Glucose restriction induces cell death in parental but not in homeodomain-interacting protein kinase 2-depleted RKO colon cancer cells: molecular mechanisms and implications for tumor therapy

A Garufi1, A Ricci2, D Trisciuoglio3, E Iorio2, G Carpinelli2, G Pistritto4, M Cirone5 and G D’Orazi*1,6

Tumor cell tolerance to nutrient deprivation can be an important factor for tumor progression, and may depend on deregulation of both oncogenes and oncosuppressor proteins. Homeodomain-interacting protein kinase 2 (HIPK2) is an oncosuppressor that, following its activation by several cellular stress, induces cancer cell death via p53-dependent or -independent pathways. Here, we used genetically matched human RKO colon cancer cells harboring wt-HIPK2 (HIPK2+/+) or stable HIPK2 siRNA interference (siHIPK2) to investigate in vitro whether HIPK2 influenced cell death in glucose restriction. We found that glucose starvation induced cell death, mainly due to c-Jun NH2-terminal kinase activation, in HIPK2+/+ cells compared with siHIPK2 cells that did not die. 1H-nuclear magnetic resonance quantitative metabolic analyses showed a marked glycolytic activation in siHIPK2 cells. However, treatment with glycolysis inhibitor 2-deoxy-D-glucose induced cell death only in HIPK2+/+ cells but not in siHIPK2 cells. Similarly, siGlut-1 interference did not re-establish siHIPK2 cell death under glucose restriction, whereas marked cell death was reached only after zinc supplementation, a condition known to re-activate misfolded p53 and inhibit the pseudohypoxic phenotype in this setting. Further siHIPK2 cell death was reached with zinc in combination with autophagy inhibitor. We propose that the metabolic changes acquired by cells after HIPK2 silencing may contribute to induce resistance to cell death in glucose restriction condition, and therefore be directly relevant for tumor progression. Moreover, elimination of such a tolerance might serve as a new strategy for cancer therapy.

Cell Death and Disease (2013) 4, e639; doi:10.1038/cddis.2013.163; published online 23 May 2013

Subject Category: Cancer Metabolism

Tumor cell proliferation and survival are basically maintained by oxygen and nutrients, especially glucose, supplied by the blood. For this reason, angiogenesis is considered closely involved in tumorogenesis.1 Some tumor cells acquire tolerance to glucose starvation (glu stv) which may depend on hypoxia condition.2–4 Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor with constitutive HIF-1β subunit and the HIF-1α subunit stabilized by low intracellular oxygen or genetic alteration. HIF-1 target genes that regulate glucose metabolism include the glucose transporter-1 (Glut-1), as well as multiple enzymes required for glycolysis.5 Homeodomain-interacting protein kinase 2 (HIPK2) is a corepressor protein that regulates the transcription of numerous proteins involved in tumor progression and development.6 We previously reported that HIPK2 represses HIF-1α transcription; thus, HIPK2 depletion induces a pseudohypoxic phenotype with HIF-1α upregulation and angiogenesis that results in increased tumor growth in vivo and in chemoresistance.7–9 This finding parallels the overexpression of HIF-1α in many human cancers, including colon, brain, breast, and so on, which is associated with poor prognosis and failure of tumor treatment.5 Hypoxia and HIF-1α have been found to downregulate HIPK2 in a negative regulatory loop,10,11 whereas zinc treatment has been shown to down-regulate HIF-1α with restoration of HIPK2 activity.12–14 HIPK2 induces cell death by activating p53-dependent and -independent pathways.9,15 HIPK2 activation by DNA damage (for example, ionizing radiation, IR, UV light) or antitumor drugs (for example, cisplatin, adriamycin, roscovitin) phosphorylates p53 at Ser46 with induction of p53 apoptotic function.15–18 HIPK2 participates in the c-Jun NH2-terminal kinase (JNK) activation and apoptosis in p53 null cells.19 Chronic HIPK2 depletion impairs p53 function by inducing p53 protein misfolding that can be reversed by zinc supplementation.20,21 P53 is a zinc-binding transcription factor that needs proper folding for DNA binding and transactivating functions

Received 24.1.13; revised 28.3.13; accepted 08.4.13; Edited by C Munoz-Fino

Keywords: HIPK2; glucose; tumor cell death; autophagy; zinc supplementation

Abbreviations: 1H-NMR, 1H-nuclear magnetic resonance; 2-DG, 2-deoxy-D-glucose; CQ, chloroquine; HIF-1, hypoxia-inducible factor 1; HIPK2, Homeodomain-interacting protein kinase 2; JNK, c-Jun NH2-terminal kinase; LC3, microtubule-associated protein light chain 3

Molecular Oncogenesis Laboratory, Department of Experimental Oncology, Regina Elena National Cancer Institute, Rome, Italy; 2Cell Biology and Neurosciences Department, Istituto Superiore di Sanità (ISS), Rome, Italy; 3Experimental Chemotherapy Laboratory, Regina Elena National Cancer Institute, Rome, Italy; 4Section of Pharmacology, Department of Neuroscience, University “Tor Vergata”, Rome, Italy; 5Department of Experimental Medicine, Istituto Pasteur-Fondazione Cenci Bolognetti, Sapienza University, Rome, Italy and 6Department of Medical, Oral and Biotechnological Sciences, University “G. d’Annunzio”, Chieti, Italy

*Corresponding author: G D’Orazi, Department of Medical, Oral and Biotechnological Sciences, University “G. d’Annunzio”, Via de Vestini, 31, Chieti 66013, Italy. Tel: +39 065 266 2529; Fax: +39 06 418 0526; E-mail: gdorazi@unich.it

© 2013 Macmillan Publishers Limited. All rights reserved. 2041-4889/13

www.nature.com/cddis
for oncosuppressor activity, it also has important roles in the regulation of cellular metabolism in cancer cells. Loss of p53 enhances aerobic glycolysis, resulting in the development of more aggressive tumors, and enhances oxidative pentose phosphate pathway (PPP) flux through p53 protein binding to glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the PPP that has an important role in biosynthesis. Interestingly, the inhibition of G6PD by p53 is independent of transcription and is a cytoplasmic, not nuclear, function of p53, probably attributed to the native conformation of p53.

Autophagy is a degradative process through which damaged organelles and misfolded proteins are targeted for disruption via the lysosomes. In cancer, autophagy may contribute to tumor cell survival. As cancer cells experience higher metabolic demands than normal cells, due to their altered glycolytic metabolism, they may depend more heavily on autophagy for survival. Therefore, inhibition of autophagy may enhance the therapeutic benefits of various cancer therapies.

In the current study, we investigated the effect of HIPK2 depletion in cancer cell response to glucose restriction. HIPK2 silencing impaired RKO colon cancer cell death under limiting glucose availability or under inhibition of glucose metabolism by 2-deoxy-D-glucose (2-DG), compared with HIPK2-proficient cells that instead underwent marked cell death. Zinc supplementation reduced HIPK2 siRNA interference (siHIPK2) cell resistance to glucose deprivation inducing cell death. Moreover, blocking the glu stv-induced autophagy increased HIPK2−/− cell death and re-established siHIPK2 cell death. These findings could be directly relevant to the documented role of HIPK2 as a tumor suppressor, because absence of HIPK2 might confer to tumor cells the metabolic adaptability necessary to survive longer in adverse environment.

Results

1H-NMR analyses detected different metabolic profiles in HIPK2-proficient compared with HIPK2-depleted cancer cells. To evaluate the effect of HIPK2 depletion on cellular bioenergetics, we compared metabolic measurements of human colorectal carcinoma-derived RKO cells that retain HIPK2 (HIPK2+/+) with their isogenic derivatives in which the HIPK2 gene had be stably knocked down by siRNA interference (siHIPK2, with HIPK2 mRNA reduction of about 70%). The siHIPK2 cell line constitutes a bona fide model of tumor progression. Seven independent (biological) replicates of HIPK2−/− and siHIPK2 cells were grown under the same optimal conditions. One-dimensional 1H-nuclear magnetic resonance (1H-NMR) spectra of metabolic extracts were measured and used in the qualitative and quantitative analysis. Representative one-dimensional 1H-NMR spectra of aqueous extracts is shown in Figure 1a with peak assignments. The qualitative analysis of the major variances in the spectra was performed directly by using principal component analysis (PCA), an unsupervised method allowing orthogonal decomposition of variance associated with the analyzed metabolites. As shown in Figure 1b, score plot of the first two principal components, PC1 and PC2, of intracellular metabolites, revealed a clear difference between the two cell lines with a confidence level of 80%. In particular, phosphocholine (PCho, 3.22 p.p.m.), myo-inositol (myo-ino 4.05 p.p.m.), lactate (Lac, 1.33 p.p.m.), and total creatine (tCr 3.04 p.p.m.) were responsible for segregation in two separate cluster in PC1 and PC2 score plot, as shown in 2D loading plot (Figure 1c). Quantitative NMR analyses showed intracellular higher levels in siHIPK2 with respect to HIPK2+/+ cells of relevant metabolites involved in glycolysis, tricarboxylic acid (TCA) cycle, and phosphatidylcholine metabolism (Figure 1d). In particular, siHIPK2 cells showed significant increase of choline-containing metabolites (tCho, P = 0.002), glycine (gly; P = 0.006), and intracellular Lac (P = 0.015) and alanine (Ala; P = 0.007), compared with HIPK2−/− cells (Figure 1d). These altered metabolic profiles might be potential novel indicators of tumor progression, as reported. Moreover, glutamate (P = 0.004) and glutamine (P = 0.007) involved in TCA cycle increased in siHIPK2 cells, while significant decrease (P = 0.007) in creatine plus phosphocreatine (tCr) levels were observed in siHIPK2 compared with HIPK2−/− cells (Figure 1d). Altogether, these data show that chronic HIPK2 depletion strongly affected metabolic profiles of RKO cells, and in particular, the significant increase in Lac, Ala, and gly suggested a marked glycolytic activation.

Silencing of HIPK2 protects tumor cells from glu stv-induced cell death. Glycolytic metabolism promotes proliferation of cancer cells, but it also protects them from cell death induced by growth factor withdrawal or nutrient starvation; therefore, we challenged HIPK2+/+ and siHIPK2 cells with glucose restriction. Cells were grown with normal glucose concentration (25 mM, mock) or in media lacking glucose (glu stv). Cell viability showed time-dependent increase of cell death in HIPK2−/− cells, whereas HIPK2 knockdown significantly protected tumor cells from glu stv-induced cell death (< 0.01; Figure 2a, left panels); as also evidenced macroscopically (Figure 2b, upper panel) and microscopically (Figure 2b, lower panel), where evident signs of cell death were present in HIPK2+/+ cells after 24 h starvation (1d stv), while siHIPK2 cells appeared still alive after 48 h starvation (2d stv). Cell proliferation analysis showed reduced cell growth of glucose-starved HIPK2+/+ cells, in agreement with the cell death induction (Figure 2a, upper/right panel); on the other hand, although siHIPK2 cells did not die in glucose-free condition, they underwent growth arrest (Figure 2a, lower/right panel). Cell death was obtained in HIPK2−/− compared with siHIPK2 cells also with reduced glucose concentration (2.5 mM, 1:10). Extension of the glu stv to 2 days further confirmed the HIPK2 requirement for starvation-induced cell death: substantial numbers of alive cells were observed in the siHIPK2 cells whereas in the HIPK2−/− ones they appeared mostly dead (Figure 2b), suggesting a greater ability of siHIPK2 cells to deal with glucose deprivation. Thus, HIPK2 depletion induced marked lactate production under normal culture condition that did not change during glu stv, compared with HIPK2−/− cells where glu stv significantly decreased lactate production (Figure 2c).

Next, we performed Annexin V/propidium iodide (PI) staining that allows the discrimination of viable cells ( Annexin


Decrease in Cr levels in siHIPK2 compared with HIPK2+/+ and principal components (PC1 and PC2) in HIPK2+/+ siHIPK2 cells. Significant increases in: Lac, P versus for segregation in two separate clusters in PC1 metabolites for different segregation between the two cell lines. In particular, PCho, 3.22 ppm; myo-inositol, 4.05 ppm; Lac, 1.33 ppm; and tCr, 3.04 ppm were responsible for segregation in two separate clusters in PC1 versus PC2 score plot. (d) Score plot of the first two principal components (PC1 and PC2) in HIPK2+/+ (dotted circle) and siHIPK2 (dashed circle) cells. Level of confidence = 90%. (e) 2D loading plot showing the critical metabolites for different segregation between the two cell lines. In particular, PCho, 3.22 p.p.m.; myo-inositol, 4.05 p.p.m.; Lac, 1.33 p.p.m.; and tCr, 3.04 p.p.m. were responsible for segregation in two separate clusters in PC1 versus PC2 score plot. (d) Intracellular concentrations (nmol/10⁶ cells) of 1H-NMR detectable metabolites in HIPK2+/+ and siHIPK2 cells. Significant increases in: Lac, P = 0.015; Ala, P = 0.007; glycine (gly), P = 0.006; tCho, P = 0.002; Glt, P = 0.004; and glutamine, P = 0.007. Significant decrease in tCr levels in siHIPK2 compared with HIPK2+/+ cells (P = 0.007).

Figure 1 1H-NMR analyses of different metabolic profiles in HIPK2-proficient (HIPK2+/+) compared with HIPK2-depleted (siHIPK2) cancer cells. (a) Representative 1H-NMR spectra (400 MHz) of aqueous extract from HIPK2+/+ and siHIPK2 cancer cells. Peak assignments: myo-inositol (myo-inositol), tau (taurine), tCho (total choline-containing metabolites), resonance including choline (Cho), phosphocholine (PCho), glycerophosphocholine (GPCho), tCr (total creatine: creatine plus phosphocreatine), GS (glutathione), Glt (glutamate), Glx (glutamate + glutamine + glutathione), Acetate, glycine (3-hydroxybutyrate), alanine (Ala); and lactate (Lac), p.p.m., parts per million. (b) Score plot of the first two principal components (PC1 and PC2) in HIPK2+/+ (dotted circle) and siHIPK2 (dashed circle) cells. Level of confidence = 90%. (c) 2D loading plot showing the critical metabolites for different segregation between the two cell lines. In particular, PCho, 3.22 p.p.m.; myo-inositol, 4.05 p.p.m.; Lac, 1.33 p.p.m.; and tCr, 3.04 p.p.m. were responsible for segregation in two separate clusters in PC1 versus PC2 score plot. (d) Intracellular concentrations (nmol/10⁶ cells) of 1H-NMR detectable metabolites in HIPK2+/+ and siHIPK2 cells. Significant increases in: Lac, P = 0.015; Ala, P = 0.007; glycine (gly), P = 0.006; tCho, P = 0.002; Glt, P = 0.004; and glutamine, P = 0.007. Significant decrease in tCr levels in siHIPK2 compared with HIPK2+/+ cells (P = 0.007).

V−/PI−), early apoptotic (Annexin V+/PI−), and late apoptotic or necrotic cells (Annexin V+/PI+). As shown in Figures 2d and e, 24 h glu stv increased the percentage of both early (about 7% compared with mock) and late apoptotic or necrotic events (about 36% compared with mock) in HIPK2+/+ cells, whereas it did not induce apoptosis or necrotic cell death in siHIPK2 cells. In agreement, PARP cleavage was observed only in HIPK2+/+ cells 24 h after glu stv, compared with siHIPK2 cells (Figure 2f). Of note, replenishment with full medium, which might mimic angiogenesis in vivo or increased dietary glucose intake, led to a time-dependent increase of siHIPK2 but also of HIPK2+/+ cell death (Supplementary Figure S1). These latter data suggest that siHIPK2 cells and also the few remaining HIPK2+/+ cells were still viable after, respectively, 48 and 24 h glu stv and capable of resuming cell proliferation as soon as nutrient supply was provided. Altogether, these results demonstrate that HIPK2 silencing protected RKO colon cancer cells from glu stv-induced cell death.

JNK activity is engaged in death induced by glucose deprivation. To underline the role of HIPK2 in glu stv-induced cell death, we attempted to analyze pathways known to be modified by HIPK2. Glucose deprivation has been shown to phosphorylate p53 at Ser46, which is a target of HIPK2 kinase activity,16,17 for apoptotic cell death.31 We found that Ser46 was slightly phosphorylated in HIPK2+/+ cells after 24 h glucose restriction and absent in siHIPK2 cells (Supplementary Figure S2), indicative of a late involvement of HIPK2/p53Ser46 in cell death in this setting. HIPK2 has been shown to induce JNK phosphorylation to trigger apoptosis.19 Thus, JNK phosphorylation in HIPK2+/+ after 16 and 24 h glu stv was markedly impaired in siHIPK2 cells (Figure 3a); JNK activity was monitored by c-Jun phosphorylation that was indeed absent in siHIPK2 cells although showed reduced c-Jun expression level (Figure 3a). The role of JNK in glu stv-induced cell death was evaluated by stable transfection of the JNK1-APF mutant (DN-JNK1)32 in RKO-HIPK2+/+ cells (Figure 3b). As shown in Figure 3c, overexpression of the inactivatable DN-JNK1 mutant in HIPK2+/+ cells markedly abolished glu stv-induced c-Jun phosphorylation and impaired cell death (Figure 3d), as also evidenced microscopically (Figure 3e).

HIPK2 is an unstable protein that undergoes degradative ubiquitination in basal condition.9,15 After induction, HIPK2 is temporarily stabilized and its activity may be regulated by caspase-mediated removal of an autoinhibitory domain, resulting in its hyperactivation.33 Here, we found a slight induction of HIPK2 expression level in HIPK2+/+ cells after 8 h glu stv, which decreased in the subsequent time points, suggestive of HIPK2 activation,9,27,33 while siHIPK2 cells...
were negative (Supplementary Figure S2). In addition, chromatin immunoprecipitation (ChIP) showed that the binding of HIPK2 on HIF-1α promoter, as previously shown,7 was strongly increased after 8 h of glu stv (Figure 3f). Altogether, these results demonstrate that JNK activation is one of the signaling pathway involved in triggering cell death in glucose-starving RKO-HIPK2+/+ cells that was abrogated by HIPK2 depletion.

Targeting glycolysis by 2-DG or siGlut-1 does not induce siHIPK2 cell death under glu stv. Targeting aerobic glycolysis is a very promising approach as anticancer treatment. Among the glycolysis inhibitor in preclinical and clinical development is the inactive analog of glucose and hexokinase inhibitor, 2-DG.34,35 Therefore, we addressed the question of whether inhibition of glycolysis could re-establish glucose-starving siHIPK2 cell death. Treatment with 2-DG
ranging between 4 and 12 mM for 48 h induced marked HIPK2+/+ cell death, compared with siHIPK2 cells that did not die (Figure 4a), as also evidenced microscopically (Figure 4b). Replenishment of cell cultures with full medium increased cell number in both cell lines (Figures 4b and c + rec), although it was more marked in siHIPK2 cells (Figure 4c + rec). Cell proliferation was also assessed by western immunoblotting of cyclin B1, which, in mammalian cells, has a critical role in the progression through mitosis.36 As shown in Figure 4c: lower panel, cyclin B1 almost completely disappeared after 2-DG treatment in both cell lines; however, replenishment of cell cultures with full medium (+ rec) efficiently restored cyclin B1 expression in both cell lines. These data indicate that HIPK2+/+ cells were more sensitive to cell death induced by glicolysis inhibition compared with siHIPK2 cells. However, both cell lines were capable of resuming cells’ proliferation after glucose supply replenishment.

Next, we attempted to evaluate Glut-1 expression, as increased Glut-1 and likely of glycolytic flux enhances, rather than impairs, cell tolerance to low glucose availability.37 We found Glut-1 upregulation in siHIPK2 cells compared with HIPK2+/+ cells both at mRNA and protein levels (Figure 4d), which was somehow expected, given that Glut-1 is both transcriptionally repressed by p5338 and activated by HIF-1,5 being both molecules, respectively, inhibited and activated by HIPK2 depletion.7,20 Then, we inhibited Glut-1 expression by transient transfection with specific siRNA (Figure 4e: inset, about 60% Glut1 mRNA reduction compared with control). However, Glut-1 silencing was not sufficient to induce siHIPK2 cell death under glucose deprivation (Figure 4e), suggesting that additional pathway other than Glut-1 upregulation might be involved in resistance to metabolic stress-induced cell death.

Zinc supplementation induces cell death in glucose-starved siHIPK2 cells. Next, we attempted to modify the siHIPK2 cell response to glucose deprivation by using zinc supplementation, which inhibits the HIF-1 activity and reverses p53 misfolding.13,20,21 Zinc treatment greatly
decreased the level of lactate production by glucose-starved siHIPK2 cells (Figure 5a) that correlated with significant increase of siHIPK2 cell death (Figure 5b). Similar siHIPK2 cell death was obtained with 2-DG in combination with zinc, whereas zinc treatment alone had almost no effect on cell viability (Figure 5b). Annexin V/PI staining confirmed that zinc supplementation in condition of glu stv induced about 55% of late apoptotic/necrotic cells (Annexin V⁻, PI⁺) (Figures 5c and d). Notably, no induction of early apoptosis (Annexin V⁻, PI⁻) was observed in the same experimental condition, and <10% of cell death was evidenced by Annexin V staining in both glucose-starved or zinc-treated siHIPK2 cells (Figure 5d). These data suggest that zinc treatment could re-establish cell death response in glucose-starving siHIPK2 cells.

Autophagy contributes to cell resistance to nutrient deprivation. Autophagy contributes to the tolerance to nutrient deprivation in colorectal cancer cells.⁴⁹ We evaluated the expression of microtubule-associated protein light chain 3 (LC3) protein that, after conversion from LC3-I to its autophagosome membrane-associated lipidated form LC3-II, is considered a cellular readout of autophagy.⁴⁰ Figure 6a shows LC3 induction upon glu stv in both cell lines; treatment with early inhibitor of autophagosome formation 3-methyladenine (3-MA)⁴⁰ did not dramatically reduce glu stv-induced LC3; on the contrary, treatment with inhibitor of autophagic protein degradation, chloroquine (CQ),⁴⁰ strongly increased LC3-II expression under glu stv compared with CQ treatment alone, in both cell lines (Figure 6a), indicating the absence of defective autophagy. Autophagy is a key function of the lysosomal compartment⁴¹ and CQ has been shown to disrupt lysosomal structure and function preventing effective autophagy degradation, leading to the accumulation of ineffective autophagosomes and cell death in cells reliant on autophagy for survival.⁴² We found that CQ significantly increased cell death in glucose-starving siHIPK2 cells (Figure 6b) and slightly increased also glucose-starving HIPK2+/+ cell death, providing evidence that autophagy can contribute to tumor cell survival in this setting. Furthermore, Annexin V/PI staining showed that CQ increased the percentage of glu stv-induced late apoptotic/necrotic cell death that was not further increased by zinc supplementation (Figure 6c); on the other hand, the lack of cell death in glucose-starving siHIPK2 cells was reversed by adding CQ or zinc single treatments, and further increased by combining CQ and zinc supplementation (Figure 6c). These results suggest that autophagy is induced by glu stv, and that its
inhibition slightly increased cell death in sensitive RKO $^{+/+}$ while re-established cell death after glu stv particularly in resistant siHIPK2 cells that could be further improved by zinc supplementation.

We finally analyzed JNK phosphorylation and found that autophagy inhibition did not reverse JNK inhibition in siHIPK2 cells (Figure 7a), suggesting the modulation of additional pathways leading to cell death. In previous studies, we found that HIPK2 depletion induces constitutive Akt phosphorylation, which contributes to cell death resistance. Akt regulates many biological processes, such as proliferation, apoptosis and growth, and is involved in tumor progression. Here, we found that Akt phosphorylation was markedly inhibited by CQ treatment in glucose-starving siHIPK2 cells (Figure 7b). Interestingly, also zinc supplementation reduced (p)-Akt in glucose-starving siHIPK2 cells (Figure 7b). These results indicate that autophagy is activated by glu stv in this setting and that autophagy inhibition abrogated signaling pathway of apoptosis resistance such as Akt that correlated with increased cell death.

**Discussion**

In this study we show that HIPK2 depletion changed RKO cancer cell response to glucose restriction; thus, whereas HIPK2 $^{+/+}$ cells underwent death HIPK2-depleted cells did not. What was the mechanism of such resistance to metabolic stress? Solid tumors are dependent on glucose, but are
HIPK2 regulates tumor survival to metabolic stress
A Garufi et al

Figure 6 Inhibition of autophagy increased cell death. (a) Equal amount of total cell extracts from HIPK2+/− and siHIPK2 cells left untreated or treated for 24 h with glucose-free medium (glu stv) alone or in combination with autophagy inhibitors 3-MA (5 mM) or CQ (25 μM) were assayed by western immunoblotting of LC3-I/II protein level. β-actin was used as protein loading control. A representative result of three independent experiments is shown. (b) HIPK2+/− and siHIPK2 cells were cultured for 24 h in glucose-free (glu stv) medium alone or in combination with CQ (25 μM), before trypan blue staining was performed. The result is the mean of three independent experiments performed in duplicate ± S.D. *P = 0.001. (c) siHIPK2 cells were cultured in glucose-free medium (glu stv) alone for 48 h or in combination with ZnCl2 (100 mM) and CQ (25 μM) before cell death analysis was performed by Annexin V/PI staining and evaluated by immunocytochemistry. Two hundred cells were counted in duplicate in two independent experiments and plotted as percentage compared with control ± S.D.

Figure 7 Akt phosphorylation is inhibited by CQ and zinc treatments. (a) Equal amount of total cell extracts from siHIPK2 cells left untreated or treated for 24 h with glucose-free medium (glu stv) alone or in combination with autophagy inhibitors 3-MA (5 mM) or CQ (25 μM) were assayed by western immunoblotting of Akt(Ser473) phosphorylation and total Akt levels. β-actin was used as protein loading control. A representative result of two independent experiments is shown.

HIPK2 is an oncosuppressor and a corepressor molecule that can stimulate gene expression upon phosphorylation of several transcription factors including p53,6,16,17 and repress transcription of several genes involved in development, apoptosis, and tumor progression such as HIF-1α.7 HIF-1 enhances glycolysis by inducing the expression of genes encoding glycolytic enzymes and glucose transporters, such as Glut-1,5 which was indeed increased in siHIPK2 cells. Similarly, p53 has important roles in the regulation of glycolysis and oxidative phosphorylation in cancer cells.23–25 Loss of p53 enhances aerobic glycolysis, resulting in the development of a more aggressive tumor.24 Given the multiplicity of molecules modulated by HIPK2, one cannot assume that only one pathway might be involved. One of the mechanisms involved in late (24 h) apoptosis/necrosis upon glu stv was p53Ser46 phosphorylation that was abolished in siHIPK2 cells. At earlier time point (16 h), JNK was activated in HIPK2+/− cells upon glucose restriction and its activity was markedly impaired by HIPK2 depletion. JNK1 has important roles in triggering apoptosis in response to cellular stress, and its activation by HIPK2 has been previously reported.9 However, the role of HIPK2 activation in JNK activation upon glucose restriction needs to be further elucidated because several signaling kinases may regulate survival during glu stv.30 To render cancer cells sensitive to glucose depletion may potentially provide an effective strategy for cancer intervention. However, Glut-1 silencing or targeting glucose metabolism with 2-DG did not induce siHIPK2 cell death in this experiment.

generally glucose-deprived owing to poor vascularization. Therefore, cancer cells undergo metabolic reprogramming to better cope with nutrient deprivation.4,45 A distinct trait of the cancer metabolism is the unscheduled activation of glycolytic enzymes in normoxic conditions, indicated by increased cellular glucose uptake, hyperglycolysis, and lactate production.46 Coupling of 1H-NMR metabolic analyses to biochemical experiments revealed that HIPK2 depletion led to increased lactate production and glycolytic metabolism that likely contributed to adaptation to hypoglycemic environment.
setting, suggesting the activation of additional pathways. Zinc supplementation inhibited siHIPK2 lactate production that correlated with increased cell death, likely by reversing p53 misfolding and by inhibiting HIF-1 activity.

Cancer cells encountering limited nutrient supplies in the growing tumor may exploit autophagy for survival; thus, autophagy contributes to the tolerance to nutrient deprivation in colorectal cancer cells. In agreement, autophagy was induced upon glu stv in both HIPK2 and siHIPK2 cells, and blocking autophagy increased glucose-starving HIPK2 cell death and re-established siHIPK2 cell death. What was the mechanism that re-established glucose-starving siHIPK2 cell death upon autophagy inhibition? Autophagy inhibition markedly impaired Akt phosphorylation in glucose-starving siHIPK2 cells, which could partly support re-establishment of siHIPK2 cell sensitivity to metabolic stress-induced cell death. The inhibition of autophagy has been shown to enhance the therapeutic benefits of various cancer therapies; however, strategies to induce autophagic cell death (that is, metformin) have also been proposed to inhibit tumor progression; therefore, modulation of autophagy for cancer therapy is still somewhat controversial.

In cancer, the importance of HIPK2 has been elucidated by HIPK2 knock-out mice that showed increased sensitivity to develop skin cancer after treatment with carcinogens. Moreover, loss of HIPK2 heterozygosity occurs in radiation-induced tumors in mouse cells and in human papillary thyroid cancers, underlining the important activity of HIPK2 as tumor suppressor. HIPK2 may be inhibited in tumors by several mechanisms such as hypoxia-induced protein degradation, gene mutation, or oncogene-mediated cytoplasmic localization. Although glucose deprivation has been shown to activate metabolic and signaling pathways inducing cell death, molecular alterations leading to HIPK2 impairment could counteract this effect increasing tumor resistance to metabolic stress. Glucose restriction can be obtained by reduced angiogenesis or by low carbohydrate diet that indeed could counteract this effect increasing tumor resistance to metabolic stress. Glucose restriction can be obtained by reduced angiogenesis or by low carbohydrate diet that indeed has been shown to slow tumor growth. On the contrary, higher dietary glycemic load associates with an increased risk of recurrence and mortality in stage III colon cancer patients. Of recurrence and mortality in stage III colon cancer patients.54

Chemicals. ZnCl2 was dissolved in DMSO, and used at 100 µM; 3-MA was dissolved in DMSO, and used at 5 mM. 2-OG was dissolved in DMSO and used at concentrations ranging between 4 and 12 mM. CO2 was dissolved in DMSO and used at 25 µm. All chemicals were from Sigma-Aldrich (St. Louis, MO, USA). ZnCl2, 3-MA, and CO2 were added to culture media 1 h before treatments.

Measurement of cell proliferation and viability. Cells numbers were determined in duplicate at different time points. Cell counts were performed using a hemocytometer by adding trypan blue to equal volume of cell suspension. The percentage of cell viability, as blue/live cells, was assayed by counting 200 cells per well three times. Bright field images were taken in a Nikon Eclipse TS100 microscope equipped with a Nikon ELWD camera (Nikon Instruments Europe BV, Amsterdam, Netherlands). For survival assay, 2 × 10^5 cells were plated on 60-mm dishes and 24 h later treated with glucose-free medium. Death-resistant cells were stained with crystal violet 24–48 h later.

Annexin V/PI staining. Apoptosis was quantified by cytometric analysis staining cells simultaneously with FITC-Annexin V and the non-vital dye PI (Immunological Sciences, Rome, Italy), following the manufacturer’s instruction. At the end of incubation with the respective reagents, samples were analyzed with a FACScan instrument (Becton Dickinson Europe Holdings SAS - Le Pont De Claix, France). About 30,000 events were acquired and gated using forward scatter and side scatter to exclude cell debris. Bivariate analysis allows the discrimination of intact cells (FITC^-PI^-), early apoptotic (FITC^-PI^+), and late apoptotic or necrotic cells (FITC^PI^+). The percentage of Annexin V+ cells relatives to the different analyses was calculated using CellQuest software (Becton Dickinson). FITC-conjugated Annexin V/PI staining (Immunological Sciences) was also visualized by Olympus BX53 fluorescence microscope (Olympus Italia Srl, Milan, Italy).

Measurement of lactate. Cells were seeded onto 35-mm tissue-culture dishes, and 24 h later washed three times in PBS before adding glucose-free media for 16 h. Lactate secretion of triplicates was measured by using the Lactate Colorimetric Assay Kit II (BioVision Research Products, Mountain View, CA, USA), according to the manufacturer’s instruction.

Western blotting. Total cell extracts were prepared by incubation in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 150 mM KCl, 1 mM dithiothreitol, 1% Nonidet P-40) and a mix of protease and phosphatase inhibitors (Sigma-Aldrich Chemical Company, Sigma-Aldrich). Samples were denatured in SDS sample buffer. Total proteins were separated by loading 20–60 µg of total cell lysates on denaturing 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 5% nonfat dry milk in PBS and incubated with primary antibodies that recognize: cyclin B1, G-actin (Santa Cruz Biotechnology, CA, USA), PARP (cleavage site-214/215, Millipore), LC3B (Sigma-Aldrich), HA (Roche S.p.A, Milan, Italy), p-JNK, JNK, p-c-Jun, c-Jun, p-Ser46, p-Akt (Ser473), p-ATK (Ser473), (p)-Akt, (p)-Ser46, (p)-Akt (Ser473), actin (all from Cell Signaling Technologies, Danvers, MA, USA). HIPK2 (kindly provided by ML Schmitz, Justus-Liebig University, Giessen, Germany), β-actin (Calbiochem, San Diego, CA, USA), and tubulin (Immunological Sciences). Secondary antibody conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA) was used to detect primary antibodies, and enzymatic signals were visualized by chemiluminescence (ECL kit, Amersham Biosciences, Freiburg, Germany).

Materials and Methods

Cell culture and treatments. Human RKO colon cancer (carrying wtp53; HIPK2^-/-^-), the RKO stably interfered for HIPK2 (siHIPK2), and the human lung cancer H1299 (p53 null) cells were routinely maintained in DMEM (Life Technology-Invitrogen) medium containing 10% heat-inactivated fetal bovine serum (Life Technology-Invitrogen, Carlsbad, CA, USA). 100 units/ml penicillin/streptomycin, and glutamine, in 5% CO2 humidified incubator at 37°C. For glu stv, cells were transferred to corresponding medium without glucose. Glucose-free DMEM was supplemented with full medium to achieve the desired concentration of glucose (full DMEM contains 25 mM glucose and was diluted in glucose-free DMEM 10-fold to achieve concentration of 2.5 mM).

Human RKO colon cancer (carrying wtp53; HIPK2^-/-^-), the RKO stably interfered for HIPK2 (siHIPK2), and the human lung cancer H1299 (p53 null) cells were routinely maintained in DMEM (Life Technology-Invitrogen) medium containing 10% heat-inactivated fetal bovine serum (Life Technology-Invitrogen, Carlsbad, CA, USA). 100 units/ml penicillin/streptomycin, and glutamine, in 5% CO2 humidified incubator at 37°C. For glu stv, cells were transferred to corresponding medium without glucose. Glucose-free DMEM was supplemented with full medium to achieve the desired concentration of glucose (full DMEM contains 25 mM glucose and was diluted in glucose-free DMEM 10-fold to achieve concentration of 2.5 mM).
Transfection and plasmids. Cells were transfected by using the LipofectaminePlus method (Invitrogen), according to the manufacturer’s specifications. The amount of plasmid DNA was equalized in each sample by supplementing with empty vector, and transfection efficiency was visualized with the use of a co-transfected GFP expression vector. For stable transfection, $4 \times 10^6$ RKO cells were transfected with the nonphosphorylatable (competitive inhibitor) HA-JNK1-APF (dominant negative, HA-JNK1) (kindly provided by Lynn E. Heasley, University of Colorado, Aurora, CO). Forty-eight hours after transfection, cells underwent selection with geneticin G418 (1 mg/ml). G418-resistant cells were pooled as mixed population 2 weeks later.

siRNA interference. Cells were plated at semiconfluent in 35-mm dishes the day before transfection. Control-siRNA and siG1-1 (Dharmacon, Thermo-Scientific, Fisher Scientific SAS, Illkirch Cedex, France) were transfected overnight using LipofectaminePlus reagent (Invitrogen).

ChIP assay. H1299 cells were plated at subconfluence in 150-mm petridishes. The day after plating, cells were washed in PBS three times and glucose-free medium was added for 8 h. Protein complexes were cross-linked to DNA in living cells grown to 60–70% confluence were trypsinized 24 h after culture medium change, counted, and assessed for viability of Professor Rita Levi Montalcini. May her outstanding contribution to science be an immortal legacy and an example for us all.

Conflict of Interest The authors declare no conflict of interest.

Acknowledgements. This study was supported by grant form Associazione Italiana per la Ricerca sul Cancro (AIRC, IG 11377, to GD) and My First AIRC grant (MAGF 11502, to DT). We are greatly indebted to Drs. L. DI Renzo and G Bossi for their helpful advice, stimulating discussion and for sharing reagents. GD dedicates this study to the memory of Professor David Givol who with a combination of intelligence, passion, vision, and organization was a precious and challenging collaborator and an unforgettable friend. We also dedicate this study to the memory of Professor Rita Levi Montalcini. May her outstanding contribution to science be an immortal legacy and an example for us all.

Author contributions GD designed the research; AG, AR, DT, EDI, GC, MC analyzed the data; and GD wrote the paper.

1. Folkman J. Can mosquito tumor vessels facilitate molecular diagnosis of cancer? Proc Natl Acad Sci USA 2001; 98: 398–400.

2. Izuishi K, Kato K, Ogura T, Kinoshita T, Esumi H. Remarkable tolerance of tumor cells to nutrient deprivation: possible new biochemical target for cancer therapy. Cancer Res 2000; 60: 6201–6207.

3. Esumi H, Izuishi K, Kato K, Hashimoto K, Kurashima Y, Kishimoto A et al. Hypoxia and nitric oxide treatment confer tolerance to glucose starvation in a 5-AF-activated protein kinase-dependent manner. J Biol Chem 2002; 277: 32791–32798.

4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646–674.

5. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 2010; 29: 625–634.

6. Rinaldo C, Prodosmo A, Siepi F, Soddu S, HIPK2: a multitailed partner for transcription factors in DNA damage response and development. Biochem Biophys Acta 2007; 1765: 411–418.

7. Nardinocchi L, Puca R, Guidoni D, Belloni AS, Bossi G, Michels C et al. Transcriptional regulation of hypoxia-inducible factor 1 alpha by HIPK2 suggests a novel mechanism to restrain tumor growth. Biochim Biophys Acta 2009; 1793: 368–377.

8. D’Orazi G, Sculli MG, Di Stefano V, Riccomini C, Frattini M, Falcioni R et al. Homeodomain-interacting protein kinase-2 restrains cystic phospholipase A2-dependent proapoptotic E2 generation in human colorectal cancer cells. Clin Cancer Res 2006; 12: 735–741.

9. D’Orazi G, Rinaldo C, Soddu S. Updates on HIPK2: a resourceful oncopressor for clearing cancer. J Exp Clin Cancer Res 2012; 31: 63.

10. Calzado MA, de la Vega L, Moller A, Bowtell DD, Schmitz ML. An inducible autoregulatory loop of HIPK2 and Sihl2 at the apex of the hypoxic response. Nat Cell Biol 2009; 11: 85–91.

11. Nardinocchi L, Puca R, D’Orazi G. HIPK2 antagonizes p53-mediated apoptosis by triggering HIPK2 degradation. Aging (Albany NY) 2011; 3: 33–43.

12. Nardinocchi L, Puca R, Sacchi A, Rechavi G, Givol D, D’Orazi G. Targeting hypoxia in cancer cells by restoring homeodomain-interacting kinase-2 and p53 activity and suppressing HIPK1-alpha. PLoS ONE 2009; 4: e8189.

13. Nardinocchi L, Pantisano V, Puca R, Porru M, Aiello A, Grasselli A et al. Zinc downregulates HIPK1 and inhibits its activity in tumor cells in vitro and in vivo. PLoS ONE 2010; 5: e10548.

14. Sheller M, Simon AJ, Rechavi G, Domany E, Givol D, D’Orazi G. Genome-wide analysis discloses reversal of the hypoxia-induced changes of gene expression in colon cancer cells by zinc supplementation. Oncotarget 2011; 2: 1191–1202.

15. Puca R, Nardinocchi L, Givol D, D’Orazi G. Regulation of p53 by HIPK2: molecular mechanisms and therapeutical implications in human cancer cells. Oncogene 2010; 29: 4378–4387.

16. D’Orazi G, Cecchinelli B, Bruno T, Mani I, Higashimoto Y, Saijo S et al. Homeodomain-interacting protein kinase-2 phosphorylates p53 at Ser46 and mediates apoptosis. Nature Cell Biol 2002; 4: 11–19.

17. Hofmann TG, Möller A, Sima H, Zentgraf H, Taya Y, Droge W et al. Regulation of p53 activity by its interaction with homeodomain-interacting protein-2. Nature Cell Biol 2002; 4: 1–10.

18. Puca R, Nardinocchi L, Sacchi A, Rechavi G, Givol D, D’Orazi G. HIPK2 modulates p53 activity towards pro-apoptotic transcription. Mol Cancer 2009; 14: 85.

19. Hofmann TG, Stoßberg N, Schmitz ML, Will H. HIPK2 regulates transforming growth factor-β-induced c-Jun NH2-terminal kinase activation and apoptosis in human hepatoma cells. Cancer Res 2003; 63: 8271–8277.

20. Puca R, Nardinocchi L, Gal H, Rechavi G, Amargiolo N, Domany E et al. Reversible dysfunction of wild-type p53 following homeodomain-interacting protein kinase-2 knockdown. Cancer Res 2008; 68: 3707–3714.

21. Puca R, Nardinocchi L, Bossi G, Sacchi A, Rechavi G, Givol D et al. Restoring wt p53 activity in HIPK2 depleted MCF7 cells by modulating methionine and zinc. Exp Cell Res 2009; 315: 67–75.
HippK2 regulates tumor survival to metabolic stress
A Garufi et al

Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)