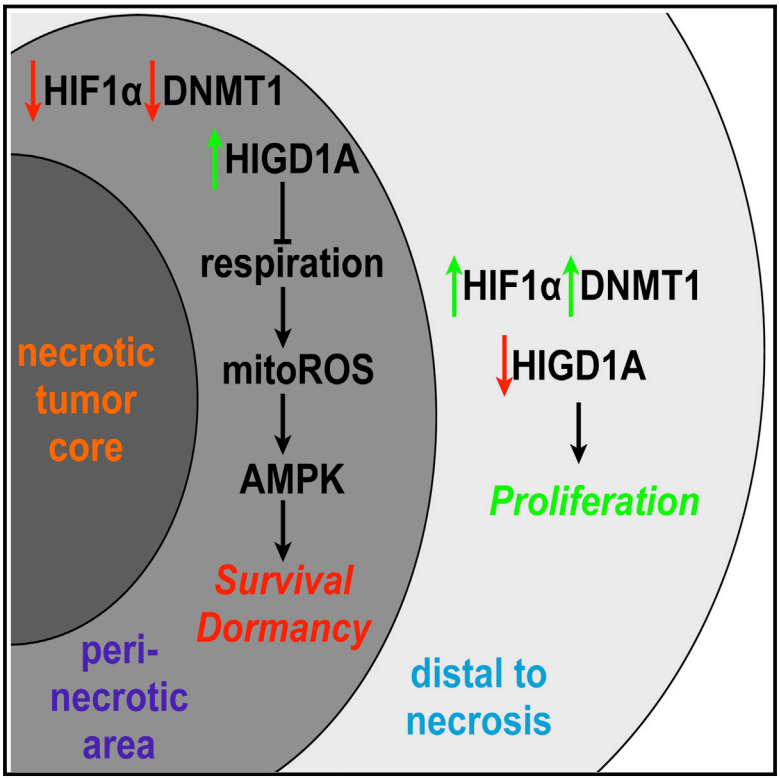
Cell Reports

Report

HIGD1A Regulates Oxygen Consumption, ROS Production, and AMPK Activity during Glucose Deprivation to Modulate Cell Survival and Tumor Growth

Graphical Abstract



Authors

Kurosh Ameri, Arman Jahangiri, ..., Manish K. Aghi, Emin Maltepe

Correspondence

emin.maltepe@ucsf.edu

In Brief

Hypoxia-inducible gene domain family member 1A (HIGD1A) is a hypoxia-inducible factor 1 (HIF-1) target found in perinecrotic tumor regions lacking HIF-1 expression. Ameri et al. now find it interacts with the electron transport chain to trigger mitochondrial ROS-dependent AMPK activation and reduces respiration and total ROS to promote survival and suppress growth.

Highlights

- HIGD1A protects from glucose deprivation but suppresses tumor growth
- HIGD1A interacts with the electron transport chain to decrease respiration
- HIGD1A can be induced independent of HIF-1 via differential methylation
- HIGD1A may play roles in metabolic regulation of tumor dormancy

Ameri et al., 2015, Cell Reports *10*, 891–899
February 17, 2015 ©2015 The Authors
http://dx.doi.org/10.1016/j.celrep.2015.01.020

CellPress

Cell Reports
Report

OPEN ACCESS
CellPress

HIGD1A Regulates Oxygen Consumption, ROS Production, and AMPK Activity during Glucose Deprivation to Modulate Cell Survival and Tumor Growth

Kurosh Ameri,[1] Arman Jahangiri,[2] Anthony M. Rajah,[1] Kathryn V. Tormos,[1] Ravi Nagarajan,[2] Melike Pekmezci,[3] Vien Nguyen,[4] Matthew L. Wheeler,[5] Michael P. Murphy,[6] Timothy A. Sanders,[1] Stefanie S. Jeffrey,[7] Yerem Yeghiazarians,[8] Paolo F. Rinaudo,[9] Joseph F. Costello,[2] Manish K. Aghi,[2] and Emin Maltepe[1,*]
[1]Department of Pediatrics/Biomedical Sciences, University of California San Francisco, San Francisco, CA 94143, USA
[2]Department of Neurological Surgery, University of California San Francisco, San Francisco, CA 94143, USA
[3]Department of Pathology, University of California San Francisco, San Francisco, CA 94143, USA
[4]Department of Biomedical Sciences, University of California San Francisco, San Francisco, CA 94143, USA
[5]Department of Microbiology/Immunology, University of California San Francisco, San Francisco, CA 94143, USA
[6]Mitochondrial Biology Unit, MRC, Cambridge CB2 0XY, UK
[7]Department of Surgery, Stanford University School of Medicine, Stanford, CA 94305, USA
[8]Department of Medicine/CVRI/Eli and Edythe Broad Center for Regeneration Medicine, University of California San Francisco, San Francisco, CA 94143, USA
[9]Department of Obstetrics, Gynecology/Reproductive Sciences, University of California San Francisco, San Francisco, CA 94143, USA
*Correspondence: emin.maltepe@ucsf.edu
http://dx.doi.org/10.1016/j.celrep.2015.01.020
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

SUMMARY

Hypoxia-inducible gene domain family member 1A (HIGD1A) is a survival factor induced by hypoxia-inducible factor 1 (HIF-1). HIF-1 regulates many responses to oxygen deprivation, but viable cells within hypoxic perinecrotic solid tumor regions frequently lack HIF-1α. HIGD1A is induced in these HIF-deficient extreme environments and interacts with the mitochondrial electron transport chain to repress oxygen consumption, enhance AMPK activity, and lower cellular ROS levels. Importantly, HIGD1A decreases tumor growth but promotes tumor cell survival in vivo. The human *Higd1a* gene is located on chromosome 3p22.1, where many tumor suppressor genes reside. Consistent with this, the *Higd1a* gene promoter is differentially methylated in human cancers, preventing its hypoxic induction. However, when hypoxic tumor cells are confronted with glucose deprivation, DNA methyltransferase activity is inhibited, enabling HIGD1A expression, metabolic adaptation, and possible dormancy induction. Our findings therefore reveal important new roles for this family of mitochondrial proteins in cancer biology.

INTRODUCTION

Heart disease, stroke, and cancer are associated with hypoxia (Semenza, 2014) and nutrient deprivation (Hardie et al., 2012). Hypoxia inducible factor 1 (HIF-1) is a widely expressed transcription factor that regulates the survival of cells during oxygen and glucose deprivation (Iyer et al., 1998; Maltepe et al., 1997; Ochiai et al., 2011; Ryan et al., 1998). HIF can also regulate tumor metabolism by repressing respiration (Kim et al., 2006; Papandreou et al., 2006) while promoting glycolysis, which enables rapid tumor cell proliferation (Vander Heiden et al., 2009). When severe, cancer cells can survive hypoxia and/or nutrient deprivation by entering a dormant state, which suppresses their growth (Bragado et al., 2012; Sosa et al., 2013). Since most cancer therapies target proliferating cells, oxygen/nutrient-deprived tumor regions frequently become resistant and contribute to tumor recurrence. New agents are therefore being developed to target these regions (Harada et al., 2012; Zhang et al., 2014). Paradoxically, chronically oxygen-starved tumor regions frequently lack HIF-1α expression (Ameri et al., 2010; Sobhanifar et al., 2005), likely due to simultaneous glucose deprivation (Catrina et al., 2004; Osada-Oka et al., 2010). However, some HIF-1 target genes such as CAIX remain either due to greater protein stability (Sobhanifar et al., 2005) or HIF-1-independent pathways (van den Beucken et al., 2009).

Oxygen or glucose deprivation promotes reactive oxygen species (ROS) production, which can trigger adaptive responses such as HIF induction (Sena and Chandel, 2012) or can induce apoptosis (Malhotra et al., 2008). Therefore, cells need to modulate both oxygen consumption and ROS production in order to survive oxygen/glucose deprivation. One pathway that cells utilize to achieve this relies on AMP-dependent protein kinase (AMPK) activation (Jeon et al., 2012). AMPK can activate multiple adaptive pathways, including antioxidant mechanisms. Interestingly, the effects of AMPK on tumor growth are complex, acting as oncogene or tumor suppressor depending on context (Hardie and Alessi, 2013).

Hypoxia-Inducible Gene Domain Family Member 1A (HIGD1A) is a survival factor regulated by HIF-1 (Wang et al., 2006). We previously demonstrated that HIGD1A is expressed in regions of severe ischemia in vivo (Ameri et al., 2013) that frequently

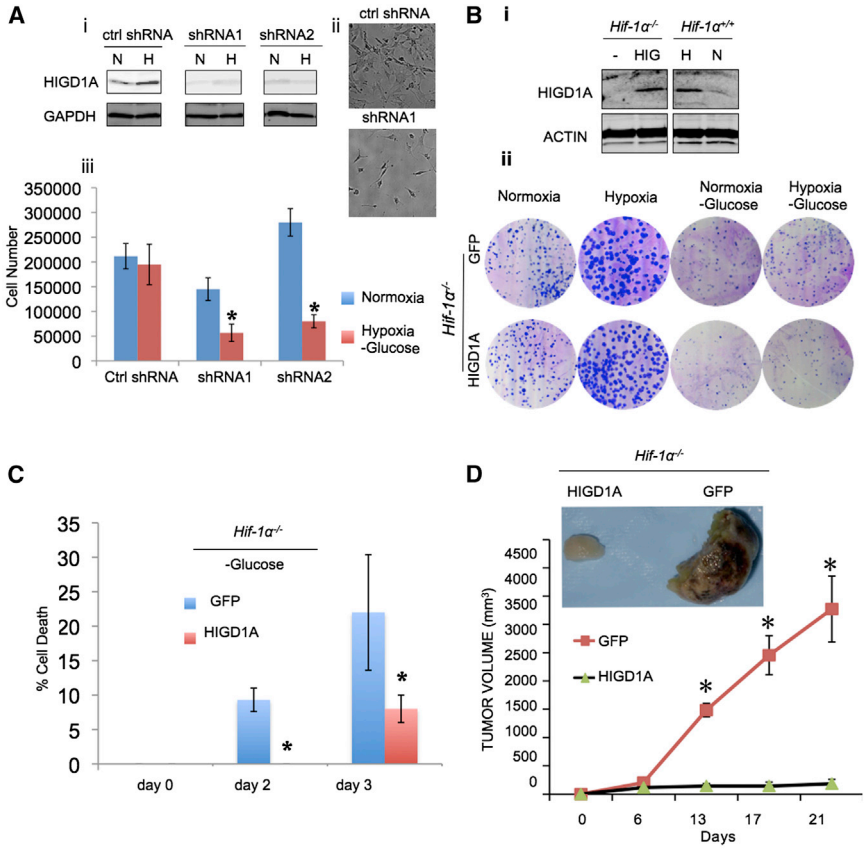


Figure 1. HIGD1A Protects against Glucose Starvation and Suppresses Tumor Growth with Diminished Apoptosis

(A) (i) Immunoblot analysis of HIGD1A levels following small hairpin RNA (shRNA)-mediated knockdown in WT MEFs (control shRNA = ctrl). (ii and iii) Phase-contrast microscopy and trypan blue exclusion count indicate that HIGD1A is necessary for survival of cells during glucose starvation/hypoxia. 20,000 cells were seeded in 6-well plates and counted after 4 days.

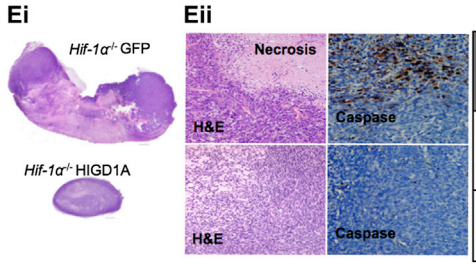
(B) (i) Immunoblot analysis comparing protein levels of HIGD1A in HIF-deficient (*Hif-1α*$^{-/-}$) MEFs stably expressing HIGD1A versus wild-type MEFs (*Hif-1α*$^{+/+}$) exposed to hypoxia. (ii) Colony formation assays showing that HIGD1A expression in HIF-deficient (*Hif-1α*$^{-/-}$) MEFs results in fewer as well as smaller colonies during combined hypoxia/glucose deprivation or glucose deprivation alone.

(C) Viability assay of *Hif-1α*$^{-/-}$ MEFs expressing HIGD1A compared with control GFP cells following 3 days of glucose deprivation.

(D) *Hif-1α* $^{-/-}$ MEFs stably expressing HIGD1A resulted in significantly smaller tumor xenografts when grown for 3 weeks subcutaneously in mice.

(E) (i) Histopathological analysis indicating lack of necrosis in *Hif-1α*$^{-/-}$ HIGD1A tumors but profound necrosis in *Hif-1α* $^{-/-}$ GFP control tumors. Cleaved-caspase-3 immunohistochemical staining shows significantly more apoptosis in *Hif-1α*$^{-/-}$ GFP control tumors (ii).

Error bars represent ± SD. *p < 0.05. Five mice per group were used for tumor growth and analysis.



		Hif-1α-/- HIGD1A	Hif-1α-/- GFP
Apoptosis (20 HPF counted)	Mean +/- SD	6.1+/-2.1	13.6+/-6.6*
	Median (min-max)	6 (3-9)	12 (6-29)
caspase (40 HPF counted)	Positive cell/ 10HPF	5.5	12*

lack detectable HIF-1 activity. To investigate this phenomenon, we interrogated the function of HIGD1A in RAS-transformed HIF-1-deficient MEFs (Ryan et al., 2000) as well as in human cancers in vitro and in vivo. Our studies identify novel functions for HIGD1A with implications for tumor cell survival and dormancy mechanisms.

RESULTS

HIGD1A Protects from Oxygen and Glucose Deprivation but Suppresses Growth

HIGD1A can protect cells from glucose- and oxygen-deprivation-induced death (Wang et al., 2006). To confirm this, we generated HIGD1A "knockdown" mouse embryonic fibroblasts (MEFs) (Figure 1Ai), which exhibited poor survival during oxygen/glucose deprivation (Figures 1Aii and 1Aiii). To isolate the function of this single HIF-1α target from other HIF-dependent effects, we generated HIF-1α-deficient MEFs (*Hif-1α*$^{-/-}$ MEFs) that stably expressed HIGD1A to levels observed in wild-type MEFs exposed to hypoxia (Figure 1Bi). Sustained HIGD1A expression in *Hif-1α*$^{-/-}$ MEFs had negligible effects on colony formation under normoxic or hypoxic conditions (Figure 1Bii). Glucose deprivation reduced colony size and number in *Hif-1α* $^{-/-}$ MEFs expressing either HIGD1A or GFP (Figure 1Bii). However, *Hif-1α* $^{-/-}$ MEFs expressing HIGD1A produced even fewer numbers of colonies that were also smaller in size during glucose- or combined glucose/oxygen deprivation (Figure 1Bii). Interestingly, HIGD1A expression protected *Hif-1α*$^{-/-}$ MEFs from death during glucose deprivation (Figure 1C), suggesting that increased cell death was not the cause for reduced colony number or size. Consistent with these results, tumors derived from *Hif-1α*$^{-/-}$ MEFs expressing HIGD1A were much smaller than control *Hif-1α*$^{-/-}$ MEF tumors (Figure 1D) and did not contain any appreciable areas of necrosis, which was widely seen in control tumors (Figure 1Ei). Furthermore, these tumors also exhibited significantly less apoptosis (Figure 1Eii). These results indicate that HIGD1A can promote cell survival during nutrient deprivation while simultaneously suppressing growth in vitro and in vivo.

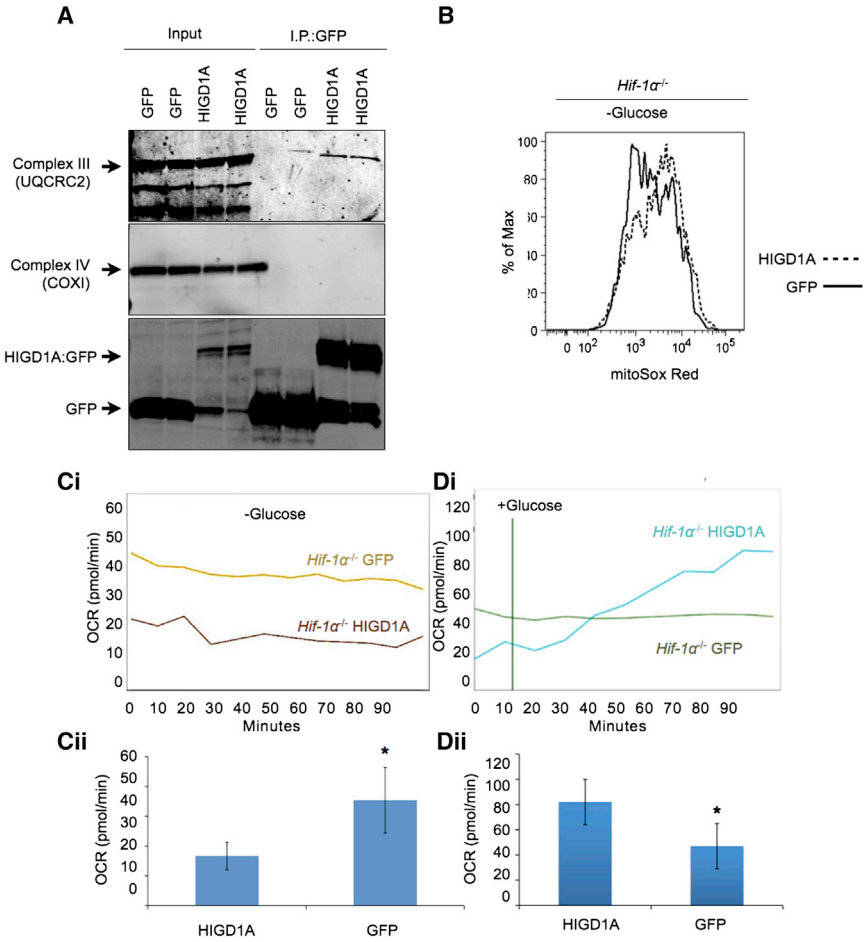


Figure 2. HIGD1A Can Regulate Mitochondrial Superoxide and Oxygen Consumption during Glucose Starvation

(A) Immunoprecipitation assay showing HIGD1A can interact with complex III subunit 2 of the respiratory chain.

(B) FACS analysis showing that HIF-deficient cells expressing HIGD1A have increased mitochondrial ROS (superoxide) during glucose starvation compared to control cells expressing GFP.

(Ci and Cii) Oxygen consumption is lower during glucose deprivation when HIGD1A is expressed in *Hif-1α*$^{-/-}$ cells.

(Di and Dii) When glucose is re-introduced to glucose-starved cells, HIGD1A-expressing cells increase their oxygen consumption at a faster rate than control GFP-expressing cells.

Error bars represent ±SD, *$p < 0.05$.

HIGD1A Interaction with the Electron Transport Chain Modulates Mitochondrial ROS Production and Oxygen Consumption

HIGD1A is an inner mitochondrial protein, and recently orthologs of HIGD1A and the related HIGD2A were shown to interact with complex IV in yeast (Chen et al., 2012; Strogolova et al., 2012). Immunoprecipitation assays with extracts derived from MEFs expressing GFP-tagged HIGD1A detected an interaction between murine HIGD1A and complex III subunit 2 of the mitochondrial electron transport chain (ETC), but not with complex IV subunit I (Figure 2A). Complex III is an important site for mitochondrial superoxide (O_2^-) production (Buetler et al., 2004; Chen and Gibson, 2008), which can be increased when the proton motive force increases, as occurs with decreased flow through the respiratory chain (Murphy, 2009). Therefore, we examined the level of mitochondrial O_2^- production via fluorescence-activated cell sorting (FACS)-mediated analysis of Mitosox Red intensity. This was increased to a greater extent in HIGD1A-expressing *Hif-1α*$^{-/-}$ MEFs during glucose starvation than their GFP-expressing counterparts (Figure 2B). Additionally, HIGD1A expression in *Hif-1α*$^{-/-}$ MEFs resulted in an approximately 2-fold reduction in cellular oxygen consumption during glucose deprivation (Figures 2Ci and 2Cii). Interestingly, following re-introduction of glucose, oxygen consumption was reversed more rapidly in cells stably expressing HIGD1A (Figures 2Di and 2Dii). These results indicate that HIGD1A expression can modulate mitochondrial ROS production and oxygen consumption during conditions of glucose deprivation via interaction with the ETC.

HIGD1A Induces AMPK Activity and Decreases Cellular ROS to Promote Survival

ROS generation during glucose deprivation can result in cell death (Gao et al., 2012; Lenin et al., 2012; Malhotra et al., 2008). AMPK can be induced by glucose deprivation and mitochondrial O_2^- production (Wu and Wei, 2012; Mackenzie et al., 2013) to reduce total ROS via the promotion of pentose phosphate shunt-mediated NADPH production (Jeon et al., 2012). Consistent with these studies, *Hif-1α*$^{-/-}$ MEFs that stably expressed HIGD1A increased pAMPK levels to a greater extent than control cells during glucose deprivation (Figure S1A), and this effect was reduced by the mitochondria-targeted antioxidant MitoQ (Kelso et al., 2001) (Figure S1B). Furthermore, this diminished total cellular ROS in HIGD1A-expressing cells during glucose starvation (Figure S1C), and a cell permeable form of the antioxidant glutathione (Graham et al., 2012) improved the viability of control *Hif-1α*$^{-/-}$ MEFs during glucose deprivation (Figure S1D). Thus, HIGD1A triggers increased mitochondrial O_2^- production to activate AMPK and decrease total cellular ROS levels to promote cell survival. To determine whether AMPK activity was necessary for the protective effect of HIGD1A during glucose deprivation, we stably transfected *Ampk1/2*$^{-/-}$ MEFs with HIGD1A or GFP as control and examined their survival during glucose starvation. AMPK-deficient cells do not reduce total cellular ROS levels and fail to induce autophagy during glucose starvation, compromising their survival (Jeon et al., 2012; Kim et al., 2011). Interestingly, HIGD1A expression did not protect *Ampk1/2*$^{-/-}$ MEFs from glucose starvation (Figure S1E), indicating that AMPK activation

is necessary for the protective effect of HIGD1A during glucose deprivation. We also examined AMPK activation in vivo utilizing tumors derived from *Hif-1α*$^{-/-}$ MEFs expressing HIGD1A previously described in Figure 1D and found that they demonstrated pAMPK immunoreactivity that was more intense as well as more diffusely distributed than control tumors (Figure S1F), consistent with the reduction of cell death. Immunoreactivity of pAMPK was ablated when treated with pAMPK blocking peptide (Figure S1G). Finally, we questioned whether the effects of HIGD1A-dependent pAMPK induction during glucose deprivation could be due to autophagy induction, since it can also be regulated by AMPK to increase cell survival during glucose starvation (Kim et al., 2011). As seen in Figure S2, autophagy induction was dispensable for the protective effects of HIGD1A.

HIGD1A Is Not Induced by HIF-1α in Hypoxic Human Cancers but Is Triggered by Additional Metabolic Stressors

We next examined the mode of regulation of HIGD1A in a variety of human cancer cell lines. Human HT1080 fibrosarcoma and HeLa cervical cancer cell lines exhibited basal levels of HIGD1A that were surprisingly not further induced by hypoxia, despite inducing HIF-1α (Figure 3A). BNIP3, another HIF-1 target mitochondrial protein (Sowter et al., 2001), was induced, however. The gene encoding HIGD1A is located on human chromosome 3p22.1, where many tumor suppressor genes reside, and these are often inactivated via epigenetic mechanisms (Bhat Singh and Amare Kadam, 2013; Buchhagen et al., 1994). From genome-wide analyses of aberrant DNA methylation in glioblastoma multiforme (GBM) (Nagarajan et al., 2014), we identified two candidate regions near the *Higd1a* promoter that exhibited GBM-specific hypermethylation. As indicated in Figure 3B, one of these regions, located upstream of the 5′ CpG island promoter, in a CpG island "shore," contains consensus core hypoxia response elements (HREs; blue underline). Two CpG sites within this region are interrogated on the Illumina HumanMethylation450 methylation array and exhibit high methylation in several cancer cells and only partial methylation in normal human NH-A astrocytes (ENCODE data), indicating that it may be a differentially methylated region (DMR, red and green CG). Chromatin immunoprecipitation sequencing (ChIP-seq) profiles from the Roadmap Epigenomics Project confirmed the presence of histone modifications associated with enhancers in both brain and breast, consistent with it harboring potential HREs. ChIP analysis confirmed that HIF-1α was able to bind the HRE within this DMR in HeLa cells during hypoxia (Figure 3C), despite its expression not being induced. Treating HeLa cells with the DNA methylation inhibitor 5-aza-2′-deoxycytidine, however, enhanced HIGD1A expression during hypoxia (Figure 3D). Reduced expression of DNA methyl transferase 1 (DNMT1) can reactivate tumor suppressors (Xiang et al., 2014; Yao et al., 2014), and glucose starvation can reduce expression of the gene encoding DNMT1 (Lin et al., 2012). Glucose starvation reduced DNMT1 expression in hypoxic HeLa cells suggesting that DNA methylation pathways are inhibited during combined oxygen/glucose deprivation (Figure 3E). Reduction of DNMT1 correlated with enhanced HIGD1A expression during glucose starvation (Figure 3F). Unlike canonical tumor suppressor genes that are sometimes strongly and permanently silenced by dense methylation across their promoter CpG islands, the *Higd1a* gene locus shows more nuanced epigenetic regulation in human cancer. DNA methylation at upstream HREs might prevent transactivation of HIGD1A via HIF-1 and thereby suppress enhanced HIGD1A expression during growth permissive hypoxic conditions but allow epigenetic activation when environmental conditions favoring HIGD1A expression are encountered.

HIGD1A Expression Is Enhanced in Severely Ischemic Tumor Regions In Vivo

We next examined whether HIGD1A expression is induced in a similar fashion in human cancers in vivo, particularly since reduced methyl cytosine levels have been reported in ischemic tumor regions due to reduced DNMT activity (Shahrzad et al., 2007; Skowronski et al., 2010). MDA-MB 231 breast cancer xenografts demonstrated severely ischemic perinecrotic regions as evidenced by strong staining with the hypoxia marker pimodinazole, along with diminished HIF-1α immunoreactivity, indicating likely glucose starvation (Figure 4A). As shown in Figure S3, lack of HIF-1α was also observed in severely ischemic myocardial regions following experimental myocardial infarction in mice, suggesting that this may be a common indicator of starvation severity. As indicated in Figures 4B and S3, these areas of ischemia demonstrated enhanced HIGD1A expression. Similar to our observations with HeLa cells, DNMT1 levels were also reduced in ischemic MDA-MB 231 cells (Figure 4C). In addition, HIGD1A expression was enhanced in vitro in ischemic MDA-MB 231 cells when compared with hypoxia (Figure 4D). Circulating tumor cells (CTCs) derived from MDA-MB 231 xenografts were previously reported to be more resistant to anoxia (Ameri et al., 2010). Both MDA-MB231 cells and their CTCs induced HIF-1α but not HIGD1A during hypoxia (Figure 4E). Interestingly, basal levels of HIGD1A were higher in CTCs. These results further confirm that hypoxic HIF-1α induction is not sufficient to enhance HIGD1A expression in human cancers in vitro or in vivo, but that additional pathways triggered by severe metabolic stressors are necessary. Finally, we analyzed HIGD1A expression patterns in glioblastoma multiforme (GBM) biopsies from patients before and after treatment with the anti-angiogenesis agent bevacizumab, which is known to induce severe tumor ischemia (Hu et al., 2012). Primary GBM biopsies exhibited hypoxic areas as evident by increased CA9 expression (Figure 4F). These areas did not demonstrate significant HIGD1A expression. However, after treatment with bevacizumab, HIGD1A was strongly induced (Figure 4F). This indicates that HIGD1A expression is prevented during physiological hypoxia and that additional metabolic stressors are needed to induce HIGD1A in human cancers in vivo.

DISCUSSION

We and others previously documented (Ameri et al., 2010; Sobhanifar et al., 2005) and confirmed once again here that some of the most metabolically compromised tumor regions found around their necrotic cores fail to induce HIF activity, potentially due to glucose starvation (Catrina et al., 2004; Osada-Oka et al., 2010). Interestingly, these regions still express the HIF-1 target

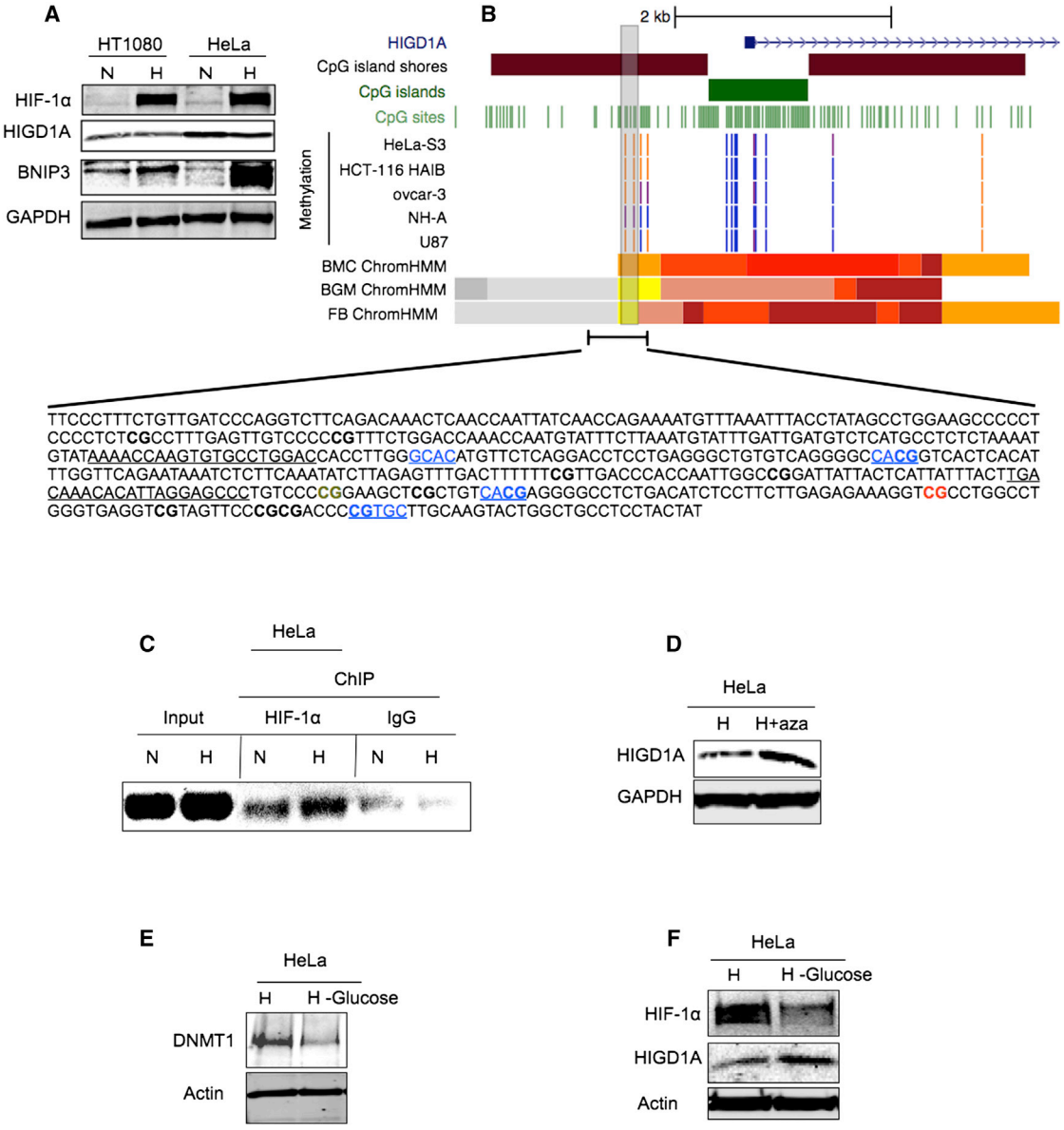


Figure 3. Expression and Regulation of HIGD1A in Cancer

(A) Immunoblot analysis of HIGD1A, HIF-1α, and BNIP3 expression in human HT1080 and HeLa cancer cell lines during normoxia or hypoxia.

(B) Data from Illumina HumanMethylation450 methylation array, and the ENCODE consortium showing high methylation level (vertical orange lines) upstream of the 5′ CpG island promoter, in a CpG island "shore." The HIGD1A CpG island itself is generally unmethylated (vertical blue and violet lines) in both cancer cell lines (U87, ovcar-3, HCT-116, HeLa) as well as in normal human astrocytes (NH-A). Two of the CpGs (within vertical gray rectangle) in the 5′ shore of that CpG island have high methylation levels in HeLa, ovcar-3, HCT-116, and U87 cell lines and partial methylation in normal human astrocytes, indicating a potential differential methylated region (DMR, red, and green CG in the sequence given). The sequence of the entire 5′ region (region chromosome 3: 42846997-42847502) including the two specific CpGs (highlighted as green and red in the sequence) of this putative DMR neighbors several HRE-core sequences (blue underlined). ChIP-seq data from the Roadmap Epigenomics Project indicate that this region is marked by histone modifications associated with enhancers (yellow and orange bars) in both brain (FB ChromHMM, BGM ChromHMM) and breast (BMC ChromHMM). Primers used for CHIP analysis in black underlined.

(C) ChIP analysis performed on normoxic (N) or hypoxic (H) HeLa cells using primers (black underlined in sequence) within the 5′ region that contains the two specific CpGs (highlighted as green and red in the sequence) of this putative DMR.

(D) Immunoblot analysis demonstrating expression of HIGD1A protein in the human cervical cancer cell line HeLa in hypoxia (H) versus hypoxia combined with the DNA methylation inhibitor (DNMT-inhibitor) 5-aza-2′-deoxycytidine (H^+aza).

(E) Immunoblot analysis showing that glucose starvation (−glucose) during hypoxia (H) reduces expression of DNMT1.

(F) Glucose starvation induces HIGD1A in hypoxic HeLa cells. H, hypoxia (1% O_2)

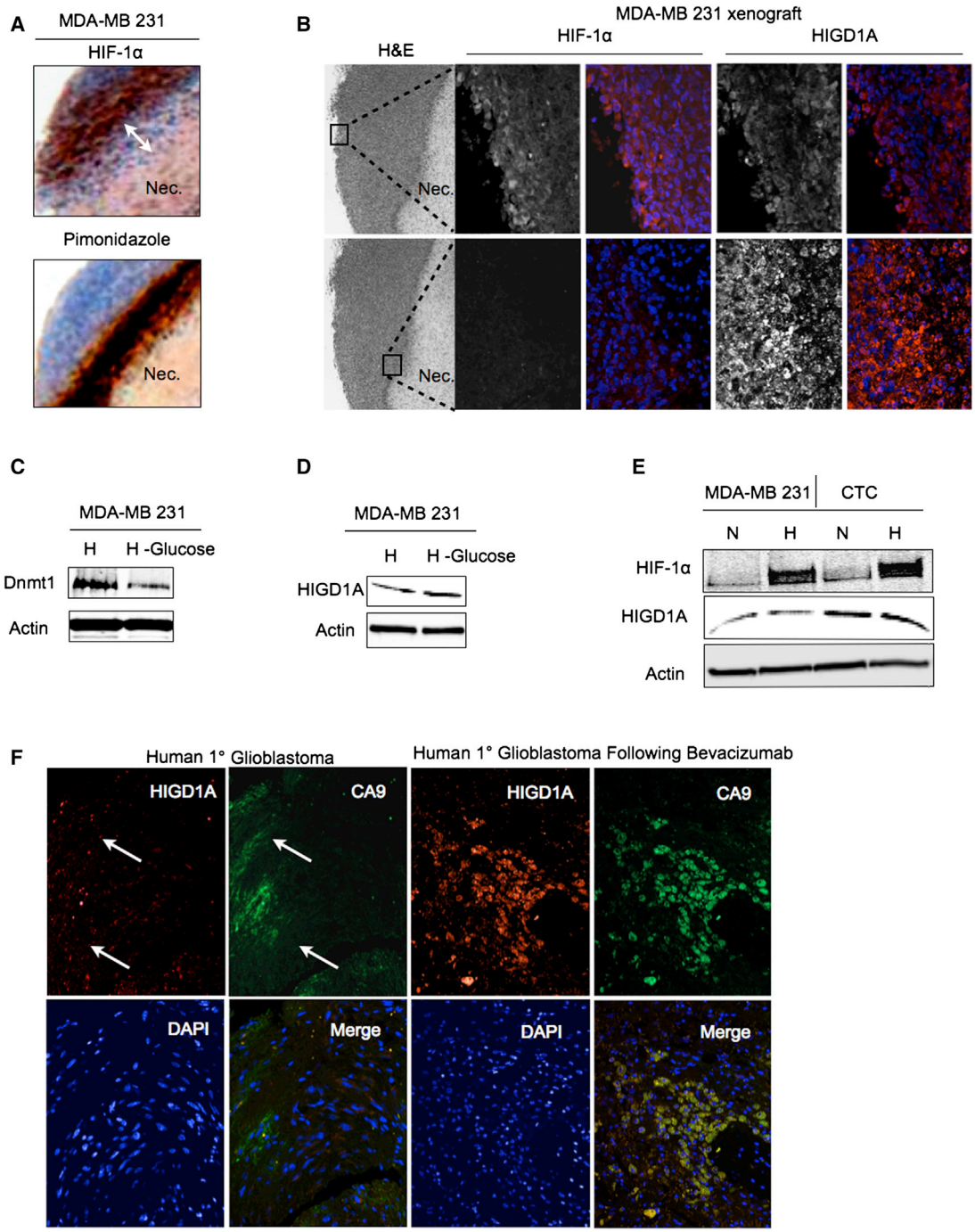


Figure 4. Expression of HIGD1A In Vivo and in Circulating Tumor Cells

(A) Pimonidazole and HIF-1α staining of MDA-MB 231 xenografts showing diminished expression of HIF-1α within perinecrotic regions where pimonidazole staining is strongest.

(B) MDA-MB 231 xenografts showing enhanced expression of HIGD1A at perinecrotic regions where HIF-1α expression is diminished.

(C) Immunoblot analysis showing that expression of DNMT1 during hypoxia (H) versus hypoxia and glucose starvation (H –Glucose).

(D) Glucose starvation during hypoxia enhances HIGD1A protein level in MDA-MB 231 cells.

(E) Immunoblot analysis of HIGD1A expression in MDA-MB231 cells from which the xenografts where made, and in CTCs derived from the xenografts via blood extraction, as a function of oxygen.

(F) Human primary glioblastoma biopsies demonstrate lack of HIGD1A induction in hypoxic regions where Ca9 induction is evident. Induction of HIGD1A is evident only after treatment with the anti-angiogenesis agent bevacizumab.

N, normoxia (21% oxygen); H, hypoxia (1% oxygen); HBS, HIF binding site; HRE, hypoxia response element; CTC, circulating tumor cell.

HIGD1A. One way that cells can survive metabolic stress is via lowering cellular ROS and oxygen consumption, which are parameters associated with quiescence and dormancy-mediated survival (Endo et al., 2014; Lagadinou et al., 2013; Lopes et al., 2010). Consistent with this, we found that HIGD1A interacts with the mitochondrial electron transport chain, modulates oxygen consumption, ROS production, and AMPK activity to promote cell survival during glucose starvation, while simultaneously suppressing tumor growth in vivo. Furthermore, anti-VEGF therapy has previously been shown to induce AMPK activity to promote tumor cell survival in vivo (Nardo et al., 2011), which we also confirm to be associated with enhanced HIGD1A activity in human GBM. Multiple studies have previously linked ROS suppression and cell survival with AMPK activation, likely via phosphatase inhibition (Davies et al., 1995; Faubert et al., 2013; Han et al., 2010; Hofstetter et al., 2012; Indraccolo, 2013; Ingebritsen et al., 1983; Jeon et al., 2012; Klaus et al., 2012; Kwan et al., 2013; Rotte et al., 2010; Wu and Wei, 2012). Our studies confirm these observations and identify HIGD1A as an important upstream component of this signaling cascade. Furthermore, HIGD1A repression is associated with tumor recurrence in breast cancers following therapy (Chanrion et al., 2008), consistent with our observations that HIGD1A expression helps repress tumor growth. These findings therefore provide novel insights into tumor cell adaptation mechanisms to extreme environments and suggest that HIGD1A may play an important role in tumor dormancy or recurrence mechanisms (Giancotti, 2013).

The ability of HIGD1A expression to be regulated epigenetically provides an attractive model whereby environmental factors can regulate HIGD1A expression independent of HIF activity to modulate tumor growth. The gene encoding HIGD1A is located on human chromosome 3p22.1, where many tumor suppressors reside, and many of which are inactivated via epigenetic mechanisms (Bhat Singh and Amare Kadam, 2013; Buchhagen et al., 1994). Our analysis of the human *Higd1a* locus indicated hypermethylation of the upstream promoter region in various cancer cell lines that was able to bind HIF-1α but not drive its hypoxic expression. This suggested that additional pathways are required to induce HIGD1A in human cancers in vivo, and we found that reducing the expression or activity of DNA methyl transferases (DNMTs) increased HIGD1A expression in vitro. This result is consistent with previous reports linking DNMT1 inhibition with tumor suppressor reactivation in response to environmental stressors in vivo (Xiang et al., 2014; Yao et al., 2014; Lin et al., 2012). Constitutive basal expression of HIGD1A might be beneficial during growth permissive hypoxic conditions without glucose deprivation. When glucose deprivation becomes severe, enhanced HIGD1A expression modulated by epigenetic mechanisms may help trigger a state of dormancy and tumor growth inhibition (LaRue et al., 2004; Sutherland, 1988). Such dormant cells are typically resistant to many therapies, enabling tumor cell survival and cancer recurrence (Indraccolo, 2013; Lin et al., 2012). Novel molecules are therefore being developed to target these dormant cells (Zhang et al., 2014). Small molecule modulators of the HIG family of mitochondrial proteins (Lindert et al., 2014) may therefore prove useful in the fight against cancer.

EXPERIMENTAL PROCEDURES

Cell Culture

MEFs, HT1080, and HeLa cells were cultured in RPMI-1640, 10% FBS, and 110 μg/ml sodium pyruvate. Glucose starvation was achieved by using glucose-free RPMI-1640. Normoxic cells were incubated at 5% CO_2 and 21% O_2 while hypoxia experiments were performed at 1% O_2 with 5% CO_2.

Oxygen Consumption and ROS Measurements

O_2 consumption measured via use of the SeaHorse Extracellular Flux Analyzer according to the manufacturer's protocol. Mitochondrial ROS measured via FACS-mediated analysis of Mito-Sox Red, and total ROS assayed by measuring Cell-Rox Deep Red fluorescence via manufacturer's instructions.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) MEF and human tumor sections were cut at 5 μm, subjected to antigen retrieval, treated with M.O.M. kit, and incubated for 1 hr at 37°C with primary antibody. Glioblastoma FFPE biopsies were cut at 16-μm sections. Sections were incubated overnight at 4°C in primary antibody. A list of antibodies and suppliers is available in the Supplemental Information. Imaging was performed with a Zeiss Imager Z.2 fluorescence microscope equipped with an Apotome and axiovision-ZEN software for optical sectioning and analysis.

Immunoprecipitation and Immunoblotting

GFP fusion proteins were immunoprecipitated with Chromotek-GFP-Trap bead according to manufacturer's recommendations. Pull-downs, as well as all other immunoblotting, were performed via SDS-PAGE and blotted onto Immobilon-FL membranes using semi-dry transfer. Membranes were blocked in blocking buffer from LI-COR Biosciences and probed with primary antibodies in LI-COR blocking buffer.

ChIP Assays

The ExactaCHIP kit was used for chromatin immunoprecipitation assays according to the manufacturer's protocol.

Tumor Models and Human Glioblastoma Samples

Tumor models and human glioblastoma samples are described in the Supplemental Experimental Procedures. All studies were approved by the UCSF Committee on Human Research and Institutional Animal Care and Use Committee.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.01.020.

ACKNOWLEDGMENTS

This research was made possible by a grant from the NIH R01 HD072455 and from the California Institute for Regeneration Medicine (grant number TB1-01194). *Hif-1α*$^{-/-}$ MEFs were provided by R. Johnson (University of Cambridge); *Ampk1/2*$^{-/-}$ MEFs were provided by B. Viollet (INSERM); *Atg5*$^{-/-}$ MEFs were provided by N. Mizushima (University of Tokyo).

Received: June 20, 2014
Revised: October 27, 2014
Accepted: January 9, 2015
Published: February 12, 2015

REFERENCES

Ameri, K., Luong, R., Zhang, H., Powell, A.A., Montgomery, K.D., Espinosa, I., Bouley, D.M., Harris, A.L., and Jeffrey, S.S. (2010). Circulating tumour cells demonstrate an altered response to hypoxia and an aggressive phenotype. Br. J. Cancer *102*, 561–569.

Ameri, K., Rajah, A.M., Nguyen, V., Sanders, T.A., Jahangiri, A., Delay, M., Donne, M., Choi, H.J., Tormos, K.V., Yeghiazarians, Y., et al. (2013). Nuclear localization of the mitochondrial factor HIGD1A during metabolic stress. PLoS ONE *8*, e62758.

Bhat Singh, R., and Amare Kadam, P.S. (2013). Investigation of tumor suppressor genes apart from VHL on 3p by deletion mapping in sporadic clear cell renal cell carcinoma (cRCC). Urol. Oncol. *31*, 1333–1342.

Bragado, P., Sosa, M.S., Keely, P., Condeelis, J., and Aguirre-Ghiso, J.A. (2012). Microenvironments dictating tumor cell dormancy. Recent Results Cancer Res. *195*, 25–39.

Buchhagen, D.L., Qiu, L., and Etkind, P. (1994). Homozygous deletion, rearrangement and hypermethylation implicate chromosome region 3p14.3-3p21.3 in sporadic breast-cancer development. Int. J. Cancer *57*, 473–479.

Buetler, T.M., Krauskopf, A., and Ruegg, U.T. (2004). Role of superoxide as a signaling molecule. News Physiol. Sci. *19*, 120–123.

Catrina, S.B., Okamoto, K., Pereira, T., Brismar, K., and Poellinger, L. (2004). Hyperglycemia regulates hypoxia-inducible factor-1alpha protein stability and function. Diabetes *53*, 3226–3232.

Chanrion, M., Negre, V., Fontaine, H., Salvetat, N., Bibeau, F., Mac Grogan, G., Mauriac, L., Katsaros, D., Molina, F., Theillet, C., and Darbon, J.M. (2008). A gene expression signature that can predict the recurrence of tamoxifen-treated primary breast cancer. Clin. Cancer Res. *14*, 1744–1752.

Chen, Y., and Gibson, S.B. (2008). Is mitochondrial generation of reactive oxygen species a trigger for autophagy? Autophagy *4*, 246–248.

Chen, Y.C., Taylor, E.B., Dephoure, N., Heo, J.M., Tonhato, A., Papandreou, I., Nath, N., Denko, N.C., Gygi, S.P., and Rutter, J. (2012). Identification of a protein mediating respiratory supercomplex stability. Cell Metab. *15*, 348–360.

Davies, S.P., Helps, N.R., Cohen, P.T., and Hardie, D.G. (1995). 5′-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. FEBS Lett. *377*, 421–425.

Endo, H., Okuyama, H., Ohue, M., and Inoue, M. (2014). Dormancy of cancer cells with suppression of AKT activity contributes to survival in chronic hypoxia. PLoS ONE *9*, e98858.

Faubert, B., Boily, G., Izreig, S., Griss, T., Samborska, B., Dong, Z., Dupuy, F., Chambers, C., Fuerth, B.J., Viollet, B., et al. (2013). AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. Cell Metab. *17*, 113–124.

Gao, H.J., Zhu, Y.M., He, W.H., Liu, A.X., Dong, M.Y., Jin, M., Sheng, J.Z., and Huang, H.F. (2012). Endoplasmic reticulum stress induced by oxidative stress in decidual cells: a possible mechanism of early pregnancy loss. Mol. Biol. Rep. *39*, 9179–9186.

Giancotti, F.G. (2013). Mechanisms governing metastatic dormancy and reactivation. Cell *155*, 750–764.

Graham, N.A., Tahmasian, M., Kohli, B., Komisopoulou, E., Zhu, M., Vivanco, I., Teitell, M.A., Wu, H., Ribas, A., Lo, R.S., et al. (2012). Glucose deprivation activates a metabolic and signaling amplification loop leading to cell death. Mol. Syst. Biol. *8*, 589.

Han, Y., Wang, Q., Song, P., Zhu, Y., and Zou, M.H. (2010). Redox regulation of the AMP-activated protein kinase. PLoS ONE *5*, e15420.

Harada, H., Inoue, M., Itasaka, S., Hirota, K., Morinibu, A., Shinomiya, K., Zeng, L., Ou, G., Zhu, Y., Yoshimura, M., et al. (2012). Cancer cells that survive radiation therapy acquire HIF-1 activity and translocate towards tumour blood vessels. Nat. Commun. *3*, 783.

Hardie, D.G., and Alessi, D.R. (2013). LKB1 and AMPK and the cancer-metabolism link - ten years after. BMC Biol. *11*, 36.

Hardie, D.G., Ross, F.A., and Hawley, S.A. (2012). AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat. Rev. Mol. Cell Biol. *13*, 251–262.

Hofstetter, C.P., Burkhardt, J.K., Shin, B.J., Gürsel, D.B., Mubita, L., Gorrepati, R., Brennan, C., Holland, E.C., and Boockvar, J.A. (2012). Protein phosphatase 2A mediates dormancy of glioblastoma multiforme-derived tumor stem-like cells during hypoxia. PLoS ONE *7*, e30059.

Hu, Y.L., DeLay, M., Jahangiri, A., Molinaro, A.M., Rose, S.D., Carbonell, W.S., and Aghi, M.K. (2012). Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. Cancer Res. *72*, 1773–1783.

Indraccolo, S. (2013). Insights into the regulation of tumor dormancy by angiogenesis in experimental tumors. Adv. Exp. Med. Biol. *734*, 37–52.

Ingebritsen, T.S., Stewart, A.A., and Cohen, P. (1983). The protein phosphatases involved in cellular regulation. 6. Measurement of type-1 and type-2 protein phosphatases in extracts of mammalian tissues; an assessment of their physiological roles. Eur. J. Biochem. *132*, 297–307.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., and Semenza, G.L. (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev. *12*, 149–162.

Jeon, S.M., Chandel, N.S., and Hay, N. (2012). AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. Nature *485*, 661–665.

Kelso, G.F., Porteous, C.M., Coulter, C.V., Hughes, G., Porteous, W.K., Ledgerwood, E.C., Smith, R.A., and Murphy, M.P. (2001). Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. J. Biol. Chem. *276*, 4588–4596.

Kim, J.W., Tchernyshyov, I., Semenza, G.L., and Dang, C.V. (2006). HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab. *3*, 177–185.

Kim, J., Kundu, M., Viollet, B., and Guan, K.L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat. Cell Biol. *13*, 132–141.

Klaus, A., Polge, C., Zorman, S., Auchli, Y., Brunisholz, R., and Schlattner, U. (2012). A two-dimensional screen for AMPK substrates identifies tumor suppressor fumarate hydratase as a preferential AMPKα2 substrate. J. Proteomics *75*, 3304–3313.

Kwan, H.T., Chan, D.W., Cai, P.C., Mak, C.S., Yung, M.M., Leung, T.H., Wong, O.G., Cheung, A.N., and Ngan, H.Y. (2013). AMPK activators suppress cervical cancer cell growth through inhibition of DVL3 mediated Wnt/β-catenin signaling activity. PLoS ONE *8*, e53597.

Lagadinou, E.D., Sach, A., Callahan, K., Rossi, R.M., Neering, S.J., Minhajuddin, M., Ashton, J.M., Pei, S., Grose, V., O'Dwyer, K.M., et al. (2013). BCL-2 inhibition targets oxidative phosphorylation and selectively eradicates quiescent human leukemia stem cells. Cell Stem Cell *12*, 329–341.

LaRue, K.E., Khalil, M., and Freyer, J.P. (2004). Microenvironmental regulation of proliferation in multicellular spheroids is mediated through differential expression of cyclin-dependent kinase inhibitors. Cancer Res. *64*, 1621–1631.

Lenin, R., Maria, M.S., Agrawal, M., Balasubramanyam, J., Mohan, V., and Balasubramanyam, M. (2012). Amelioration of glucolipotoxicity-induced endoplasmic reticulum stress by a "chemical chaperone" in human THP-1 monocytes. Exp. Diabetes Res. *2012*, 356487.

Lin, H.Y., Kuo, Y.C., Weng, Y.I., Lai, I.L., Huang, T.H., Lin, S.P., Niu, D.M., and Chen, C.S. (2012). Activation of silenced tumor suppressor genes in prostate cancer cells by a novel energy restriction-mimetic agent. Prostate *72*, 1767–1778.

Lindert, S., Maslennikov, I., Chiu, E.J., Pierce, L.C., McCammon, J.A., and Choe, S. (2014). Drug screening strategy for human membrane proteins: from NMR protein backbone structure to in silica- and NMR-screened hits. Biochem. Biophys. Res. Commun. *445*, 724–733.

Lopes, A.S., Lane, M., and Thompson, J.G. (2010). Oxygen consumption and ROS production are increased at the time of fertilization and cell cleavage in bovine zygotes. Hum. Reprod. *25*, 2762–2773.

Mackenzie, R.M., Salt, I.P., Miller, W.H., Logan, A., Ibrahim, H.A., Degasperi, A., Dymott, J.A., Hamilton, C.A., Murphy, M.P., Delles, C., and Dominiczak, A.F. (2013). Mitochondrial reactive oxygen species enhance AMP-activated

protein kinase activation in the endothelium of patients with coronary artery disease and diabetes. Clin. Sci. *124*, 403–411.

Malhotra, J.D., Miao, H., Zhang, K., Wolfson, A., Pennathur, S., Pipe, S.W., and Kaufman, R.J. (2008). Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. Proc. Natl. Acad. Sci. USA *105*, 18525–18530.

Maltepe, E., Schmidt, J.V., Baunoch, D., Bradfield, C.A., and Simon, M.C. (1997). Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. Nature *386*, 403–407.

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. Biochem. J. *417*, 1–13.

Nagarajan, R.P., Zhang, B., Bell, R.J., Johnson, B.E., Olshen, A.B., Sundaram, V., Li, D., Graham, A.E., Diaz, A., Fouse, S.D., et al. (2014). Recurrent epimutations activate gene body promoters in primary glioblastoma. Genome Res. *24*, 761–774.

Nardo, G., Favaro, E., Curtarello, M., Moserle, L., Zulato, E., Persano, L., Rossi, E., Esposito, G., Crescenzi, M., Casanovas, O., et al. (2011). Glycolytic phenotype and AMP kinase modify the pathologic response of tumor xenografts to VEGF neutralization. Cancer Res. *71*, 4214–4225.

Ochiai, D., Goda, N., Hishiki, T., Kanai, M., Senoo-Matsuda, N., Soga, T., Johnson, R.S., Yoshimura, Y., and Suematsu, M. (2011). Disruption of HIF-1α in hepatocytes impairs glucose metabolism in diet-induced obesity mice. Biochem. Biophys. Res. Commun. *415*, 445–449.

Osada-Oka, M., Hashiba, Y., Akiba, S., Imaoka, S., and Sato, T. (2010). Glucose is necessary for stabilization of hypoxia-inducible factor-1alpha under hypoxia: contribution of the pentose phosphate pathway to this stabilization. FEBS Lett. *584*, 3073–3079.

Papandreou, I., Cairns, R.A., Fontana, L., Lim, A.L., and Denko, N.C. (2006). HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab. *3*, 187–197.

Rotte, A., Pasham, V., Eichenmüller, M., Bhandaru, M., Föller, M., and Lang, F. (2010). Upregulation of Na+/H+ exchanger by the AMP-activated protein kinase. Biochem. Biophys. Res. Commun. *398*, 677–682.

Ryan, H.E., Lo, J., and Johnson, R.S. (1998). HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J. *17*, 3005–3015.

Ryan, H.E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J.M., and Johnson, R.S. (2000). Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res. *60*, 4010–4015.

Semenza, G.L. (2014). Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Annu. Rev. Pathol. *9*, 47–71.

Sena, L.A., and Chandel, N.S. (2012). Physiological roles of mitochondrial reactive oxygen species. Mol. Cell *48*, 158–167.

Shahrzad, S., Bertrand, K., Minhas, K., and Coomber, B.L. (2007). Induction of DNA hypomethylation by tumor hypoxia. Epigenetics *2*, 119–125.

Skowronski, K., Dubey, S., Rodenhiser, D., and Coomber, B. (2010). Ischemia dysregulates DNA methyltransferases and p16INK4a methylation in human colorectal cancer cells. Epigenetics *5*, 547–556.

Sobhanifar, S., Aquino-Parsons, C., Stanbridge, E.J., and Olive, P. (2005). Reduced expression of hypoxia-inducible factor-1alpha in perinecrotic regions of solid tumors. Cancer Res. *65*, 7259–7266.

Sosa, M.S., Bragado, P., Debnath, J., and Aguirre-Ghiso, J.A. (2013). Regulation of tumor cell dormancy by tissue microenvironments and autophagy. Adv. Exp. Med. Biol. *734*, 73–89.

Sowter, H.M., Ratcliffe, P.J., Watson, P., Greenberg, A.H., and Harris, A.L. (2001). HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. Cancer Res. *61*, 6669–6673.

Strogolova, V., Furness, A., Robb-McGrath, M., Garlich, J., and Stuart, R.A. (2012). Rcf1 and Rcf2, members of the hypoxia-induced gene 1 protein family, are critical components of the mitochondrial cytochrome bc1-cytochrome c oxidase supercomplex. Mol. Cell. Biol. *32*, 1363–1373.

Sutherland, R.M. (1988). Cell and environment interactions in tumor microregions: the multicell spheroid model. Science *240*, 177–184.

van den Beucken, T., Koritzinsky, M., Niessen, H., Dubois, L., Savelkouls, K., Mujcic, H., Jutten, B., Kopacek, J., Pastorekova, S., van der Kogel, A.J., et al. (2009). Hypoxia-induced expression of carbonic anhydrase 9 is dependent on the unfolded protein response. J. Biol. Chem. *284*, 24204–24212.

Vander Heiden, M.G., Cantley, L.C., and Thompson, C.B. (2009). Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science *324*, 1029–1033.

Wang, J., Cao, Y., Chen, Y., Chen, Y., Gardner, P., and Steiner, D.F. (2006). Pancreatic beta cells lack a low glucose and O2-inducible mitochondrial protein that augments cell survival. Proc. Natl. Acad. Sci. USA *103*, 10636–10641.

Wu, S.B., and Wei, Y.H. (2012). AMPK-mediated increase of glycolysis as an adaptive response to oxidative stress in human cells: implication of the cell survival in mitochondrial diseases. Biochim. Biophys. Acta *1822*, 233–247.

Xiang, J., Luo, F., Chen, Y., Zhu, F., and Wang, J. (2014). si-DNMT1 restore tumor suppressor genes expression through the reversal of DNA hypermethylation in cholangiocarcinoma. Clin Res Hepatol Gastroenterol *38*, 181–189.

Yao, J., Zhou, B., Zhang, J., Geng, P., Liu, K., Zhu, Y., and Zhu, W. (2014). A new tumor suppressor LncRNA ADAMTS9-AS2 is regulated by DNMT1 and inhibits migration of glioma cells. Tumour Biol. *35*, 7935–7944.

Zhang, X., Fryknäs, M., Hernlund, E., Fayad, W., De Milito, A., Olofsson, M.H., Gogvadze, V., Dang, L., Påhlman, S., Schughart, L.A., et al. (2014). Induction of mitochondrial dysfunction as a strategy for targeting tumour cells in metabolically compromised microenvironments. Nat. Commun. *5*, 3295.