uncharacterized mechanisms leading to cell survival at hypoxic conditions. Knowing the mechanisms behind the atypical hypoxic adaptation in SCLC cells may open up for new strategies to target aggressive SCLC.

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## Conflict of interest

The authors declare no conflict of interest.

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**Table 1**

Sequences of DNA primers used in Q-PCR experiments

| Primers | Primer sequence |
|---|---|
| *BNIP3* | f: 5'-AAAATATTCCCCCCAAGGAGTTC-3' |
| | r: 5'-ACGCTCGTGTTCCTCATGCT-3' |
| *BNIP3L* | f: 5'-GGCCTGTGAAGTGGTGTATTGT-3' |
| | r: 5'-ATTATAATCTGCTGGTGGCATT-3' |
| *CA9* | f: 5'-CCAGGCCTCACTGTGCAACT-3' |
| | r: 5'-TCGCCCAGTGGGTCATCT-3' |
| *GLUT1* | f: 5'-CTTCTATCCCAGGAGGTGGCTAT-3' |
| | r: 5'-AATGGAGCCTGACCCCTAGAG-3' |
| *GLUT3* | f: 5'-GCCGCTGCTACTGGGTTTTA-3' |
| | r: 5'-GGGACTTTCAGGGCAAAATG-3' |
| *HIF1A* | f: 5'-TTCCAGTTACGTTCCTTCGATCA -3' |
| | r: 5'-TTTGAGGACTTGCGCTTTCA -3' |
| *HIF2A* | f: 5'-GTGCTCCCACGGCCTGTA-3' |
| | r: 5'-TTGTCACACCTATGGCATATCACA-3' |
| *HK2* | f: 5'-GCCGGGCTGAGATTCTTCT-3' |
| | r: 5'-GCGACGTGTCCGGTTGTC-3' |
| *HPRT1* | f: 5'-TGACACTGGCAAAACAATGCA-3' |
| | r: 5'-GGTCCTTTTCACCAGCAAGCT-3' |
| *OCT4* | f: 5'-GAGAACCGAGTGAGAGGCAACC-3' |
| | r: 5'-CATAGTCGCTGCTTGATCGCTTG-3' |
| *SERPINB9* | f: 5'-CTTCGGCATTTGGGAATTGT-3' |
| | r: 5'-GGTCTCTCTCCGCTGACATTG-3' |
| *sXBP1* | f: 5'-CTGAGTCCGAATCAGGTGCAG-3' |
| | r: 5'-ATCCATGGGGAGATGTTCTGG-3' |
| *TBP* | f: 5'-CACGAACCACGGCACTGATT-3' |
| | r: 5'-TTTTCTTGCTGCCAGTCTGGAC-3' |
| *UBC* | f: 5'-ATTTGGGTCGCGGTTCTTG-3' |
| | r: 5'-TGCCTTGACATTCTCGATGGT-3' |
| *VEGF* | f: 5'-AGGAGGAGGGCAGAATCATCA-3' |
| | r: 5'-CTCGATTGGATGGCAGTAGCT-3' |

## Figure legends

**Figure 1.** Lack of HIF-2α protein whereas HIF-1α protein is present adjacent to necrotic regions in human SCLC specimens.

**A** and **B**, Immunohistochemical characterization of HIF-1α and HIF-2α in human SCLC and NSCLC specimens, selected tumors from the tissue microarray immunostainings summarized in C. In **B**, HIF-2α-positive infiltrating immune cells (arrowheads) are shown. Scale bars in **A**, 200 μm and in **B**, 50 μm. **C**, Summary of the HIF-1α and HIF-2α immunostaining of 35 specimens from SCLC patients and 9 specimens from NSCLC patients organized in a tissue microarray. **D**, SK-N-BE(2)c neuroblastoma cells grown at 21% and 1% oxygen for 4 h and 72 h were used to verify the specificity of the HIF-1α and HIF-2α antibody, respectively, used in **A-C**.

**Figure 2.** No or extremely low levels of HIF-2α mRNA in SCLC cells.

Q-PCR analyses of HIF-1α and HIF-2α mRNA levels in SCLC and NSCLC cells grown at 21% and 1% oxygen for 4 h and 72 h. All expression data were normalized to three reference genes (*HPRT1, UBC, TBP*). Data are one of two representative experiments; error bars show the standard deviation within triplicates. * indicates $p < 0.05$, ** $< 0.01$, *** $< 0.001$ in a 2-tailed unpaired Student's t test, $C_t$ levels above the detection cutoff point of 32 are not taken under consideration.

**Figure 3.** No detectable HIF-2α but sustained HIF-1α protein expression at prolonged hypoxia in SCLC cells.

**A**, HIF-1α and HIF-2α western blot analyses of whole cell lysates (80 μg) from SCLC and NSCLC cells cultured at 21% and 1% oxygen for 4-96 h. SK-N-BE(2)c neuroblastoma (NB) cells (BE(2)c) grown at 21% and 1% oxygen for 4 h were used as positive control for HIF-1α

and grown for 72 h as a control for HIF-2α. Representative blots of two independent experiments are shown and actin was used as a loading control. **B**, Immunocytochemical staining of HIF-2α in U-1690 SCLC and SK-N-BE(2)c neuroblastoma cells cultured at 21% and 1% oxygen for 72 h. Bars, 50 μm.

**Figure 4.** HIF-1α and HIF-2α protein expression patterns in immortalized alveolar epithelial cells and A549 NSCLC cells.

HIF-1α and HIF-2α western blot analyses (80 μg) of P2G, P2GH and A549 cells grown at either 21% or 1% oxygen for 4-96 h. Neuroblastoma SK-N-BE(2)c cells (Be(2)c) grown at 21% and 1% oxygen for 4 h were used as positive control for HIF-1α and grown for 72 h as a control for HIF-2α. Actin was used as a loading control and one of two representative experiments is shown.

**Figure 5.** SCLC cells survive well at low oxygen levels.

**A-C**, SCLC cells and **D**, NSCLC cells were cultured at varying oxygen levels for 72 h, thereafter the numbers of viable and dead cells were counted after staining with trypan blue. The experiment was performed in triplicate and **A** and **D** are mean from three experiments and **B** and **C** are one of two representative experiments. The arrows indicate the number of cells seeded at day 0 and error bars show the standard deviation. Significant differences in viable cells compared to 21 % oxygen are shown. **E** and **F**, Cell proliferation analyses of non-synchronized U-1690 cells grown at varying oxygen levels using CFSE staining. **E**, The unfilled lines show unlabeled cell populations at day 0 and 4, whereas filled lines demonstrate CFSE-labeled cells, data are one of three representative experiments. **F**, The relative levels of non-cycling cells (gates from E) and data are mean from three experiments. Significant differences in relative levels of non-cycling cells compared to 21 % oxygen are shown. **G**,

Cell cycle phase distribution was analyzed using propidium iodide in non-synchronized U-1690 cells after 72 h of culture at different oxygen pressures. Data are mean from three experiments. All statistical analyses were performed using 2-tailed unpaired Student's t test, * indicates $p < 0.05$, ** < 0.01, *** < 0.001.

**Figure 6.** Known hypoxia-inducible genes are moderately induced in SCLC cells at hypoxia. The relative mRNA expression levels of indicated genes were analyzed by Q-PCR in SCLC and NSCLC cells. The cells were grown at 21% and 1% oxygen for 4 and 72 h and neuroblastoma (NB) SK-N-BE(2)c cells (BE(2)c) grown at 21% and 1% oxygen for 72 h were included for comparison. All expression data were normalized to three reference genes (*HPRT1, UBC, TBP*). Data are one of two representative experiments; error bars show the standard deviation within triplicates. * indicates $p < 0.05$, ** < 0.01, *** < 0.001 in a 2-tailed unpaired Student's t test on up-regulated genes, $C_t$ levels above the detection cutoff point of 32 are not taken under consideration.

**Figure 7.** SCLC cells survive well at hypoxia despite reduced HIF-1α expression.
SCLC cells were transfected with siRNA against HIF-1α (si-H1α) at 21%, 1% and 0.1% oxygen for 72 h. An unspecific siRNA was used as control (si-C), samples treated with lipofectamine alone (C) are also shown. Neuroblastoma (NB) cells SK-N-BE(2)c cells (BE(2)c) grown at 21% and 1% oxygen for 4 h and 72 h were included for comparison.
**A**, Q-PCR and western blot analyses (80 µg) of HIF-1α and HIF-2α in U-1690 cells. All mRNA expression data were normalized to three reference genes (*HPRT1, UBC, TBP*). Data are one of three representative experiment; error bars show the standard deviation within triplicates. SDHA was used as a loading control for western blot and data are one of three representative experiments. **B**, The numbers of viable and dead cells were counted after

staining the cells with trypan blue and the experiments was performed in triplicates. Data are mean from three experiments; error bars show the standard deviation. Statistical analyses of viable and dead cells were performed. The arrows indicate the number of cells seeded at day 0. **C**, Western blot analyses of HIF-1α and HIF-2α (60 µg and 40 µg, respectively). SDHA was used as a loading control, and for unknown reason SDHA have migrated different in U-1690 compared to BE(2)c cells. One of three representative experiments is shown. **D-E**, Q-PCR analyses of spliced XBP1 in untransfected and si-RNA-transfected SCLC cells. The expression data were normalized to three reference genes (*HPRT1, UBC, TBP*). Data are one of two representative experiments; error bars show the standard deviation within triplicates. Statistical analyses were performed using 2-tailed unpaired Student's t test, * indicates $p < 0.05$, ** < 0.01, *** < 0.001. The following comparisons were done: panels A-B, control-siRNA- vs. si-HIF-1α-treated cells; panel D, 1% vs. 0.1%; panel E, 21% control- vs 0.1% control-treated cells or control-siRNA- vs. si-HIF-1α-treated cells.

## Supplemental figure legends

**Supplemental Figure S1.** Kinetics of HIF-1α and HIF-2α mRNA and proteins in a large-cell morphological variant of SCLC cells (SCLC*, U-1568) grown at 21% and 1% oxygen for 4-96 h.

**A**, mRNA expression levels of HIF-1α and HIF-2α were analyzed by Q-PCR and as a comparison the SCLC cells U-1690 and the NSCLC cells A549 were included. All expression data were normalized to three reference genes (*HPRT1, UBC, TBP*). Data are one of two representative experiment; error bars show the standard deviation within triplicates. * indicates $p < 0.05$, ** < 0.01, *** < 0.001 in a 2-tailed unpaired Student's t test, $C_t$ levels above the detection cutoff point of 32 are not taken under consideration. **B**, HIF-1α and HIF-2α protein levels (80 µg) were analyzed by immunoblotting. SK-N-BE(2)c neuroblastoma (NB) cells (BE(2)c) cultured at 21% and 1% oxygen for 4 h were used as positive control for HIF-1α and grown for 72 h as a control for HIF-2α. Blots from one of two representative experiments are shown.

**Supplemental Figure S2.** HIF-1α-dependent induction of genes in SCLC cells.

U-1690 cells were transfected with siRNA against HIF-1α (si-H1α) at 21% and 1% oxygen for 72 h. An unspecific siRNA was used as control (si-C), samples treated with lipofectamine alone (C) are also shown. All expression data were normalized to three reference genes (*HPRT1, UBC, TBP*). **A**, Relative mRNA expression levels of BNIP3, CA9, GLUT1 and VEGF were analyzed by Q-PCR. Data are mean from three experiments; error bars show the standard deviation. Statistically significant differences between hypoxic-treated cells (*, $p<0.05$ and **, $p<0.01$) were determined by ANOVA followed by Duncan's multiple range test. **B**, Q-PCR analyses of HIF-2α mRNA levels. NSCLC cells grown at 21% oxygen for 72

h were included for comparison. Data are one of two representative experiment; error bars show the standard deviation within triplicates.

**Supplemental Figure S3.** No increased autophagy in hypoxic SCLC cells.

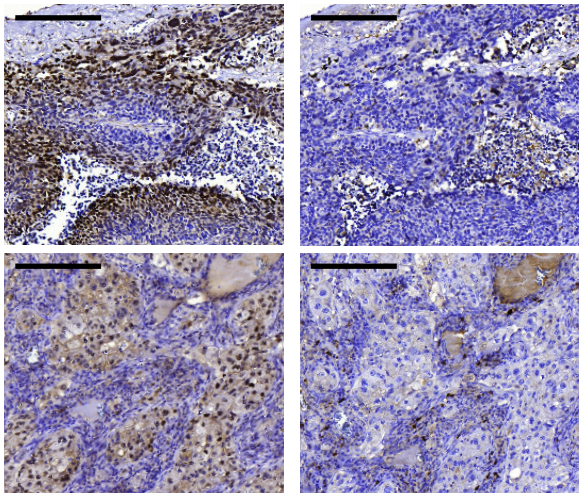
**A**, Western blot analyses of LC3-II protein levels in whole cell lysates from U-1690 SCLC cells cultured at 21% and 1% oxygen for 24 h. Cells treated with 10 nM Bafilomycin A1 (H+ATPase inhibitor) for 4 h were used as positive control for LC3-II expression. Actin was used as a loading control and results from one of two representative experiments are shown. **B**, Q-PCR analyses of BNIP3 and BNIP3L and the expression data were normalized to three reference genes (*HPRT1, UBC, TBP*). Data are one of two representative experiments; error bars show the standard deviation within triplicates. **C**, U-1690 cells transfected at 21% and 1% oxygen for 72 h with a siRNA against BNIP3L, an unspecific siRNA (si-C) or lipofectamine alone (C). The number of viable and dead cells were counted and the experiments were performed in triplicates. The arrow indicates the number of cells seeded at day 0. Q-PCR analyses of BNIP3L and the expression data were normalized to three reference genes (*HPRT1, UBC, TBP*). One of three representative experiments is shown; error bars show the standard deviation. Statistical analyses between control-siRNA- vs. si-RNA-treated cells were performed using 2-tailed unpaired Student's t test, * indicates $p < 0.05$, ** $< 0.01$, *** $< 0.001$.

**Supplemental Figure S4.** No significantly increased NF-κB activity in hypoxic SCLC cells. NF-κB luciferase reporter activity was measured in U-1690 and U-1906 cells cultured at 21% or 1% oxygen for 1-4 h. TPA stimulation (100 ng/ml) over night was used as positive control. Data are representative of two independent experiments; error bars show the standard

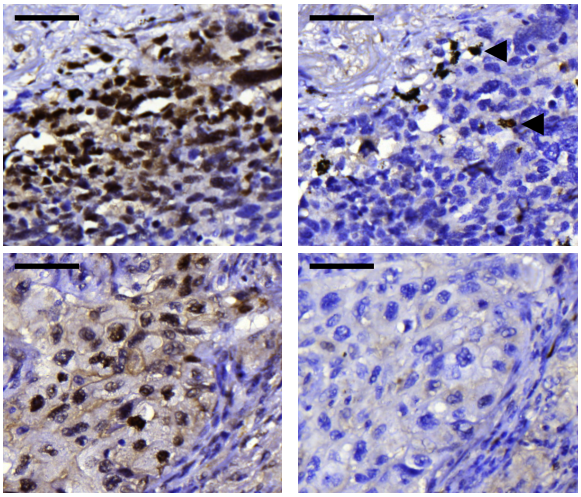
deviation within triplicates. Statistical analyses were performed using 2-tailed unpaired Student's t test.

Figure 1

A




B




C

| HIF-1α and HIF-2α positive staining | | |
|---|---|---|
| **Lung specimens** | **HIF-1α** | **HIF-2α** |
| Small cell lung carcinoma | 20/31 | 1/35 |
| Large cell carcinoma | 2/3 | 1/3 |
| Squamous cell carcinoma | 3/3 | 2/3 |
| Adenocarcinoma | 1/3 | 2/3 |

D

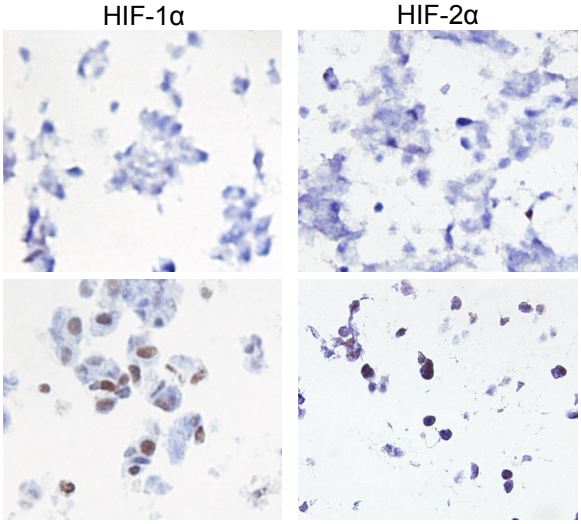
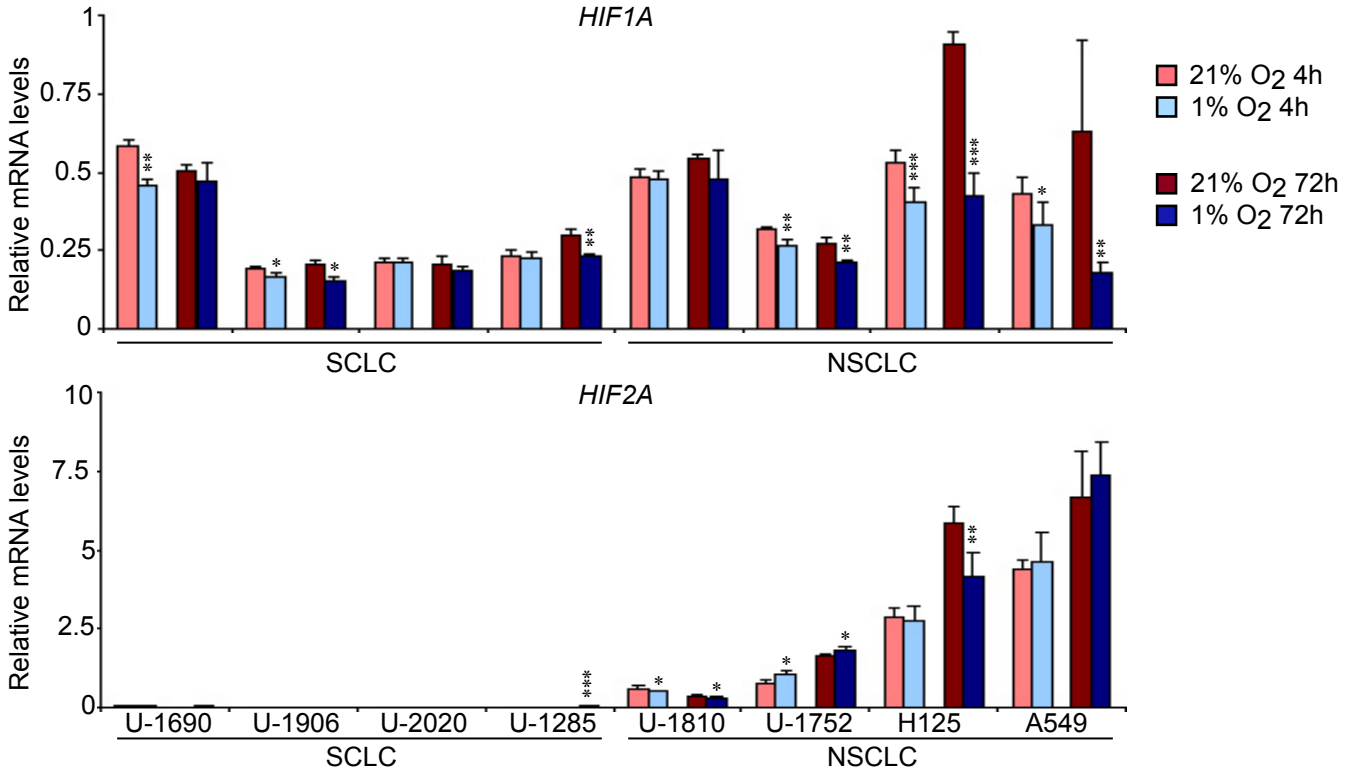


Figure 2


HIF1A
Relative mRNA levels
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0.75
0.5
0.25
0
SCLC
NSCLC
21% O2 4h
1% O2 4h
21% O2 72h
1% O2 72h
HIF2A
Relative mRNA levels
10
7.5
5
2.5
0
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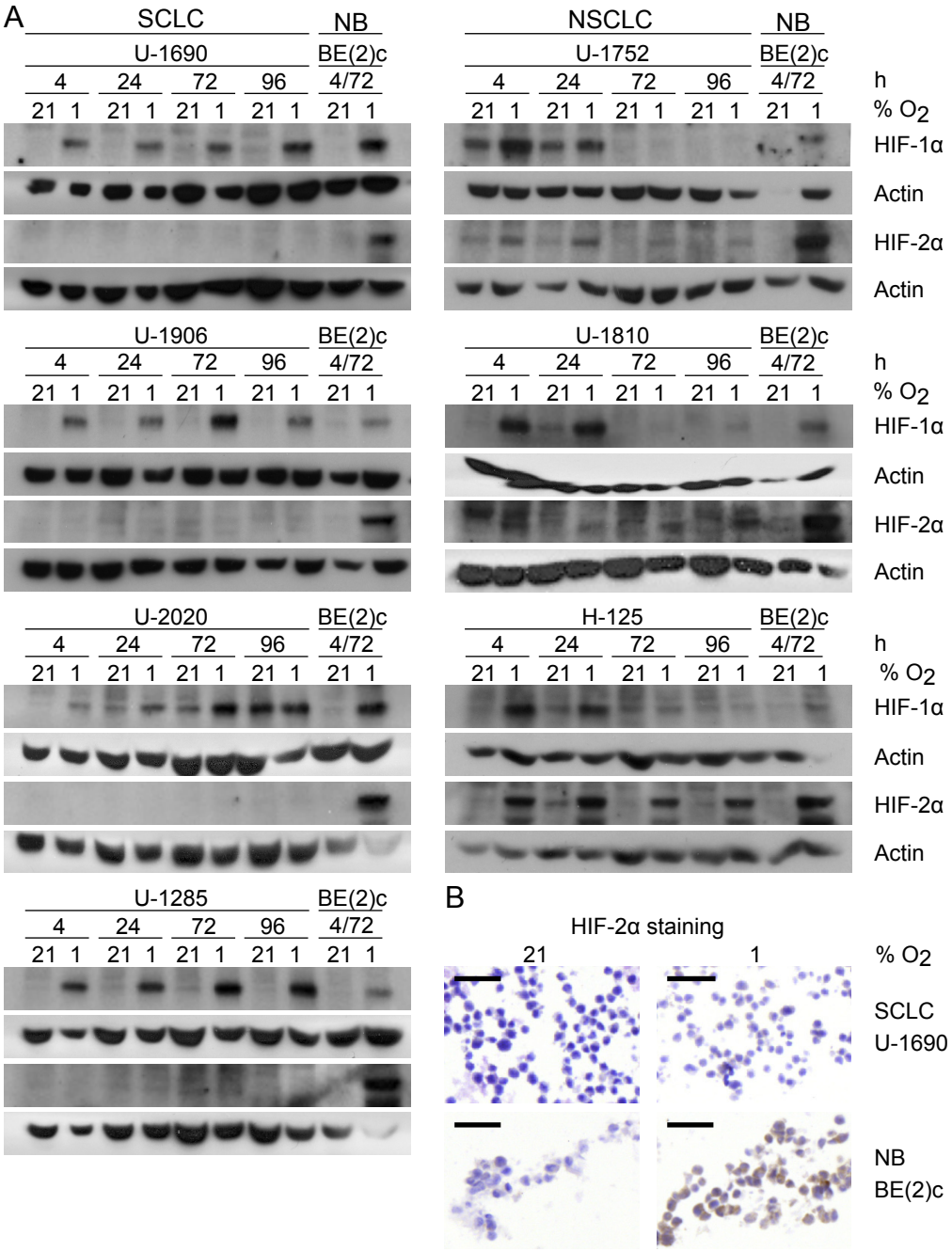
U-1906
U-2020
U-1285
U-1810
U-1752
H125
A549
SCLC
NSCLC

Figure 3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SCLC
NB
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BE(2)c

Figure 4

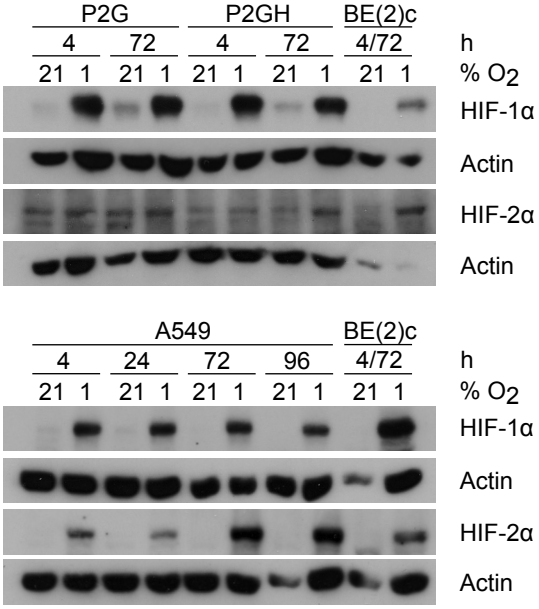

P2G
P2GH
BE(2)c
4
72
4
72
4/72
h
21 1 21 1 21 1 21 1 21 1
% O2
HIF-1α
Actin
HIF-2α
Actin
A549
BE(2)c
4
24
72
96
4/72
h
21 1 21 1 21 1 21 1 21 1
% O2
HIF-1α
Actin
HIF-2α
Actin

Figure 5

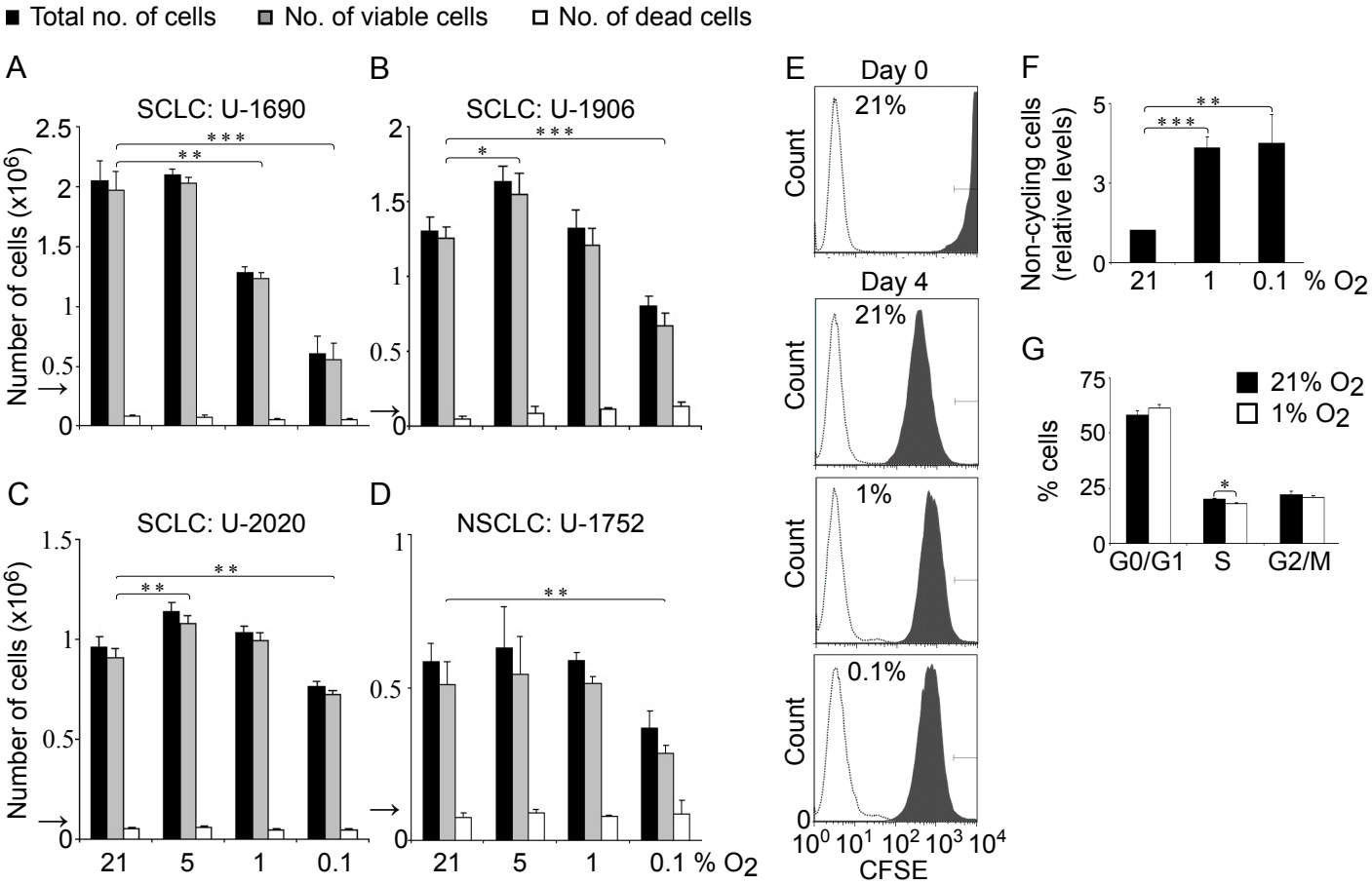

Total no. of cells
No. of viable cells
No. of dead cells
A
SCLC: U-1690
Number of cells (x10^6)
B
SCLC: U-1906
C
SCLC: U-2020
D
NSCLC: U-1752
21
5
1
0.1
% O2
E
Day 0
21%
Day 4
21%
1%
0.1%
Count
CFSE
F
Non-cycling cells (relative levels)
% O2
G
21% O2
1% O2
% cells
G0/G1
S
G2/M

Figure 6

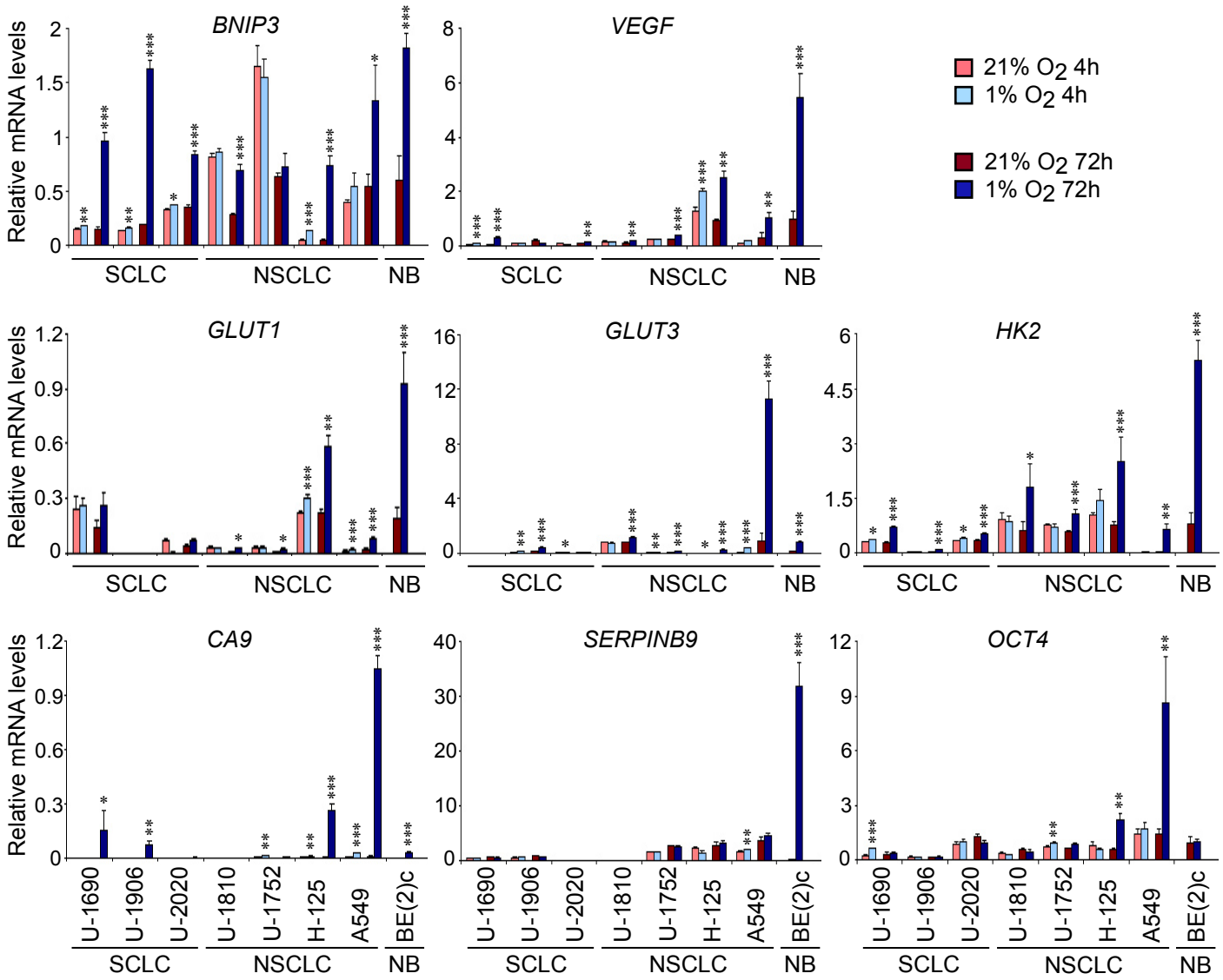
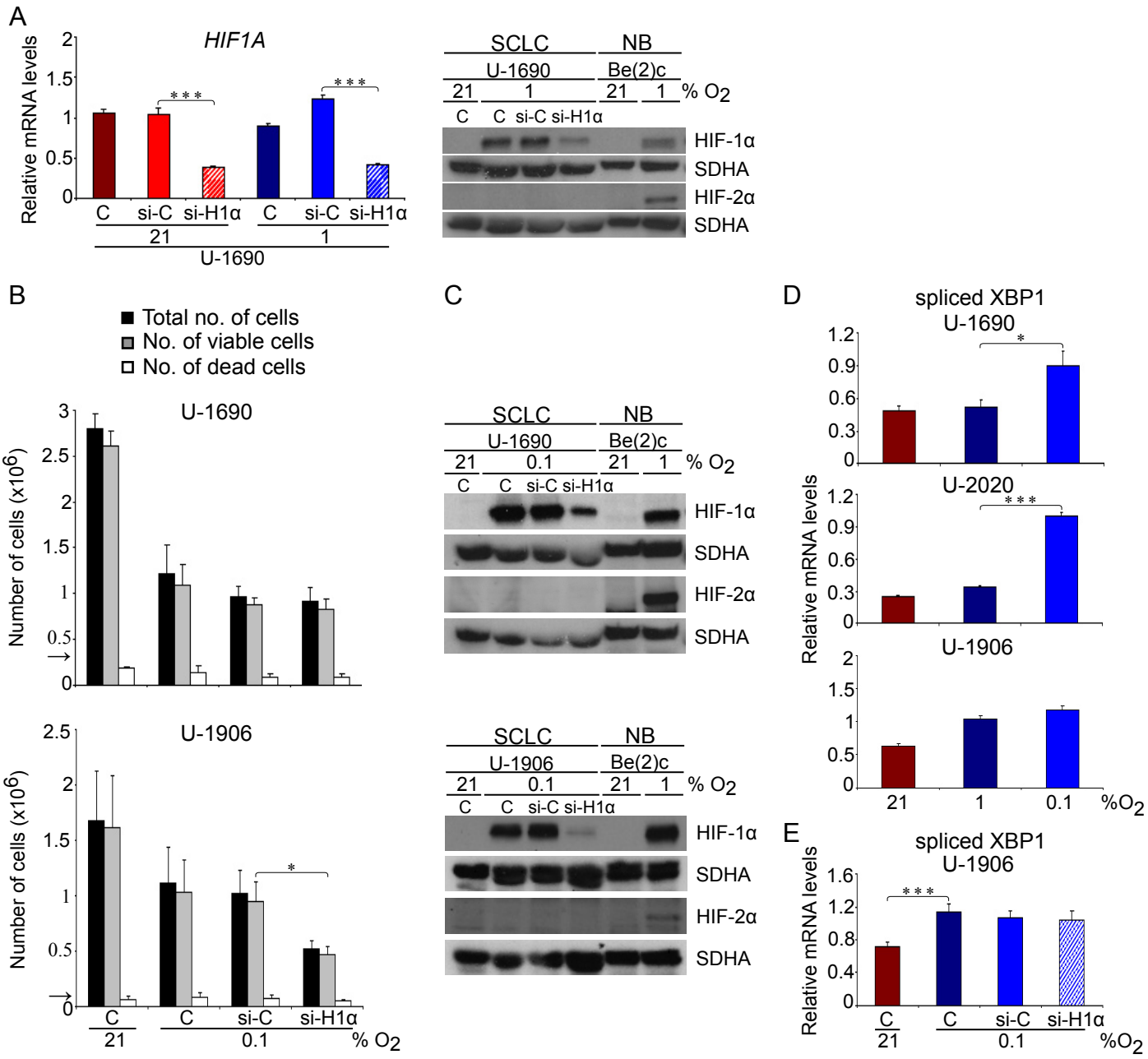
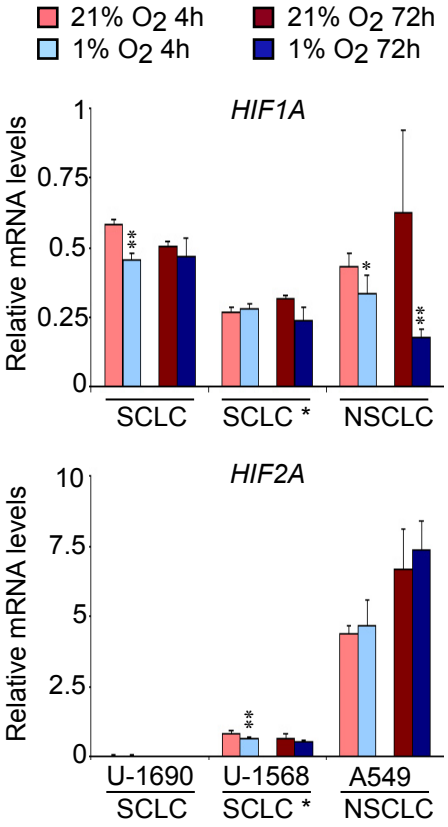

BNIP3
VEGF
GLUT1
GLUT3
HK2
CA9
SERPINB9
OCT4
Relative mRNA levels
21% O2 4h
1% O2 4h
21% O2 72h
1% O2 72h
SCLC
NSCLC
NB
U-1690
U-1906
U-2020
U-1810
U-1752
H-125
A549
BE(2)c

Figure 7

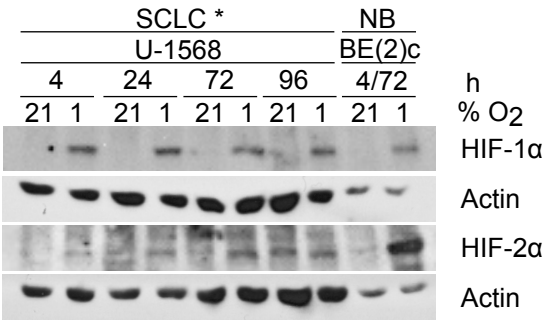

A
HIF1A
Relative mRNA levels
C si-C si-H1α C si-C si-H1α
21 1
U-1690
SCLC NB
U-1690 Be(2)c
21 1 21 1 % O2
C C si-C si-H1α
HIF-1α
SDHA
HIF-2α
SDHA
B
Total no. of cells
No. of viable cells
No. of dead cells
U-1690
U-1906
Number of cells (x10^6)
C C si-C si-H1α
21 0.1 % O2
C
SCLC NB
U-1690 Be(2)c
21 0.1 21 1 % O2
C C si-C si-H1α
HIF-1α
SDHA
HIF-2α
SDHA
SCLC NB
U-1906 Be(2)c
21 0.1 21 1 % O2
C C si-C si-H1α
HIF-1α
SDHA
HIF-2α
SDHA
D
spliced XBP1
U-1690
U-2020
U-1906
Relative mRNA levels
21 1 0.1 %O2
E
spliced XBP1
U-1906
Relative mRNA levels
C C si-C si-H1α
21 0.1 %O2

Supplemental Figure S1

A

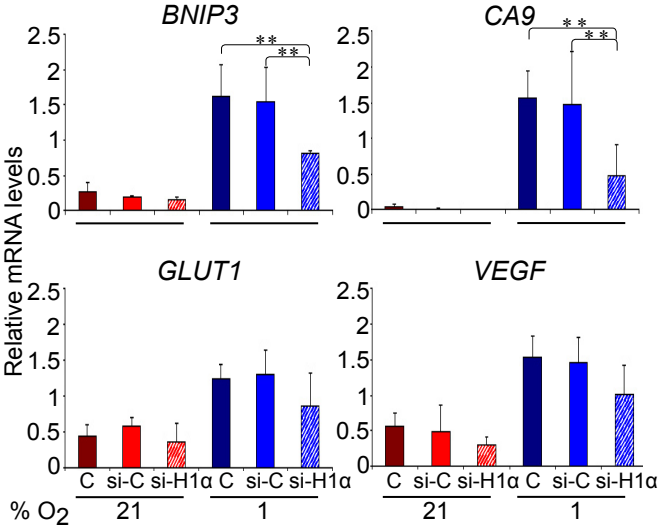



B

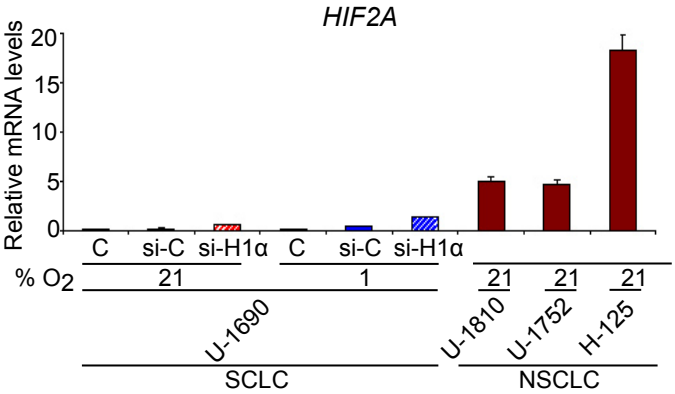


Supplemental Figure S2

A

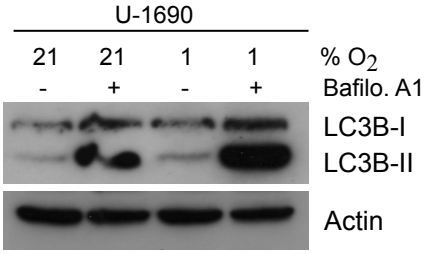



B

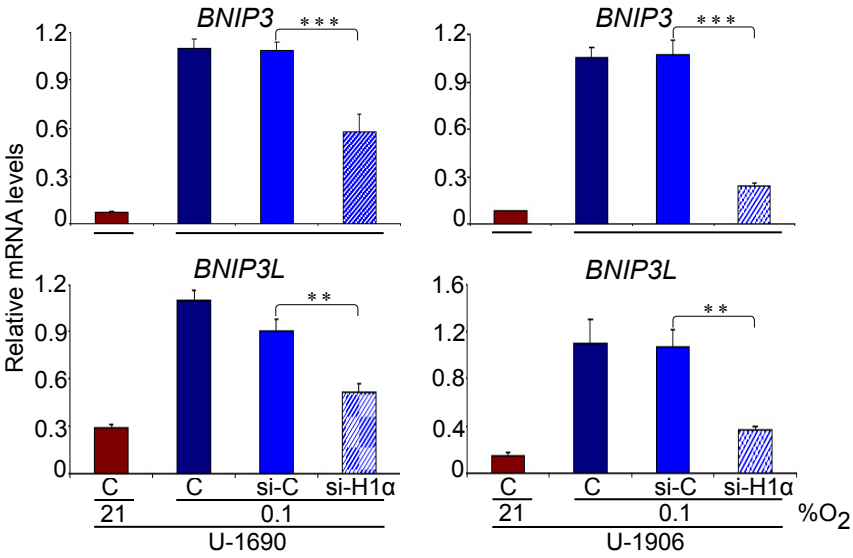


Supplemental Figure S3

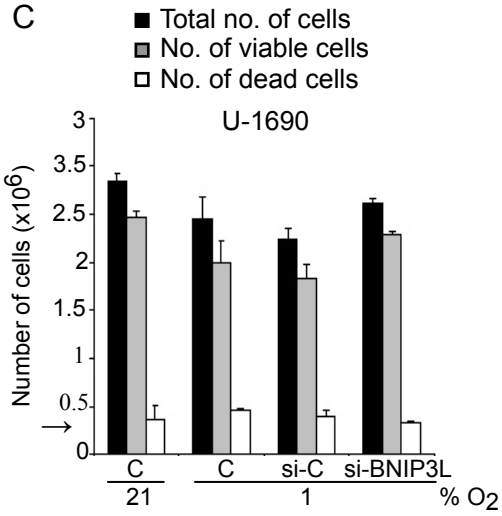
A

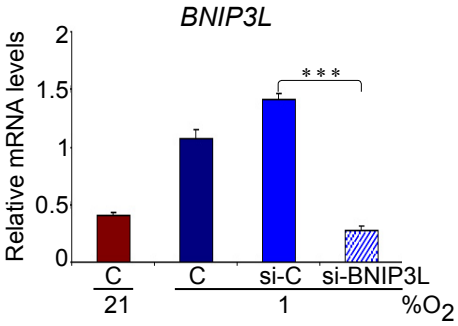
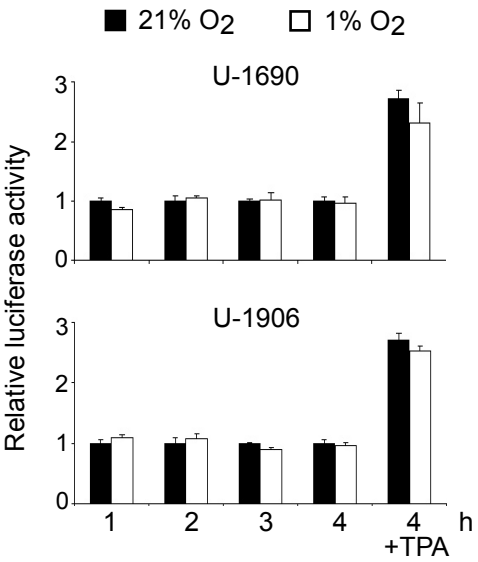



B




C






Supplemental Figure S4