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Original Research

Glutamine deprivation counteracts hypoxia-induced chemoresistance

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Abstract

The microenvironment of solid tumors is a key determinant of therapy efficacy. The co-occurrence of oxygen and nutrient deprivation is a common phenomenon of the tumor microenvironment and associated with treatment resistance. Cholangiocarcinoma (CCA) is characterized by a very poor prognosis and pronounced chemoresistance. A better understanding of the underlying molecular mechanisms is urgently needed to improve therapy strategies against CCA. We sought to investigate the importance of the conditionally essential amino acid glutamine, a centrally important nutrient for a variety of solid tumors, for CCA. Glutamine levels were strongly decreased in CCA samples and the growth of established human CCA cell lines was highly dependent on glutamine. Using gradual reduction of external glutamine, we generated derivatives of CCA cell lines which were able to grow without external glutamine (termed glutamine-depleted (GD)). To analyze the effects of coincident oxygen and glutamine deprivation, GD cells were treated with cisplatin or gemcitabine under normoxia and hypoxia. Strikingly, the well-established phenomenon of hypoxia-induced chemoresistance was completely reversed in GD cells. In order to better understand the underlying mechanisms, we focused on the oncogene c-Myc. The combination of cisplatin and hypoxia led to sustained c-Myc protein expression in wildtype cells. In contrast, c-Myc expression was reduced in response to the combinatorial treatment in GD cells, suggesting a functional importance of c-Myc in the process of hypoxia-induced chemoresistance. In summary, these findings indicate that the mechanisms driving adaption to tumor microenvironmental changes and their relevance for the response to therapy are more complex than expected.

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Introduction

Significant differences between the metabolic properties of cancer cells and those of normal cells have already been described almost a century ago [1]. Besides the dependency on high rates of aerobic glycolysis, a deregulated uptake and utilization of amino acids is considered a hallmark of cancer [2]. In addition to glucose, the non-essential amino acid glutamine is the most important primary metabolite to support neoplastic proliferation [3,4]. Its significance is not only based on its ability to fuel a variety of growth-promoting pathways through the donation of nitrogen and carbon atoms [5] but also on its central function in chromatin modification and

Abbreviations: CCA, cholangiocarcinoma, eCCA, extrahepatic cholangiocarcinoma, iCCA, intrahepatic cholangiocarcinoma, GD, glutamine-deprived, MALDI-FTICR, matrix-assisted laser desorption ionization Fourier transformation ion cyclotron mass spectrometry imaging, ECAR, extracellular acidification rate, OCR, oxygen consumption rate

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the regulation of cell signaling and anti-oxidative defense [6,7]. Although glutamine can be *de novo* synthesized from glutamate, it can become conditionally essential in phases of stress or extensive growth [8]. Especially rapidly dividing tumor cells show an increased demand for glutamine [4,6,9]. As a result, the process of tumor growth is often characterized by elevated glutamine consumption. Particularly in the core area of solid tumors, which usually show a highly heterogeneous microenvironment with a lack of functional vasculature, consumption depletes the local supply and cells might have to face periods of glutamine deprivation. Since glutamine deprivation might not be an exclusive event, but rather exist coincidentally with other microenvironmental abnormalities like hypoxia, adaptive mechanisms might be more complex than expected [10].

Cholangiocarcinoma (CCA) is the most common biliary tract cancer and the second most common primary hepatic malignancy. The prognosis of CCA is poor, given that the majority of patients are diagnosed at an advanced disease stage. While surgical resection is the only potentially curative treatment, systemic chemotherapy with gemcitabine and cisplatin has become the reference regimen in the treatment of patients with advanced, unresectable CCA [11]. Considering that one of the main challenges in the treatment of CCA is the poor response to chemotherapy, it is pivotal to understand the underlying mechanism for the development of resistances to anti-cancer drugs. It is well established that many metabolic pathways in cancer cells are functionally linked to major signaling cascades. Those interactions can directly affect cancer cell susceptibility and aggressiveness [12]. Intriguing publications demonstrated that a dysregulated glutamine metabolism mediates therapeutic resistance [13] and that the susceptibility of cancer cells to apoptotic triggers is determined by extracellular glutamine levels [14]. Based here on, the hypothesis emerged that limiting glutamine availability could be a potential strategy to prevent the development of chemoresistance [15,16]. Indeed, a large number of studies has shown that many tumor cell types fail to proliferate in the absence of glutamine, a phenomenon referred to as glutamine addiction [17]. However, recent experimental data suggest that several tumor cell types may reside in an environment where glutamine is profoundly limited, considering that many tumor cells do not necessarily die from glutamine withdrawal [6]. Whether survival of CCA cells is dependent on external glutamine supply has not yet been examined, nor whether glutamine deprivation affects the therapeutic response of CCA cells. In this study, we investigated the effect of long-term glutamine deprivation with coincident hypoxia on cytostatic drug susceptibility of CCA cells. By gradually reducing external glutamine availability, we established two extrahepatic CCA (eCCA) cell line variants that were able to overcome their addiction to glutamine supply. These cells showed an altered sensitivity to cisplatin and gemcitabine under hypoxia as compared to their parental counterparts, arguing for an interconnection between the molecular mechanisms that govern adaptation to glutamine deprivation and the hypoxic response that was not previously appreciated.

Materials and methods

Clinical specimen collection

All experimental procedures were approved by the Ethics Committees of Aachen University Hospital (Permission No: EK206-09). Written consent was obtained from all participants. Four female, native iCCA patients with an age between 61 and 82 (71 9.05) years at surgery, a grading of 2 or 3 and a tumor stage between 1a and 2 were included in the study. A female patient who received a portal vein embolization (PVE) with associated liver partition and portal vein ligation for staged hepatectomy (ALPPS) prior to surgery was used as control specimen. Tissue specimens were acquired by surgical resection in the Department of General Surgery, University Hospital RWTH Aachen. Samples were immediately frozen in liquid nitrogen and stored at -80 °C.

Tumor cell lines

Extrahepatic CCA cell lines EGI-1 and TFK-1 were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany). EGI-1 cells were cultivated in DMEM low glucose (1%) and TFK-1 cells were grown in RPMI 1640 medium, both supplemented with 10% standard fetal calf serum and 1% penicillin/streptomycin. GD variants were cultured in the appropriate medium variant without glutamine. All cells lines were maintained in a humidified tissue culture incubator with 5% CO_2.

Antiproliferative drug treatment

Antiproliferative drugs were purchased from Selleckchem (Houston, USA). Stocks of cisplatin were prepared in dimethylformamide (DMF) and stocks of gemcitabine in dimethyl sulfoxide (DMSO). Cells were seeded and directly placed under normoxic or hypoxic (1% O_2) conditions. 24 h after seeding, the medium was replaced by cytostatic drug-bearing medium. The medium was changed every 36 h to ensure optimal drug potency. Control cells were vehicle-treated with the appropriate dose of DMSO or DMF.

Immunoblot

Cells were either lysed in RIPA buffer or nuclear extraction buffer. Proteins were size-fractionated by SDS-PAGE and wet-transfer blotted onto a nitrocellulose membrane. Immunoblotting was performed using antibodies to ATM (Abcam, Cambridge, England, 1:2000), β-Tubulin (ThermoFisher Scientific, Waltham, USA, 1:2000), c-Myc (Abcam, 1:2000), γH2AX (Novus Biologicals, Centennial, USA, 1:2000), HIF-1α (Cayman Chemical, Ann Arbor, USA, 1:650), HIF-2α (Novus Biologicals, 1:2000) and YY1 (Santa Cruz Biotechnology, Dallas, USA, 1:1000). 5% milk in TBS-T buffer was used for antibody dilution and blocking. Densitometrical quantification of Western blot signals was assessed using ImageJ software (NIH, Bethesda, USA) and relative protein levels were obtained by normalization to Tubulin signal.

Metabolic flux analysis

Bioenergetic parameters were analyzed by using the Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, USA). 40,000 cells/well were seeded in their appropriate media variant. One hour prior to measurement, the medium was changed to assay medium. Seahorse XF assay media was supplemented with 10 mm glucose and either contained or lacked 2 mM Glutamax (pH adjusted to 7.3). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were real-time monitored in an incubation chamber at 37 °C. The optimal concentration of compounds has been determined empirically and was defined as 5 M oligomycin, 0.5 M carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone and 0.1 M rotenone plus antimycin A. All reagents were purchased from Sigma-Aldrich (St. Louis, USA). Values were normalized based on cell numbers by using CyQUANT NF Cell Proliferation Assay Kit (ThermoFisher Scientific) according to the manufacturers protocol.

Mass spectrometry imaging

10 m thick cryosections were mounted onto ITO-coated glass slides (DELTA Technologies Ltd., Loveland, CO, USA). On-tissue derivatiza-

tion was performed using the reagent p-N,N,N-trimethylammonioanilyl N'-hydroxysuccinimidyl carbamate iodide (TAHS) followed by application of the matrix (2,5-Dihydroxybenzoic acid) as described in [18]. Subsequently, the samples were analyzed by Matrix-assisted laser desorption ionization Fourier transformation ion cyclotron mass spectrometry imaging (MALDI-FTICR-MSI), performed with a high-resolution instrument (Solarix, 9.4 T, Bruker Daltonics, Bremen, Germany), equipped with a Smartbeam II ND:YAG UV laser. The experiment was performed with a laser frequency of 2000 Hz, a laser power of 18% and 50 shots per pixel. Data were acquired in absorption mode in the mass range of 100-550 *m/z*, using 1 million data points per pixel and a mass resolution of $1.5e^5$ at 200 *m/z*. Spectra were acquired with a 75 m step size and raster width. Data acquisition and processing was performed using FlexImaging 4.1, ftmsControl, Flexcontrol and DataAnalysis form Bruker Daltonics (Bruker Daltonics, Bremen, Germany). To distinguish off-tissue from on-tissue pixels, a total ion current filter was applied and spectra of all samples were normalized by root mean square (RMS). The human metabolome database was used for ID assignment of *m/z* values in conjunction with MS/MS fragmentation of the glutamine peak. After imaging, hematoxylin and eosin histological staining of the same tissue sections was accomplished by removing the MALDI matrix by washing with EtOH followed by H&E staining of the same tissue section. Images were recorded by using a Mirax slide scanner (Zeiss, Oberkochen, Germany).

Statistical analysis

GraphPad Prism 5.0 statistical software (GraphPad Software, La Jolla, USA) was used for all analyses. Parametric data were analyzed using students t-test. The analysis of growth curves and respiration parameters was performed using one-way ANOVA analysis with Bonferroni's multiple comparisons test. Results are presented as mean standard deviation (SD). The number of replicates is given in the respective figure legend. Statistical significance was assigned if $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

Results

Glutamine depletion is common in human CCA

To investigate whether glutamine deprivation occurs in human CCA, we performed a spatial metabolite analysis of four native human CCA tissue specimens by using MALDI-MSI and compared the localized metabolite levels with H&E stains to provide structural information of the tissue (Figure 1). A PVE/ALPPS-treated patient served as positive control, given that this procedure cuts off the portal inflow and results in nutrient depletion [19]. Pair-wise analysis of tumor and adjacent benign tissue revealed an inhomogeneous distribution of glutamine in all analyzed samples. While only one specimen displayed increased glutamine levels inside the tumor, we found glutamine to be strongly depleted in the tumor core region of the remaining samples, suggesting that glutamine-depletion might be a common event in CCA.

In vitro growth of human CCA cells depends on external glutamine supplementation

To elucidate the importance of cellular consumption with respect to the observed glutamine depletion, we next analysed whether CCA cells require external supply of glutamine for survival. We cultured two eCCA (EGI-1 and TFK-1) and two iCCA cell lines (CC-LP-1 and SNU-1079) and analyzed cell numbers in dependence on glutamine concentrations (Supplementary Figure 1). All tested cell lines showed concentration-dependent growth retardation, while deprivation of glutamine was found to either induce an almost entire cessation of proliferation or cell death. These findings suggest an *in vitro*-addiction to glutamine and support the hypothesis of a high relevance of glutamine for CCA cells.

Based on the pronounced reaction to glutamine withdrawal, we chose the cell lines TFK-1 and EGI-1 for long-term starvation experiments. A simple experimental setting with a gradual reduction of external glutamine concentrations was used to mimic a slow microenvironment-associated deprivation of glutamine in the process of tumor growth (Figure 2A). Considering the results presented in Supplementary Figure 1, we defined the initial medium concentrations of glutamine as the concentrations whereby 50% of the cells were still viable after 72 h: 1 mM for EGI-1 and 0.5 mM for TFK-1. The cells obtained in this selection process are henceforth referred to as EGI-1 GD (glutamine-deprived) and TFK-1 GD. The total time required to establish cell growth under completely glutamine-free conditions was 10 months for EGI-1 and 11 months for TFK-1 cells. To assess the cellular capability of adaption to glutamine withdrawal via a slow deprivation process, we first analyzed proliferation of eCCA GD cell lines compared to their parental counterparts. Even though both GD cell lines were capable of adapting to glutamine-independent growth, individual differences were noted. Compared to the parental cell lines in glutamine-supplied medium, TFK-1 GD showed a significantly lower while EGI-1 GD showed a significantly higher cell number at day 7 (Figure 2B). We furthermore compared morphological traits and found cell

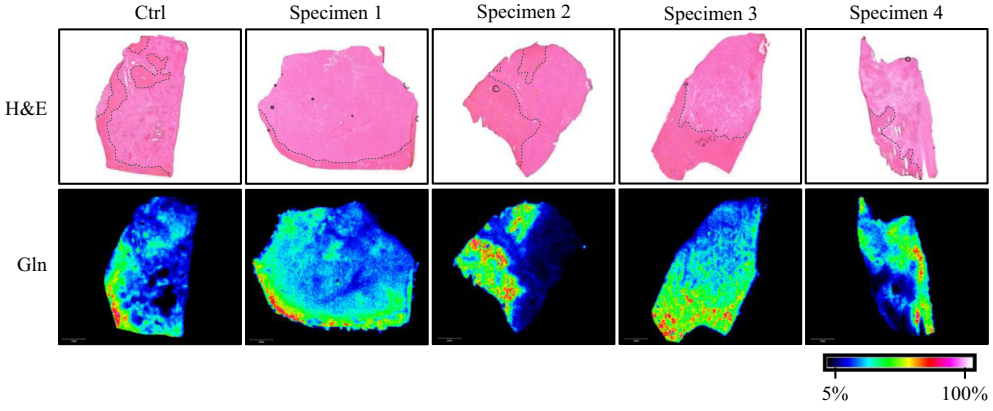

Figure 1. Human CCA patients display glutamine-poor regions inside the tumor. Specimen were analyzed for the spatial distribution of glutamine (Gln [M + TAHS]x + : (323.1714 m/z) in tumor and adjacent benign tissue by using high-resolution MALDI-FTICR-MSI and were hematoxylin and eosin (H&E)-stained. The color scale represents the absolute intensity of detected ions. A patient with a portal vein embolization (PVE) was used as control specimen (Ctrl).

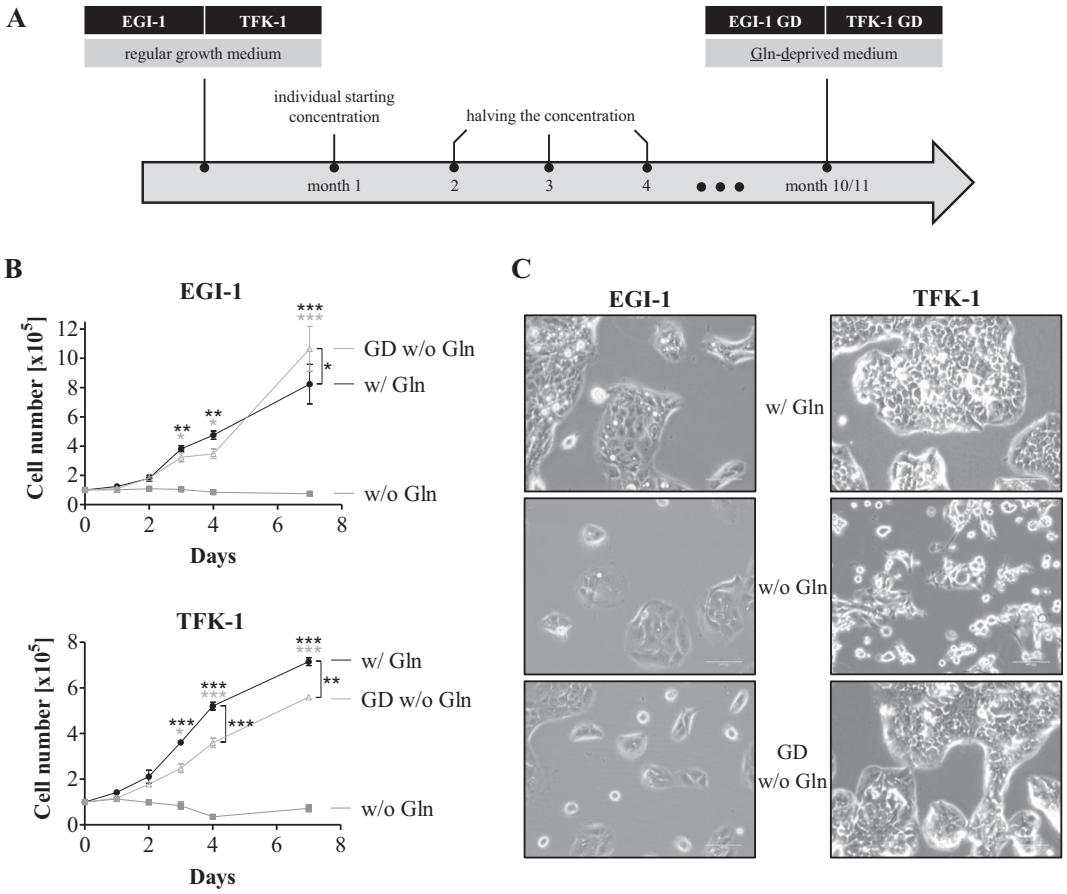

Figure 2. eCCA cells can overcome glutamine addiction by gradual reduction of external glutamine concentrations. A) Timeline of gradual glutamine depletion of EGI-1 and TFK-1 cells. The concentration of available glutamine in growth medium was monthly decreased by 50% to a final concentration of 0 mM. Cell lines obtained in this selection process are referred to as EGI-1 GD and TFK-1 GD and were standardly cultivated in glutamine-deprived growth medium. B) EGI-1 GD and TFK-1 GD show proliferation close to their parental counterparts. eCCA cells were seeded in regular glutamine-supplied growth medium and medium lacking glutamine. eCCA GD cells were seeded in medium lacking glutamine. Cells were cultivated for 7d and cell numbers were counted at the indicated time points using Trypan Blue staining. Color-coded significances placed above the time points represent the statistical comparison of growth curves to the reference (eCCA cells in glutamine-deprived medium). C) eCCA GD variants show morphological traits close to their parental counterparts. eCCA GD cell lines were seeded as described in Figure 2B. Bright field microscopy images were recorded after 72 h cultivation (200x magnification). Data represent mean SD of three individual experiments performed in triplicate.

line-specific differences in response to glutamine depletion (Figure 2C). Independent on glutamine availability, no morphological differences were detectable between EGI-1 variants. While glutamine-deprived TFK-1 cells showed substantial signs of cellular stress, we did not notice any difference in morphological appearance between the supplemented parental cell line and TFK-1 GD. These results suggest that a slow nutrient reduction, e.g. during the natural course of tumor growth, could enable CCA cells to overcome their addiction to glutamine.

Glutamine-deprived cells show substantial changes in cellular metabolism

To gain a better understanding of the metabolic state of both GD cell derivatives, we next compared their basic bioenergetic profile to the parental cell lines (Figure 3A and B). Measurement protocols for oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as well as ideal compound concentrations had been optimized in previous experiments (data not shown). Glutamine availability neither affected basal respiration, ATP-linked respiration nor basal glycolytic rates in EGI-1 cells. As we furthermore did not detect significant differences between EGI-1 and EGI-1 GD cells, we concluded that glutamine might be less essential for these cells to support sufficient ATP production. However, maximal respiratory capacity and non-mitochondrial respiration were significantly reduced in EGI-1 GD cells compared to the parental cell line. Intriguingly, TFK-1 cells showed a distinct susceptibility to glutamine deficiency. All respiration-linked parameters were significantly decreased in glutamine-deprived TFK-1 cells when compared to the nourished cell line, suggesting that glutamine metabolism is of central importance for this cell line. In medium lacking glutamine, both TFK-1 GD and the parental cell line showed increased basal glycolytic rates, which argue for a shift to anaerobic metabolism in case glutamine is not available. Interestingly, re-supplementation of glutamine had a strong effect on almost all tested parameters in TFK-1 GD cells while EGI-1 GD cells remained broadly unaffected. We then assayed whether the increased mitochondrial function in response to glutamine re-supplementation correlates with the cells proliferative potential by analyzing glutamine-dependent growth of both GD cell lines. As can be seen in Figure 3C, proliferation of EGI-1 GD cells remained unchanged while TFK-1 GD showed significantly increased numbers of viable cells when supplemented with glutamine. We concluded that in TFK-1 GD cells, metabolic reprogramming during adaption did not eliminate the need for glutamine to ensure maximal proliferation which

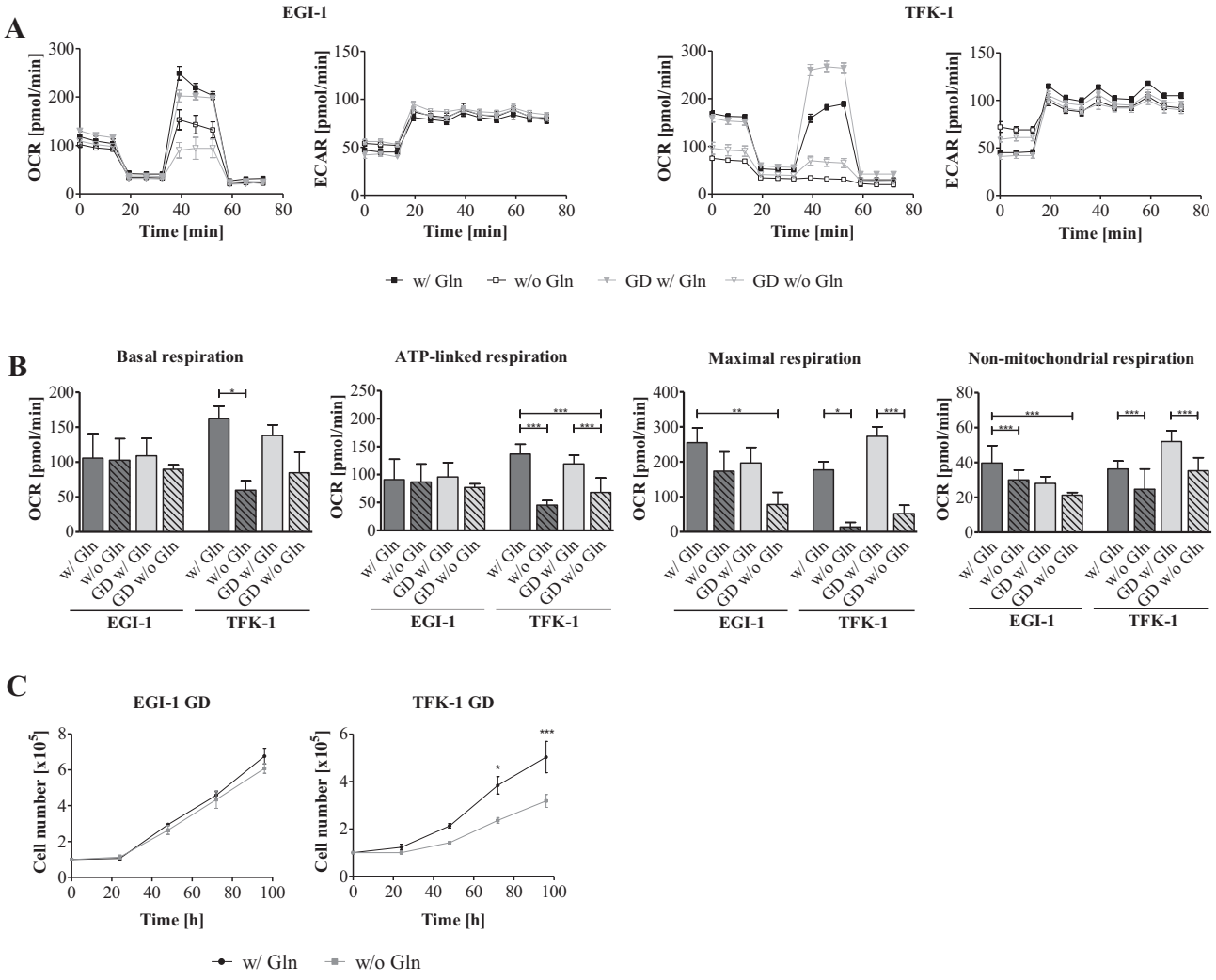

Figure 3. Glutamine availability broadly affects mitochondrial respiration and proliferative capacity in TFK-1 cell line variants. A) Bioenergetic profiles of eCCA GD cell lines and their parental counterparts were analyzed depending on glutamine availability by using an extracellular flux analyzer. Cells were seeded overnight in their regular growth medium. One hour prior to measurement the growth medium was changed to the indicated assay medium variant. Mitochondrial stress test was used to measure oxygen consumption rate (OCR, left). Data are presented as mean + SD of six replicates per group. B) Quantification of respiration parameters is presented as mean SD of six replicates per group. C) Re-supplementation of glutamine increases proliferation of TFK-1 GD cells. eCCA GD cells were cultivated in glutamine-deprived or -supplemented growth medium for 96 h. Live cells were determined via Trypan Blue exclusion. Data are presented as mean SD of two individual experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. One-Way ANOVA with Bonferroni post hoc test.

is in agreement with the reduced proliferation compared to the nourished parental cell line (Figure 2B). In summary, glutamine metabolism between CCA cell line variants displays substantial heterogeneity.

Hypoxia-induced chemoresistance is lost in glutamine-deprived CCA cells

Nutrient and oxygen limitation are common features of solid tumors and numerous studies have shown that an immediate deprivation of glutamine increases the susceptibility of tumor cells to anti-cancer drugs [20] while hypoxia has an opposing effect [21]. Against this background, we analyzed the effect of long-term glutamine deprivation with coincident hypoxia on therapy sensitivity of CCA cell lines. As a first step, we prepared dose-response curves of cisplatin and gemcitabine for both GD variants and their parental counterparts under normoxic and hypoxic conditions (Figure 4). Based on drug exposure at 72 h, comparative IC_{50} doses were defined as shown in Table 1. When comparing drug susceptibility of both GD cell lines and their parental counterparts, we found that the GD variants showed varying sensitivity to both drugs under normoxia, while under hypoxia the expected increase in IC_{50} values was not detected. For comparison purposes, we used working doses of 500 nM cisplatin and 10 nM gemcitabine for the treatment of all cell line variants.

To mimic a co-occurrence of glutamine deprivation and oxygen limitation, both eCCA GD cell lines were placed under hypoxic conditions for 24 h and were treated with cisplatin or gemcitabine for 96 h in total while maintaining reduced oxygen levels. Efficacy of the treatment was monitored by analyzing the DNA damage-associated proteins Ataxia Telangiectasia Mutated (ATM) and γH2AX, as both proteins are associated with cisplatin- and gemcitabine-induced DNA damage (Figure 5A) [22–27]. In both eCCA cell lines, cytostatic drug treatment increased γH2AX and ATM expression under normoxic conditions, while both proteins were found decreased under hypoxia. Given that the reduced activation of DNA damage proteins represents the well-known phenomenon of hypoxia-induced chemoresistance, we were intrigued to note that while

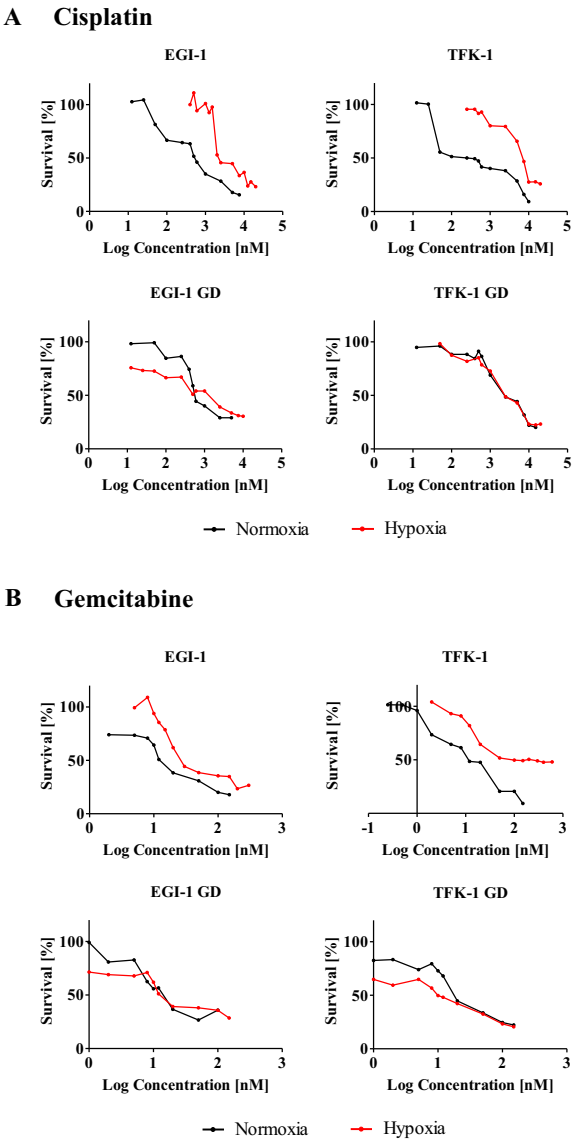

Figure 4. eCCA GD variants show varying sensitivity to chemotherapy. Dose-response curves of cisplatin and gemcitabine were prepared for eCCA and eCCA GD cell lines under normoxic and hypoxic conditions. Medium was changed to drug-supplemented medium 24 h after seeding. After 72 h drug exposure, viable cells were counted using Trypan Blue staining. Data are presented as mean of two replicates per time point, error bars were excluded for the purpose of clarity.

Table 1. Glutamine withdrawal prevents hypoxia-induced chemoresistance. IC_{50} values for cisplatin and gemcitabine were calculated from dose-response curves presented in Figure 4.

	Cisplatin [nM]		Gemcitabine [nM]	
	Normoxia	Hypoxia	Normoxia	Hypoxia
EGI-1	501.5	1874.2	11.8	40.1
TFK-1	647.8	3812.4	5.9	216.9
EGI-1 GD	469.2	521.3	14.2	13.9
TFK-1 GD	1452.1	1545.2	15.5	7.1

hypoxia did in fact increase γH2AX levels in cisplatin-treated GD cell lines, hypoxia failed to do so upon gemcitabine treatment. In both GD cell lines, we furthermore found fairly high ATM levels in all settings, which remained unchanged by cytostatic drug treatment or reduced oxygen levels. Quantification via densitometry proved that the hypoxia-induced chemoresistance in the parental cell lines had been eliminated in both GD cell lines, suggesting that the underlying resistance mechanisms could be affected by the process of adaption to glutamine withdrawal (Figure 5B). We used the same experimental setting to examine whether the increased DNA damage in GD cells under hypoxia is associated with a measurable decrease in proliferation (Figure 5C). While cultivation under hypoxia led to an expected rescue of proliferation in both cytostatic drug-treated eCCA cell lines, viable cell numbers remained unchanged in GD cells treated with cisplatin. In gemcitabine-treated cells, hypoxia significantly increased the number of TFK-1 GD cells but had no effect on EGI-1 GD cells, again demonstrating heterogeneity of these CCA cell lines. These results indicate that the increased DNA damage in eCCA GD cells has a biologically relevant impact on cell proliferation and furthermore suggest that coincident glutamine deprivation is able to counteract hypoxia-induced resistance to cisplatin while the effect on resistance to gemcitabine is cell line-dependent. As outlined above, we decided to use one defined working concentration for drug treatments. Given that GD variants showed varying sensitivity, we had to ensure that the observed effects do not result from ineffective drug concentrations. Therefore, we analyzed ATM and γH2AX levels in response to considerably higher doses of cisplatin and gemcitabine, but could not detect any dose-dependent changes regarding treatment effects (Supplementary Figure 2).

The hypoxic response is not significantly altered in GD cells

Given that hypoxia is a major regulator of both drug resistance and DNA damage response mechanisms, we hypothesized that the observed effects are explained by a modified cellular response to hypoxia obtained during the process of adaption to glutamine withdrawal. First, we analyzed proliferation of both GD cell lines and their parental counterparts depending on oxygen availability and found hypoxia to inhibit growth of all tested cell lines. Surprisingly, the effect was particularly strong in the parental cell lines. We exemplary quantified viable cell numbers under hypoxic conditions after 72 h, compared them to normoxia and found 53.26% survival in EGI-1 and 43.81% in TFK-1 versus 80.34% survival in EGI-1 GD and 73.83% in TFK-1 GD cells (Figure 6A). These findings suggest that the adaption process to glutamine withdrawal is accompanied by a reduced sensitivity to oxygen limitation. Since the cellular response to hypoxia is mainly regulated by transcription factors of the HIF family, we compared stabilization of HIF-1α and HIF-2α between GD cells and their parental counterparts. As expected, we found distinct stabilization of HIF-1α in all cell lines when cultivated under hypoxic conditions. No difference between untreated and cytostatic drug-treated cells was observed. Intriguingly, hypoxia-induced HIF-2α stabilization was remarkably low under control conditions in both eCCA GD cell lines when compared to the parental counterparts and was found increased by concomitant drug treatment (Figure 6B). In summary these data suggest an interconnection between the hypoxic response and the molecular mechanisms that govern adaptation to glutamine deprivation. However, with regard to the eliminated hypoxia-induced cisplatin resistance in both GD variants, it is questionable whether the slightly varying basic response to hypoxia does explain the effect. Therefore, we looked for a central mediator which is important for cancer cell survival and furthermore engaged in both the hypoxic response and glutamine metabolism: the protein c-Myc. While the c-Myc gene is a highly amplified oncogene among various human cancer types [28], the encoded protein is a transcription factor which affects a variety of genes that coordinate energy metabolism with biomass production in preparation for DNA replication and proliferation [29].

Expression of c-Myc protein is differentially regulated in GD cells

In a first step, we wanted to investigate the general importance of c-Myc for glutamine metabolism of CCA cells, so we analyzed c-Myc protein

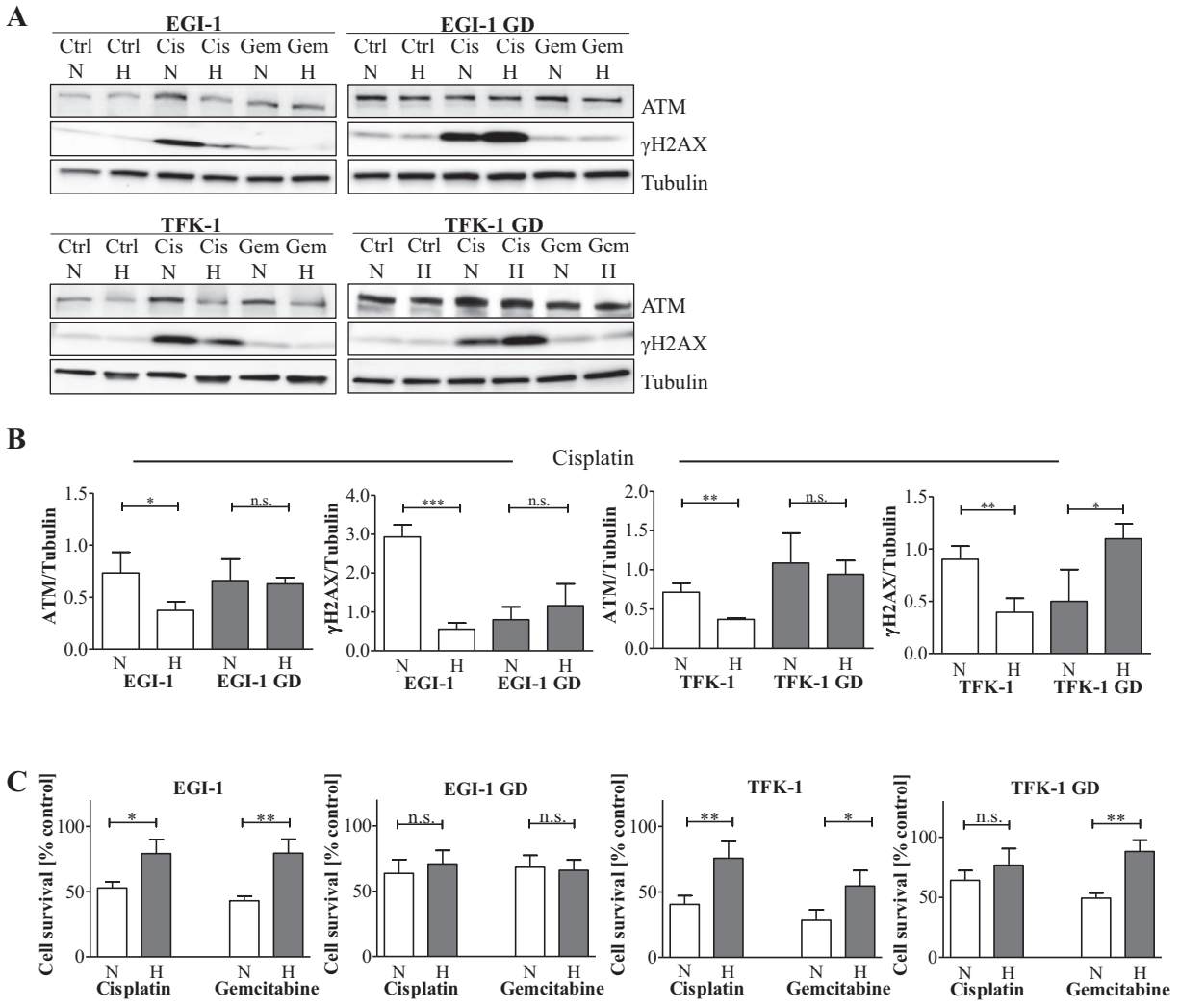

Figure 5. The adaption process to glutamine deprivation eliminates hypoxia-induced chemoresistance to cisplatin. A) Cisplatin-induced DNA damage is reduced under hypoxic conditions in eCCA cell lines but increased in eCCA GD cell lines. Cells were cultivated overnight either under normoxic (N) or hypoxic (H, 1% O_2) conditions and were treated with cisplatin [500 nM] or gemcitabine [10 nM] for 72 h. Data are shown as representative immunoblots of two or three independent experiments with detection of ATM, γH2AX and Tubulin as loading control. B) Quantification of relative ATM and γH2AX protein levels in cisplatin-treated eCCA cell line variants presented in Figure 5A. Data are shown as mean SD of two or three independent experiments. C) Hypoxia leads to a rescue of proliferation in cisplatin-treated eCCA cell lines but not eCCA GD variants. Cells were treated as described in A. Viable cells were determined via Trypan Blue exclusion. Data are presented as mean SD of two individual experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. One-Way ANOVA with Bonferroni post hoc test.

levels in EGI-1 and TFK-1 cells in dependence on external glutamine concentrations. In both cell lines, glutamine depletion caused a reduction of c-Myc protein levels (Figure 7A), thereby confirming the well-known link between c-Myc and glutamine metabolism [30] in CCA cells. We next sought to comparatively analyze basal c-Myc protein expression in both GD cell lines and their parental counterparts, given that both parental cell lines showed almost entire absence of c-Myc under glutamine withdrawal. Surprisingly, we could not detect a difference in c-Myc levels between the nourished parental cell lines and the glutamine-deprived GD variants (Figure 7B) and theorized that adaption to the normal c-Myc level could contribute to survival in the GD cell lines. Finally, the question was whether c-Myc could explain the finding of the eliminated hypoxia-induced chemoresistance to cisplatin, so we analyzed c-Myc protein levels after treatment with cisplatin in dependence on oxygen availability (Figure 7C). Under control conditions, all cell lines showed a reduction of c-Myc under hypoxia, in line with the published literature [31,32]. However, in both parental cell lines, cisplatin treatment caused a rescue of c-Myc levels under hypoxia, whereas c-Myc was still reduced in both eCCA GD cell lines. Based on several publications which demonstrated c-Myc to promote cell growth and proliferation, we theorized that upregulation of c-Myc under hypoxia promotes survival in cisplatin-treated eCCA cells, while this survival mechanism is lost in both GD variants (Figure 8). This finding would imply that the molecular regulation of c-Myc is affected during the adaption process to glutamine withdrawal.

Discussion

Similar to normal tissues, tumor vasculature supplies nutrients and oxygen to sustain survival of neoplastic growth. Impaired blood supply to the tumor can result in the emergence of hypoxic regions which are likely to show decreased concentrations of nutrients such as amino acids and glucose [33]. Tumor cells which are confronted with these challenging conditions may adjust to the setting, given that they are extremely plastic

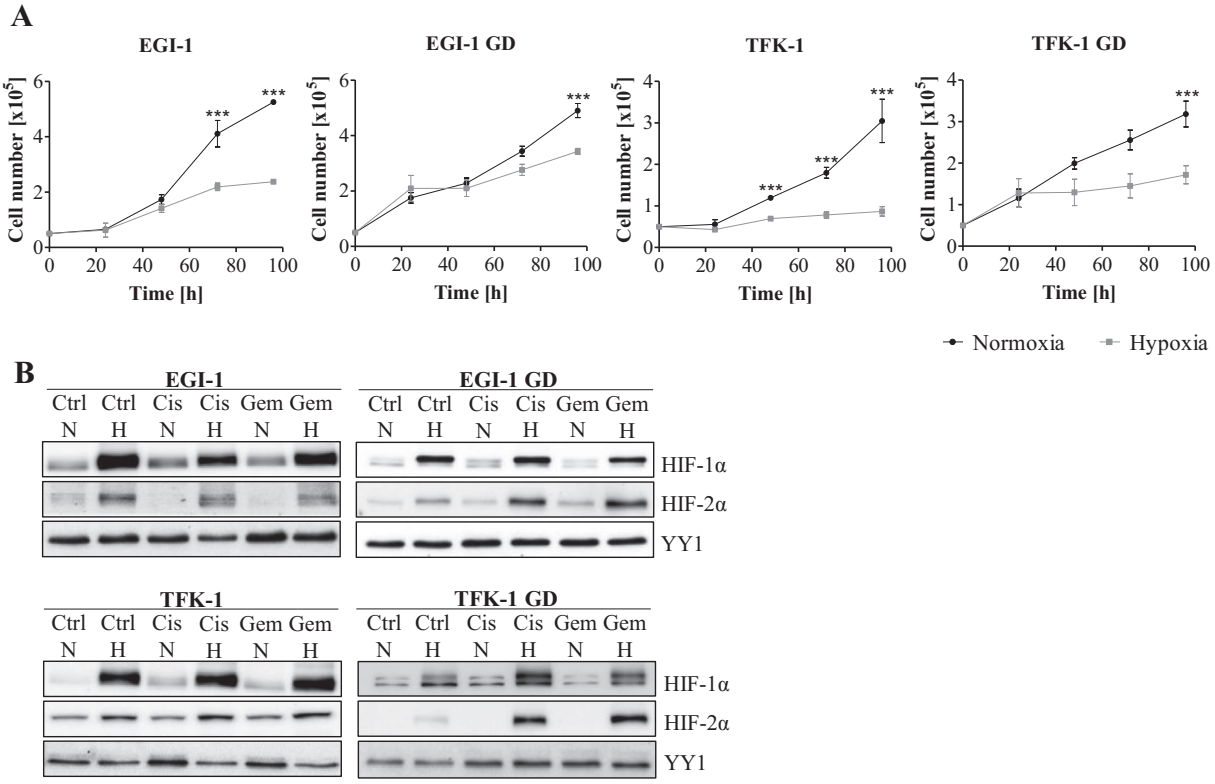

Figure 6. The adaption process to glutamine withdrawal affects the response to hypoxia. A) eCCA GD cell lines show a slightly reduced sensitivity to hypoxia when compared to their parental counterparts. Cells were cultivated in their regular growth medium under normoxia or hypoxia (1% O_2) for 96 h. Cell numbers were counted daily using Trypan Blue staining. Results are shown as means SD of two individual experiments performed in triplicate. B) eCCA GD cell lines show increased hypoxia-induced HIF-2α stabilization in response to cytostatic drug treatment. Cells were treated as described in Figure 4A. Data are shown as representative immunoblots of two individual experiments with detection of HIF-1α, HIF-2α and YY1 as loading control. *** $p < 0.001$. One-Way ANOVA with Bonferroni post hoc test.

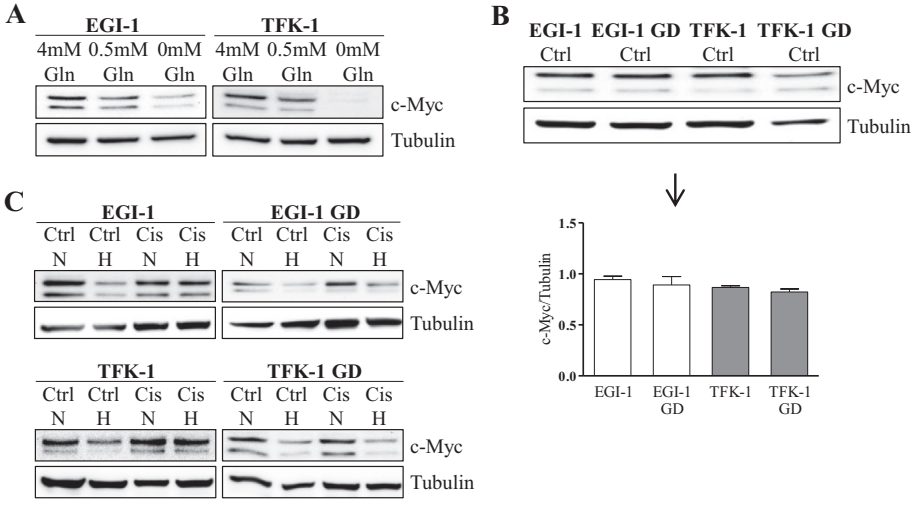

Figure 7. The adaption process to glutamine withdrawal determines the cellular regulation of c-Myc. A) c-Myc protein levels are decreased in response to glutamine deprivation in eCCA cell lines. EGI-1 and TFK-1 cells lines were cultivated with different glutamine concentrations for 24 h. Data are shown as immunoblots with detection of c-Myc and Tubulin as loading control. B) c-Myc levels of eCCA GD variants are adjusted to the basal level of their nourished parental counterparts. Densitometric analysis of relative c-Myc levels revealed no difference between the groups. Cells were cultivated under control conditions for 24 h. Data are shown as representative immunoblots of two individual experiments with detection of c-Myc and Tubulin as loading control. C) Cisplatin rescues c-Myc protein levels under hypoxia in eCCA cell lines but not in eCCA GD cell line variants. Cells were cultivated overnight either under normoxic or hypoxic (1% O_2) conditions and were treated with the established IC50 dose for cisplatin or gemcitabine for 72 h. Data are shown as representative immunoblots of two individual experiments with detection of c-Myc and Tubulin as loading control.

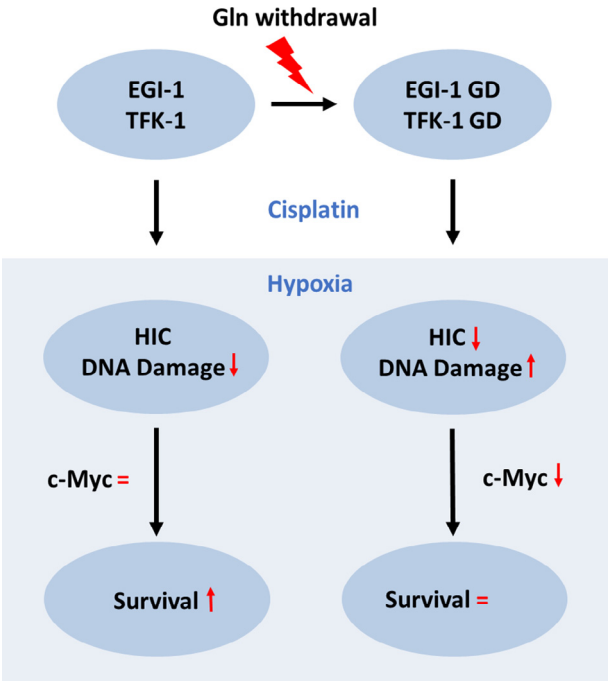

Figure 8. c-Myc potentially contributes to survival in parental CCA cell lines by sustaining cell growth and proliferation. Schematic summarization of the interaction between glutamine (Gln) withdrawal, chemotherapy and hypoxia, and their effect on c-Myc protein expression. HIC = hypoxia-induced chemoresistance.

and have evolved advanced mechanisms to adapt to environmental challenges [34,35]. In the core region of many solid tumor types, glutamine can be considerably decreased when compared to the adjacent benign tissue [36] or the periphery of the tumor [37]. However, no studies were published yet focusing on the distribution of glutamine inside CCA and adjacent tissue. To the best of our knowledge, this is the first time demonstrating that human CCA patients show a strong depletion of glutamine inside the tumor. Although this small pilot study provides an indication that glutamine depletion might be a relevant event in the progression of CCA, there are certain limitations which need to be considered. Our data confirm the notion of CCA as a heterogeneous tumor type [38,39] and the spatial resolution of MALDI MSI does not allow to relate the data to one specific cell type. Further investigation will be required to define whether this depletion might reflect increased consumption of the tumor and whether the results are applicable to eCCA.

Under nutrient depletion, cancer cells can acquire mutations which activate the cellular ability to utilize alternative ways to obtain necessary nutrients and furthermore enable the access to conventional as well as to unconventional nutrient sources [2]. In the scope of this project, we did not identify the mechanisms which enabled both eCCA cell lines to ensure proliferation under long-term glutamine withdrawal. However, to our knowledge, this is the first time showing that CCA cells can overcome their addiction to glutamine while almost recapturing the proliferative capacity of their nourished parental counterparts. This finding supports several studies which demonstrated that deprivation of glutamine suppresses tumor cell growth but rather promotes a reversible cell cycle arrest than the induction of apoptosis [40]. The adaption process was associated with a reduced sensitivity to cytostatic drug treatment under normoxia, assuming that a subpopulation of cancer cells might survive under glutamine-deprived conditions and has the potential to be increasingly aggressive. Reduced drug sensitivity in response to long-term glutamine deprivation is contrary to the observations due to acute depletion, since short-term deficiency was found to significantly increase cancer cell susceptibility to alkylating agents [41] and 3-bromopyruvate chemotherapy [20].

Double-deprivation stress through cyclic hypoxia and nutrient starvation was demonstrated to induce aggressiveness and resistance to chemotherapeutic agents in human cervical carcinoma and glioblastoma as well as in murine melanoma cells [42,43]. To investigate whether a setting of long-term glutamine starvation and hypoxia has a comparable impact on different CCA cells, we developed a simple experimental model and found that synergy of both conditions eliminates hypoxia-induced chemoresistance to cisplatin. Unexpectedly, both adapted GD cell lines showed a decreased sensitivity to hypoxia, given that many cancer cells use compensatory mechanisms by utilization of alternative fuels such as glutamine to support proliferation under hypoxic pressure [44]. We furthermore found increased hypoxia-mediated HIF-2α protein expression in response to cisplatin treatment in both GD cell lines. Evidences suggest that HIF-2α is involved in the intracellular response to oxidative stress by protecting cellular and mitochondrial components though activation of its target genes including Sod2 [45,46]. Since cisplatin exposure induces the formation of reactive oxygen species inside the cell and it is reasonable to assume that GD cell lines lack the major antioxidant molecule glutathione, induction of HIF-2α could play a role in the maintenance of mitochondrial homeostasis. However, glutathione levels as well as the exact role of HIF-2α in GD cells remains an open question and needs to be in investigated in future studies.

The eliminated hypoxia-induced chemoresistance to cisplatin in both GD cell lines was associated with a reduced c-Myc protein expression when compared to the parental counterparts. In general, c-Myc contributes to energy-consuming cellular processes, assuming that high c-Myc levels might be harmful in phases of metabolic stress. We could confirm several studies which have shown that c-Myc protein expression is downregulated in response to stressful conditions such as nutrient depletion or hypoxia [31,40]. Hypoxia-dependent inhibition of c-Myc is primarily based on the impact of HIF proteins on a number of c-Myc-interacting proteins (reviewed in [47]). It has been shown that downregulation of c-Myc protein under dual oxygen and nutrient deprivation is independent of HIF-1α, which strengthens our results, as we could not detect a difference in HIF-1α expression in cisplatin-treated and untreated GD cell lines. Surprisingly, c-Myc protein expression in both untreated GD cell lines was rescued to the basal level of the nourished parental counterparts, suggesting that the adaption process was able to re-establish the balance between needed and supplied fuels and the cellular capability of using them.

A general correlation between c-Myc expression and platinum resistance is known for years [48,49] but glutamine metabolism as potential link has recently been demonstrated in ovarian cancer [50]. The study provides evidence that upregulation of glutamine metabolism is an early event in response to cisplatin treatment and that platinum resistance is induced by high c-Myc levels through increased utilization of glutamine. Although resistance in this study was not accomplished by hypoxia, the data provide an intriguing indication that combinatory treatment with platinum-based drugs and targeting of glutamine metabolism might be a potential therapeutic strategy to prevent resistance mechanisms. Given that both GD cell lines did not show hypoxia-induced chemoresistance to cisplatin and reduced c-Myc levels, future studies should focus on the question whether a therapeutic inhibition of c-Myc could reverse resistance in both parental CCA cell lines. In summary, the findings presented in this study warrant further investigation of the efficacy of drugs targeting glutamine metabo-

lism on long-term deprived cancer cells and highlight an interesting cross-talk between c-Myc, glutamine metabolism and platinum resistance in CCA cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2019.10.004.

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