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Hypoxia

The autophagy associated gene, ULK1, promotes tolerance to chronic and acute hypoxia

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ABSTRACT

Background and purpose: Tumor hypoxia is associated with therapy resistance and malignancy. Previously we demonstrated that activation of autophagy and the unfolded protein response (UPR) promote hypoxia tolerance. Here we explored the importance of ULK1 in hypoxia tolerance, autophagy induction and its prognostic value for recurrence after treatment.

Material and methods: Hypoxic regulation of ULK1 mRNA and protein was assessed *in vitro* and in primary human head and neck squamous cell carcinoma (HNSCC) xenografts. Its importance in autophagy induction, mitochondrial homeostasis and tolerance to chronic and acute hypoxia was evaluated in ULK1 knockdown cells. The prognostic value of ULK1 mRNA expression was assessed in 82 HNSCC patients.

Results: ULK1 enrichment was observed in hypoxic tumor regions. High enrichment was associated with a high hypoxic fraction. In line with these findings, high ULK1 expression in HNSCC patients appeared associated with poor local control. Exposure of cells to hypoxia induced ULK1 mRNA in a UPR and HIF1α dependent manner. ULK1 knockdown decreased autophagy activation, increased mitochondrial mass and ROS exposure and sensitized cells to acute and chronic hypoxia.

Conclusions: We demonstrate that ULK1 is a hypoxia regulated gene and is associated with hypoxia tolerance and a worse clinical outcome.

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Poor tumor oxygenation decreases therapy efficacy as hypoxic cells are more resistant to radio- and chemotherapy [1]. Tumor hypoxia is the result of an abnormal vascular network and is characterized by extreme inter- and intratumor heterogeneity [2]. Gradients in oxygen are observed at distances from perfused vessels, ranging from normal oxygen (~5%) close to the vessel to anoxia in the peri-necrotic region, this type of hypoxia is generally referred to as diffusion limited (or chronic) hypoxia. Hypoxia also arises as a consequence of transient changes in perfusion [3,4], so called acute hypoxia, its characterized by periodic cycles of hypoxia and reoxygenation [5,6]. Targeting important mediators of hypoxia tolerance could provide means to reduce tumor hypoxia and improve current cancer therapy [7,8]. Severe hypoxia causes endoplasmic reticulum (ER) stress, thereby activating the unfolded protein response (UPR). The UPR is mediated by three parallel ER stress sensors; PKR-like ER kinase (PERK), IRE1, and activating transcription factor 6 (ATF6) [9]. Previously we demonstrated that during chronic hypoxia, PERK dependent phosphorylation of eIF2α and subsequent ATF4 expression causes transcriptional induction of microtubule-associated protein 1 light chain β (MAP1LC3b, LC3b hereafter). This induction increases the capacity to exert a cytoprotective lysosomal degradation mechanism called autophagy [10]. Herein, organelles or protein aggregates become engulfed into LC3b-coated autophagosomes. Fusion with a lysosome effectuates degradation by lysosomal hydrolases and causes degradation of the autophagosomal content. Furthermore, we previously demonstrated that autophagy inhibition, using a lysosomotropic compound called chloroquine (CQ), sensitized cells to chronic and acute hypoxia resulting in increased radiosensitivity of tumors [10].

Unc51-like kinase 1 (ULK1) and its yeast homolog atg1 are important in nutrient starvation-induced autophagy and cell survival [11–14]. Recently, Pike et al. [15] demonstrated that during chronic hypoxia, ULK1 is transcriptionally induced as consequence of UPR activation and that ULK1 is important for mitochondrial

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homeostasis. Previously we demonstrated that autophagy is important in cellular and mitochondrial homeostasis during acute hypoxia. Hence, autophagy inhibition causes mitochondrial accumulation and exposes acute hypoxic cells to elevated ROS levels and decreased survival [16]. Considering this, we hypothesized that protective autophagy in cells exposed to acute/cycling hypoxia is mediated through ULK1 activation.

Materials and methods

Tumor model

Primary tumor xenografts derived HNSCCs, a model which is extensively used to identify predictive biological tumor characteristics [17], were maintained as described previously [18] and subsequently stained using anti-ULK1 (Abcam, ab65056), anti-pimonidazole (Chemicon) and 9F1 (rat monoclonal antibody against mouse endothelium; Dept. of pathology, Radboud University Nijmegen) antibodies. The ratio of ULK1 signal density in pimonidazole positive against pimonidazole negative tumor regions was calculated after signal quantification.

Clinical study

ULK1 gene expression was evaluated in a head and neck cancer patient cohort of the Netherlands Cancer Institute, which was described previously [19]. Material was available for 84 patients treated with concurrent chemo-radiation and local-recurrence was the main endpoint. ULK1 gene expression was assessed with quantitative PCR (qPCR) as described below and used to median-dichotomize the patient cohort. Characteristics of patients with low and high ULK1 expression are listed in Suppl. Table 1 and differences between these patient groups were assessed with a χ^2-test or Student's *t*-test. Differences in time to local recurrence between the patient groups with low and high ULK1 expression were assessed with Kaplan–Meier survival analysis and univariate Cox proportional hazard ratio modeling followed by the Wald test (survival package v2.37-4).

Cell models

Our studies included HT29 and HCT116 (colorectal adenocarcinoma), MCF7 (mammary adenocarcinoma) and U373-MG (glioblastoma–astrocytoma) cells. Cells were maintained as described by ATCC. siRNA mediated knockdown of UPR components was performed as described previously [10]. Viral particles were produced as described previously for pLKO.1 vectors [20] and pRS vectors [21]. ULK1 was targeted using 5′-TTTCCGTGTGCTCTTGCTC-3′ encoding shRNA.

Hypoxia

Chronic hypoxia exposure was performed by incubating cells in a hypoxic chamber (MACS VA500 microaerophilic workstation; Don Whitley Scientific) [10], and cycling hypoxia as described previously [16,22].

Immunofluorescence

Cells were fixed with 4% paraformaldehyde, permeabilized, blocked and subsequently stained with an anti-ULK1 antibody, anti-rabbit Alexa Fluor 488 (Invitrogen, A-11008) and Hoechst33342 (Sigma–Aldrich, B2261).

Quantitative real-time PCR

mRNA was isolated with NucleoSpin RNA II kit (Bioke) and reverse transcribed using I-script (BIO-RAD). Primers used are listed in Suppl. Table 2. Expression was normalized to 18S unless reported differently.

Immunoblot

Cells were lysed and processed as described previously [23]. After transfer, proteins were probed with antibodies against ULK1 (Santa-Cruz, sc-33182), MAP1LC3b (Cell Signaling, 2775S), CA IX (M75, [24]), p62 (BD Biosciences, 610832) and Actin (MP Biomedicals, 8961001). Bound antibodies were visualized using HRP-linked anti-rabbit (Cell Signaling, 7074S) or anti-mouse (Cell Signaling, 7076S) antibodies.

Clonogenic assay

Single cells were seeded and exposed to chronic or acute hypoxia. After colony formation, colonies were fixed with 0.4% methylene blue (Sigma, M4159) in 70% ethanol. Colonies (>50 cells) were counted.

Flow cytometry

Cells were incubated with 20 μM dihydrorhodamine-123 or 200nM MitoTracker Green (Life Technology, D632; M-7514) during oxygenation phase of the last cycle or for 30 min in the incubator, respectively, and then analyzed by flow cytometry (BD FACS Canto II).

Statistics

Data were analyzed using GraphPad Prism. Student's *t* test was used for single comparisons. Multiple testing was done using repeated measures ANOVA with a Bonferroni post hoc test. *P*-value < 0.05 was considered statistically significant.

Results

ULK1 is primarily expressed in hypoxic tumor regions

Previously we demonstrated that autophagy is activated in hypoxic tumor areas. We therefore determined the prevalence and distribution of ULK1 in 14 genetically different primary human HNSCC xenografts. The advantage of these models is that the tumor microenvironment is conserved throughout passaging and therefore closely reflects the original tumor [18]. We observed that ULK1 was expressed predominantly in pimonidazole positive tumor regions (Fig. 1A). According to the ratio ULK1 signal density in pimonidazole positive divided by pimonidazole negative tumor regions (i.e., enrichment factor), ULK1 was enriched in 12 out of 14 xenografts (ratio > 1; Fig. 1B). Furthermore, the quartile of tumors with highest ULK1 enrichment displayed the highest hypoxic fraction (Fig. 1C; $p < 0.05$), suggesting a potential role for ULK1 in mediating hypoxia tolerance and preservation of the hypoxic fraction. As high levels of hypoxia are associated with poor patient outcome in HNSCC [7,25], we investigated the prognostic value of ULK1 mRNA, as assessed by qPCR, in a cohort of 84 HNSCC patients treated with a combination of radiotherapy and cisplatin [19]. Patients with high ULK1 expression had a poorer local and locoregional control compared to the patients with low ULK1 expression, which showed a trend toward significance in this small cohort (Fig. 1D). This corresponds with previous studies that showed prognostic potential of ULK1 expression in breast cancer patients [15] and esophageal SCC patients [26].

The tumor microenvironment includes many other stresses besides hypoxia. We therefore examined whether hypoxia alone is sufficient to induce ULK1 expression. In microarray analyses that

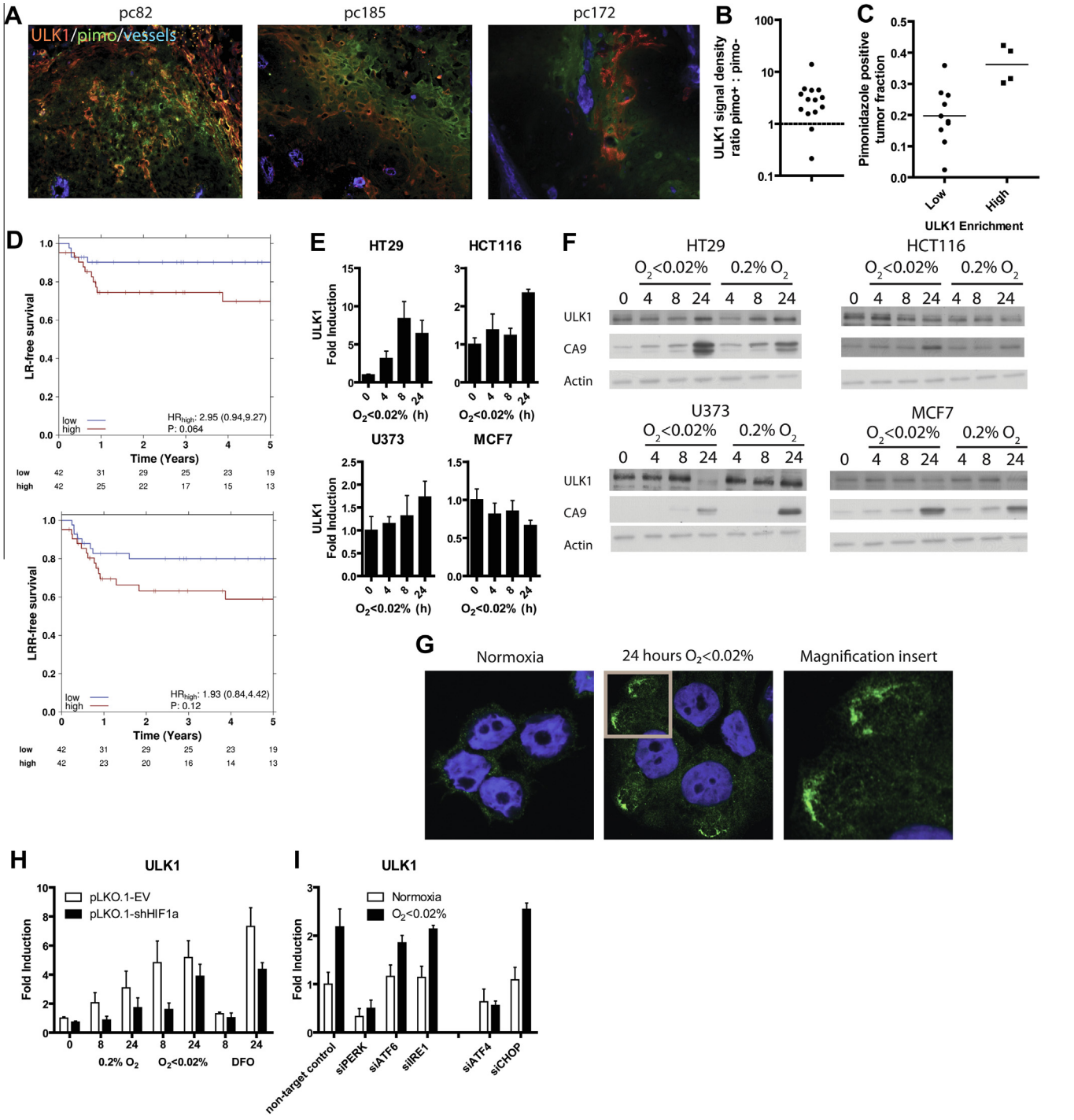

Fig. 1. ULK1 is a hypoxia regulated protein. (A and B) ULK1 protein is expressed in pimonidazole positive tumor areas of 12 out of 14 HNSCC primary xenografts. (C) Enrichment vs. hypoxic fraction. (D) ULK1 mRNA is associated with local and locoregional recurrences (LR; LRR) in HNSCC patients HR: Hazard ratio. (E) ULK1 mRNA and (F) protein expression in HT29, HCT116, U373-MG and MCF7 cells after hypoxia exposure. (G) ULK1 immunocytochemistry in HT29 cells. Transcriptional regulation of ULK1 during hypoxia is dependent on both HIF1α (H) and PERK/ATF4 signaling (I). E, H, I $n = 3$, mean ± SEM.

were previously performed in our lab, ULK1 mRNA expression was increased after exposure to severe hypoxia (Suppl. Fig. 1A) [27]. Although these severe hypoxic conditions are not the most prevalent in human cancers, these are associated with worse survival after therapy [2]. Targeting pathways, typically activated and required for survival during severe hypoxia sensitizes tumors to irradiation [28]. To validate our microarray findings, ULK1 mRNA was analyzed in independent samples after hypoxia exposure. We observed that ULK1 mRNA was increased in 3 out of 4 cell lines. Only in MCF7, which already displayed highest basal expression, no increase was observed (Fig. 1E and Suppl. Fig. 1B). Immunoblot analysis showed increased protein expression in HT29 cells and decreased expression in HCT116, MCF7 and U373-MG cells during hypoxia (Fig. 1F), suggesting increased turnover of ULK1 protein during hypoxia. As observed at the mRNA level, basal ULK1 protein was highest in MCF7 cells (Suppl. Fig 1C). Interestingly, immunofluorescent staining

revealed redistribution and clustering of ULK1 after hypoxia in HT29 (Fig. 1G) and MCF7 cells (Suppl. Fig. 1D).

Next, we analyzed which hypoxia-associated response pathways contribute to the upregulation of ULK1 gene expression. Knockdown of HIF1α reduced transcriptional upregulation during moderate and severe hypoxia. Furthermore, iron chelator desferrioxamine (DFO) mediated stabilization of HIF1α, which was sufficient to increase ULK1 gene expression (Fig. 1H, Suppl. Fig. 1E). Although ULK1 upregulation was completely dependent on HIF1α signaling under moderate hypoxia, after prolonged (24 h) exposure to severe hypoxia HIF1α knockdown did not change ULK1 mRNA upregulation. Severe hypoxia leads to rapid activation of the UPR. Knockdown of the three parallel UPR arms revealed dependency on PERK and not ATF6 or IRE1 signaling for ULK1 induction. Knocking down two transcription factors dependent on PERK signaling, ATF4 and CHOP, confirmed that ULK1 is transcriptionally dependent on ATF4 (Fig. 1I) [15].

ULK1 increases hypoxia tolerance and increases hypoxia induced autophagy

Expression of ULK1 in hypoxic tumor regions, the prognostic value of ULK1 mRNA in HNSCC and the redistribution suggest a role in the response to hypoxia. To examine whether ULK1 promotes hypoxia tolerance, we generated HT29 and MCF7 ULK1 knockdown cells (shULK1) (Fig. 2A). Hypoxia tolerance, as assessed by clonogenic survival, revealed decreased survival after exposure to severe hypoxia in both cell lines (Fig. 2B and C and Suppl. Fig. 2). ULK1 knockdown did not lead to decreased clonogenic survival or proliferation under normal oxygen concentrations (data not shown). Since autophagy can increase hypoxia tolerance [28], we investigated the importance of ULK1 in autophagy. In both HT29 and MCF7 cells, ULK1 knockdown decreases autophagic flux (LC3b-II + CQ/actin) moderately during severe hypoxia (Fig. 2D). Considering that autophagy is involved in clearance of mitochondria we examined whether ULK1 knockdown would lead to mitochondrial accumulation. As assessed by flow cytometry, we observed that ULK1 knockdown resulted in elevated mitochondrial content, during both normoxic and hypoxic conditions (Fig 2E; $p < 0.05$).

ULK1 deficiency sensitizes cells to cycling hypoxia

Previously we showed that autophagy was required for a rapid reduction in mitochondrial mass during cycling hypoxia. Inhibition of autophagy, and thus failure to reduce mitochondria, resulted in increased exposure of cells to ROS and reduced cellular survival [16]. As ULK1 knockdown resulted in mitochondrial accumulation, we investigated its role in cycling hypoxia. Similar to exposure to prolonged hypoxia, exposure to repeated cycles of hypoxia and reoxygenation resulted in transcriptional upregulation of ULK1 mRNA (Fig. 3A). This was also UPR dependent as overexpression of a non-phosphorylatable mutant eIF2α or wild-type GADD34 prevented this upregulation (Suppl. Fig. 3). Further, ULK1 knockdown sensitizes cells to cycling hypoxia compared to control cells (Fig. 3B; $p < 0.05$ for 3 cycles). As shown previously, cycling hypoxia increases ROS exposure and reduces cellular survival [16]. Although ULK1 knockdown cells were exposed to more ROS during normal conditions it was surprising, regarding the increased mitochondrial mass, that during cycling hypoxia ULK1 knockdown and control cells were equally exposed to ROS (Fig. 3C). As observed previously, pre-treatment of cells with the ROS scavenger *N*-acetyl cysteine increased clonogenic survival. Surprisingly, ROS scavenging was not sufficient to completely prevent cell death after cycling hypoxia in ULK1 deficient cells (Fig. 3D; $p < 0.05$ for all). These data indicate that the increased sensitivity of ULK1 knockdown cells to cycling hypoxia is not due to increased ROS exposure but another unknown mechanism.

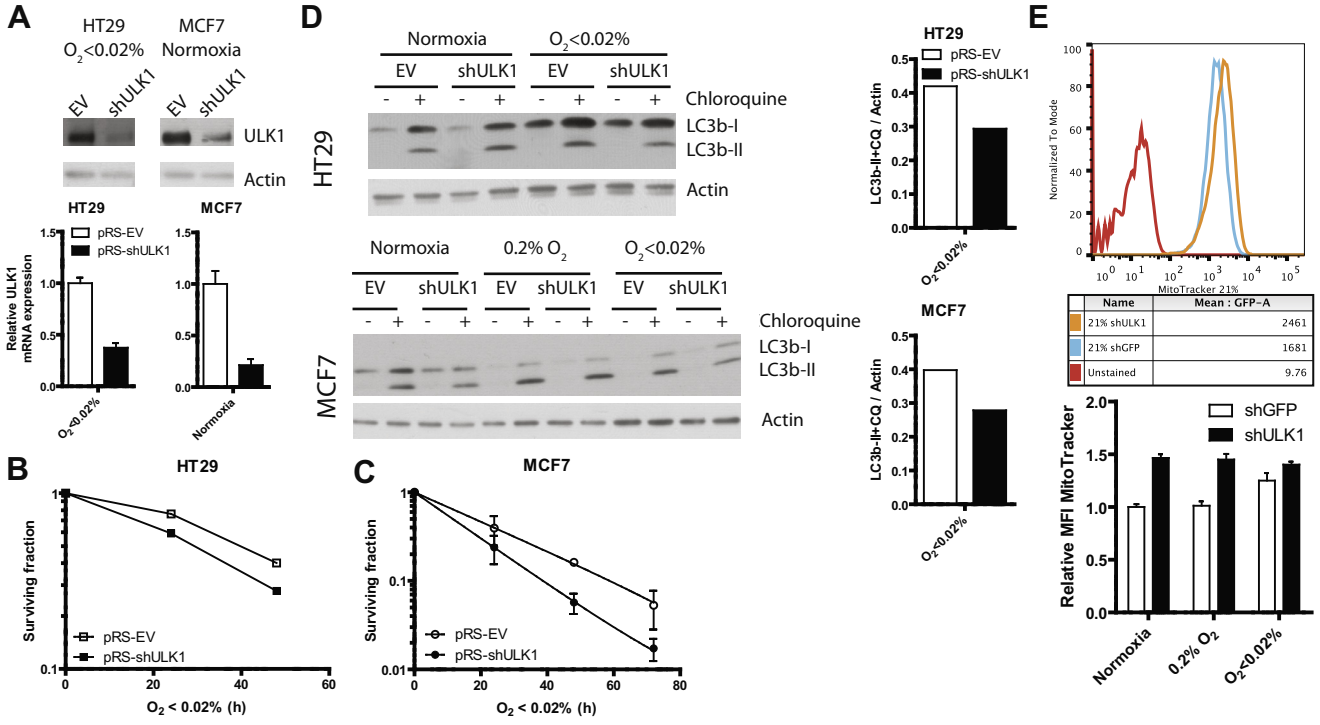

Fig. 2. ULK1 deficiency sensitizes cells to hypoxia. (A) ULK1 knockdown cells were exposed to severe hypoxia and assessed for clonogenic survival (B) HT29 (representative image, additional experiments are shown in Suppl. Fig 2) and (C) MCF7 cells. (D) Autophagic flux is decreased in ULK1 knockdown cells. Graphs indicate LC3b-II flux. (E) Mitochondrial mass as determined by MitoTracker green in HT29 cells. MFI: mean fluorescent intensity. A, B, C, E $n = 3$, mean ± SEM.

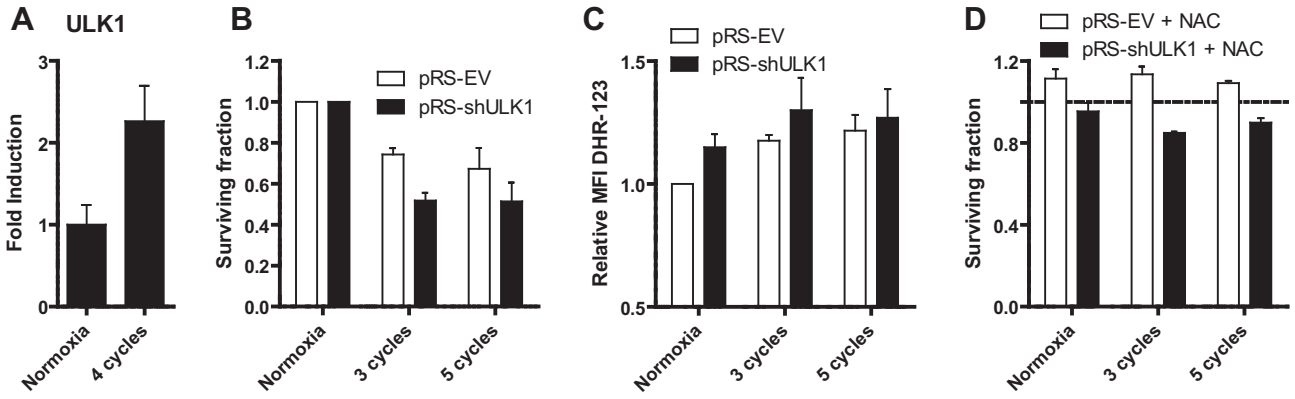

Fig. 3. ULK1 deficiency sensitizes cells to cycling hypoxia. (A) ULK1 mRNA determined by qPCR after exposure to cycling hypoxia. (B) Clonogenic survival, (C) ROS production and (D) clonogenic survival with NAC addition after cycling hypoxia. A–D $n = 3$, mean ± SEM.

Discussion

Hypoxia is a common feature of solid tumors that compromises successful treatment. Although, hypoxia is used synonymously for various types of oxygenation conditions in tumors, a clear distinction has to be made between diffusion-limited hypoxia (chronic hypoxia) and perfusion-limited (acute/cycling) hypoxia. Both are implicated in therapy resistance. Better understanding of mechanisms that promote hypoxia tolerance and the discovery of potential targets for treating these cells is therefore highly desired.

Our lab previously demonstrated that autophagy, increases the ability of hypoxic cells to survive. We show that ULK1 is protein regulated by protective UPR and HIF1α pathways during hypoxia. Accordingly, ULK1 expression was predominantly localized in hypoxic tumor areas and that high enrichment was associated with a high hypoxic fraction. *In vitro* clonogenic assays with human cancer cell lines demonstrated that during both chronic and acute/cycling hypoxia ULK1 contributes to survival, supporting the *in vivo* findings that ULK1 promotes hypoxia tolerance. We observed accumulation of mitochondria and elevated ROS levels in ULK1 knockdown cells; however, in contrast to control cells, addition of ROS-scavengers did not completely prevent cell death in ULK1 deficient cells. This indicates that ULK1 mediates protection to cycling hypoxia through another mechanism. In this paper, we present data indicating that ULK1 is a hypoxia regulated gene which promotes survival during hypoxia, suggesting its potential value as a therapeutic target. Whether targeting ULK1 is sufficient to reduce tumor hypoxia and sensitizing tumors to irradiation is worth assessing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2013.06.015.

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