Published in final edited form as:

Biochem Biophys Res Commun. 2010 April 16; 394(4): 1075–1081. doi:10.1016/j.bbrc.2010.03.129.

NUCLEOCYTOPLASMIC SHUTTLING OF HEXOKINASE II IN A CANCER CELL

Catherine L. Neary^{a,#} and John G. Pastorino^a

^a Department of Molecular Biology, School of Osteopathic Medicine, University of Medicine and Dentistry of New Jersey, Stratford, New Jersey 08084

Abstract

In yeast, the hexokinase type II enzyme (HXKII) translocates to the nucleus in the presence of excess glucose, and participates in glucose repression. However, no evidence has suggested a nuclear function for HXKII in mammalian cells. Herein, we present data showing nuclear localization of HXKII in HeLa cells, both by immunocytochemistry and subcellular fractionation. HXKII is extruded from the nucleus, at least in part, by the activity of the exportin 1/CrmA system, as demonstrated by increased nuclear expression and decreased cytoplasmic expression after incubation with leptomycin B, a bacterially-derived exportin inhibitor. Furthermore, cytoplasmic localization of HXKII is dependent on its enzymatic activity, as inhibiting HXKII activity using 2-deoxy-D-glucose (2DG) increased nuclear localization. This effect was more significant in cells incubated in the absence of glucose for 24 hours prior to addition of 2DG. Regulated translocation of HXKII to the nucleus of mammalian cells could represent a previously unknown glucose-sensing mechanism.

Keywords

Hexokinase 2; nucleocytoplasmic transport; exportin; 2-deoxyglucose

INTRODUCTION

The Warburg effect describes the particular reliance of cancer cells on glycolysis for energy. Increased glycolysis and acid resistance have been posited to be an essential part of carcinogenesis, conferring a significant growth advantage, as well as promoting typical tumor progression [1]. Cancer detection was revolutionized by the use of positron electron tomography and ¹⁸fluorodeoxyglucose, a glucose analog that is trapped in cells due to phosphorylation by hexokinases [2,3]. Hexokinases (HXK; for review, see [4]) have four isoforms: HXKI, found in all tissues but at high levels in the brain [5]; HXKII, found in skeletal muscle, the mammary gland, the liver and in adipose tissue [6,7]; HXKIII, found at low levels in all tissues but at high levels only in specific cell types within certain tissues [8]; and HXKIV, also known as glucokinase (GK), found primarily in the liver and pancreas, specifically in beta cells, but also in the hypothalamus and gut [9].

[#]Address correspondence to: Catherine L. Neary, Department of Molecular Biology, 2 Medical Center Drive SC316, Stratford, New Jersey 08084, Tel: 856-566-6373; Fax: 856-566-6291; nearycl@umdnj.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

In humans, GK is a known glucose sensor [10] that affects not only glycolysis and glycogen production, but also insulin secretion by pancreatic beta cells. In fact, substantial evidence has provided a very strong link between GK expression and the expression of many different glucose-responsive genes [11], to the point that this is considered a well-established mechanism of action of this enzyme in the liver and pancreas [9]. GK translocates to the nucleus of liver cells when glucose levels are low [12], and human GK has a nuclear export sequence [13] that is recognized by human exportins. Regulation of nuclear import and export is responsible for the regulation of multiple pathways [14,15]. Passage of large proteins through the proteinaceous nuclear pores is accomplished by binding to members of the importin family of proteins, which also have members that mediate nuclear export [14,16].

HXXKII is overexpressed in many cancer cells [17], which could be an adaptation to increased glycolysis or a necessary genetic modification occurring during carcinogenesis [1]. Binding of HXXKII to the mitochondria, through attachment to the voltage-dependent anion channel (VDAC) [18], protects against apoptosis in cancer cells [19], while detaching HXXKII from VDAC, promotes apoptosis [20], suggesting that increased glycolysis may not be the only effect of increased hexokinase expression. In yeast, HXXKII participates in glucose repression [21], in partnership with Mig1 protein [22,23]. As part of this mechanism, HXXKII translocates to the nucleus, where it binds in a complex with Mig1. Yeast HXXKII is exported through Xpo1, a member of the importin family homologous to human CRM1 (chromosome region maintenance) [24]. However, there is no previously published evidence demonstrating nucleocytoplasmic shuttling of HXXKII in a regulated manner in mammalian cells.

Establishing the localization of the hexokinase family members under different metabolic conditions could provide vital clues as to their altered functions under these conditions. Herein, we examine the localization of HXXKII by immunocytochemistry and subcellular fractionation. To characterize the movement of HXXKII in the cell, we added leptomycin B (LMB) [25], an inhibitor of CRM1, and a glucose analog, 2-deoxy-D-glucose (2DG), that is phosphorylated by HXXKII but cannot proceed through glycolysis, inhibiting HXXKII by its accumulation.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Two different anti-HXXKII antibodies from Cell Signaling Technologies (Danvers, MA) were used: C64G5 for immunocytochemistry; and the polyclonal HXXKII antibody for Western blotting. Secondary antibodies conjugated to fluorophores were purchased from Invitrogen (Carlsbad, CA), or to horseradish peroxidase (HRP), from Millipore (Billerica, MA).

Cell Culture and Treatments

HeLa cells obtained from American Type Culture Collection (Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin in a humidified atmosphere with 5% CO₂/95% air. For most experiments, cells were plated at least 24 h prior to treatment. For immunocytochemistry, cells were grown in 35 mm dishes on coverslips (Fisher Scientific, Fairlawn, NJ) sterilized with ultraviolet light for 5 min.

Prior to performing the staining experiments, LMB (EMD Biosciences, Gibbstown, NJ) toxicity was tested at three different doses; 20 ng/mL was chosen as it did not kill the cells within 24 h. To inhibit HXXKII activity, 10 mM 2DG was added. For cell culture in the absence of glucose, medium was prepared using Dulbecco's Modified Eagle's Medium Base. Phenol red (11 mg/L) was added, and glucose, fructose or galactose was added to a final concentration

of 4.5 g/L. The medium was supplemented with 15 mM HEPES, 1 mM sodium pyruvate and 2 mM L-glutamine (Mediatech, Manassas, VA), then the pH brought to 7.4 using 1 N sodium hydroxide (Fisher). The medium was sterilized using a 500 mL filter system with a 0.22 μ m filter (Corning, Corning, NY), and supplemented with penicillin-streptomycin. Prior to use in experiments, 10% FBS was added.

Immunocytochemistry

Following the treatment period, cells were washed twice with phosphate-buffered saline (PBS; Mediatech), and then the coverslips were removed from the dishes. All subsequent steps were done on the bench-top, at room temperature. The cells were fixed with 3.7% formaldehyde in PBS for 5 min and permeabilized with 0.2% Triton X-100 for 20 min. Non-specific binding was blocked with 5% goat serum and 1% bovine serum albumin (BSA) in PBS for 30 min. Following another wash, the cells were incubated for 1 h in the primary antibody solution (1:1600 rabbit anti-HXKII and 1% BSA in PBS). The cells were incubated in the secondary antibody solution (1:1000 Alexa488-conjugated goat anti-rabbit IgG and 1% BSA in PBS) for 1 h in the dark. To detect the nuclei, coverslips were incubated with 2 μ g/mL Hoechst 33258 (Invitrogen) in PBS for 15 minutes. The coverslips were mounted on glass slides (Fisher) using 80% glycerol in PBS. The stained cells were viewed on an Olympus IX51 microscope (Olympus, Center Valley, PA). Images were captured using a Hamamatsu C10600-10B camera (Hamamatsu, Bridgewater, NJ) and SlideBook software (Intelligent Imaging Innovations, Denver, CO). For quantification, each cell in the acquired images was selected, with the nucleus selected as a separate area, and the intensity determined by the SlideBook software. After subtracting the background, the nuclear intensity was divided by the whole cell intensity, to express the nuclear intensity as a percentage of the cellular staining. These percentages were then plotted as a distribution, to best reflect the variation found within populations of cells.

Nuclear Extraction

Nuclear extraction was performed using the Nuclear Extract kit from Active Motif (Carlsbad, CA) according to the manufacturer's instructions with modifications. All steps were performed on ice. Cells plated in 100 mm dishes were washed once with ice-cold PBS, then once with ice-cold PBS containing HALT protease inhibitor cocktail (Pierce, Rockford, IL). The cells were scraped into 1 mL ice-cold PBS-HALT, centrifuged at 1300 $\times$ g for 10 min at 4°C, and the cytoplasmic fraction isolated using the kit. Lysis of the nuclear fraction was performed for 45 min, longer than the 30 min recommended in the kit protocol. Whole cell lysates were prepared by washing a 35 mm dish of cells with ice-cold PBS-HALT, then scraping into 500 μ L ice-cold PBS-HALT. The cells were centrifuged at 1300 $\times$ g for 10 min at 4°C. The supernatant was removed by aspiration and discarded. Prior to the protein assay, the pellets were resuspended in 50 μ L Mammalian Protein Extraction Reagent (Pierce) containing HALT protease inhibitors. As a positive control, a HeLa nuclear extract was purchased from Active Motif.

Protein concentrations were determined using the Coomassie Plus reagent (Pierce) microplate assay, according to the manufacturer's instructions, reading the absorbances on a Synergy HT-1 plate reader (BioTek Instruments, Winooski, VT) using KC4 software (BioTek Instruments). To prepare the samples for separation on a gel, sufficient Laemmli sample buffer (Bio-Rad, Hercules, CA; supplemented with 54 mg/mL dithiothreitol) was added to a final volume of 25 μ L/well. The samples were boiled for 5 min prior to loading on the gel.

Western Blotting

Protein samples (40 μ g/well) were loaded onto Novex 10% Tris/Glycine precast gels in the XCell II module with MagicMark XP (all from Invitrogen) as the molecular weight marker. The ImmunoBlot PVDF membranes (Bio-Rad) were prepared for blotting according to the

manufacturer's protocol. The gels were transferred in the XCell II Blot module (Invitrogen) according to the manufacturer's instructions for 2 h at room temperature in transfer buffer (25 mM Tris base, 192 mM glycine, 0.05% sodium dodecyl sulfate; Fisher), with distilled deionized water filling the tank. To confirm that the transfer was effective, the blots were stained with Ponceau S prior to blocking. The membrane containing the transferred proteins was blocked for 1 h at room temperature in 3% Carnation Instant Dry Milk (Nestle, Solon, OH) in Tris-buffered saline containing 0.05% Tween 20 (TBST). The blot was incubated in primary antibody solution (1:1000 rabbit anti-HXKII in 0.5% milk in TBST) overnight at 4°C with rocking in an Enviro-Genie SI-1200 (Scientific Industries, Bohemia, NY). After washing in TBST, the blot was incubated in secondary antibody solution (1:10000 goat anti-rabbit HRP in 0.5% milk in TBST) for 1 h at room temperature. The secondary antibody detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). The blot was visualized in the BioSpectrum Multi-Spectrum Imaging System (UVP, Upland, CA) using the manufacturer-provided software. The resulting images were cropped, rotated if necessary, and inverted (to produce black bands on a white background) using Photoshop software (Adobe, San Jose, CA). If necessary, blots were stripped using Restore Plus (Pierce) according to the manufacturer's instructions.

Immunoprecipitation

Immunoprecipitation was performed using the Nuclear Complex Co-IP kit (Active Motif) according to the manufacturer's protocol. Samples (350 µg/reaction diluted in IP Buffer from the Active Motif kit) were pre-cleared using Protein G Agarose beads from the Protein G Immunoprecipitation kit (Sigma). After centrifugation to remove the beads, the samples were incubated overnight at 4°C, with mixing by rotation, with the same HXKII antibody used for Western Blotting (1:50 dilution) in columns from the Sigma kit. Fresh Protein G Agarose beads were added the following day, and the resulting solution was incubated overnight at 4°C, with mixing by rotation. The samples were then centrifuged at 12000×g for 30 s at 4°C. The column (containing the beads) was washed three times with IP wash buffer containing 1 mg/mL BSA (from the Active Motif kit), then washed three times with IP wash buffer without BSA. The beads were resuspended in Laemmli Buffer and boiled for 5 min. After cooling, the column was centrifuged at 12000×g for 30 s, and the effluent loaded on a gel for Western blotting as described above.

RESULTS AND DISCUSSION

Hexokinase II is localized in the nucleus of HeLa cells

Immunocytochemistry revealed the presence of HXKII in the nucleus of HeLa cells (Fig. 1A). The left panel shows the distribution of HXKII in the cell. The nuclear staining is even, with no distinguishable pattern suggesting association with particular nuclear structures. The cytoplasmic staining, in contrast, is distinctly punctate, suggesting association with specific cytoplasmic structures, most likely the mitochondria. An overlay of DAPI staining with the HXKII staining reveals colocalization in the nuclear region, confirming that the staining is nuclear (Fig. 1A, right panel). To further confirm the nuclear localization of HXKII in these cells, the cells were fractionated and the cytoplasmic and nuclear extracts separated by SDS-PAGE, and subjected to Western blotting (Fig. 1B). HXKII was found in both the nuclear and cytoplasmic extracts. Furthermore, HXKII was found in a commercially available HeLa nuclear extract (Fig. 1B, lane 1). To confirm the efficacy of the extraction procedure, blots were stripped and re probed for β-tubulin (data not shown).

To confirm nuclear localization of HXKII, nuclear fractions of HeLa cells were subjected to immunoprecipitation (Fig. 1C). HXKII was found in the samples in which antibody was included, but not in the control sample, incubated without antibody. HXKII was found in the

effluent from the control samples, but not the effluent from the antibody-containing samples, suggesting complete depletion of HXKII from the nuclear fraction by immunoprecipitation. No HXKII was found in the first washes of the pellet (data not shown).

HXKIII has been found previously to accumulate in the perinuclear region [8], although how its presence there is regulated is unknown. The most obvious question raised by these results is to clarify whether HXKII is inside the nucleus or merely associated with nuclear envelope tightly enough to remain associated with the nuclear fraction during subcellular fractionation. The nuclear fractionation results show clearly that HXKII is found in the nucleus of HeLa cells.

Hexokinase II is exported from HeLa nuclei by exportin

To address the regulation of HXKII nuclear localization, we first examined the mechanism by which HXKII is exported from the nucleus. The CrmA/exportin pathway is the best-known regulated mechanism for nuclear export of proteins that shuttle between the nucleus and cytoplasm. The bacterially-derived compound LMB inhibits exportin, the key member of this pathway. Exposure of HeLa cells to a non-lethal dose of LMB for as little as 4 h resulted in increased nuclear HXKII staining in some cells; by 24 h, the difference was substantial (Fig. 2A–B). Subcellular fractionation confirmed the accumulation of HXKII in the nucleus of cells exposed to LMB (Fig. 2C).

These LMB results argue against perinuclear localization, since there is no evidence that exportins release proteins from the cytoplasmic surface of the nuclear envelope. Furthermore, the fact that cytoplasmic levels decrease when nuclear levels increase confirms that LMB is preventing nuclear export. The yeast HXKII, which does not bind to mitochondria, has a nuclear localization sequence [26], an internal sequence located between lysine-6 and methionine-15 (KKPQARKGSM). Human HXKII does not have this sequence [27]. It does, however, have a putative monopartite NLS (¹⁴⁴KEKK¹⁴⁷) and bipartite NLS (⁴²⁴KRLHKAVRRLV⁴³⁵). Interestingly, there are also multiple putative nuclear export sequences, although their presence does not absolutely prove binding to exportins [15].

Inhibiting Hexokinase II enzymatic activity increases nuclear HXKII

Next, we addressed the role HXKII enzymatic activity plays in its subcellular localization. HeLa cells incubated in the presence of glucose and the glucose analog 2DG had slightly increased nuclear HXKII (Fig. 3A, top two images). However, in the absence of glucose, the presence of either fructose or galactose had little effect on the localization of HXKII (Fig. 3A, left panels). When cells incubated in fructose or galactose for 24 h were exposed to 2DG for 4 h, HXKII was localized exclusively (fructose) or primarily (galactose) in the nucleus (Fig. 3A, right panels). Quantitation of the intensity of the nuclear staining under these conditions confirms the results seen in the images (Fig. 3B).

Interestingly, Western blotting of whole cell extracts from cells treated with 2DG after incubation in glucose, fructose, or galactose revealed altered expression of HXKII. For cells incubated in the presence of glucose, addition of 2DG for 4 h reduced HXKII expression (Fig. 3C). Incubation in the presence of either fructose or galactose reduced HXKII expression, while addition of 2DG to cells incubated with either of these sugars increased HXKII expression; for fructose, the level did not return to that seen with glucose, while it appeared similar to glucose-exposed cells for cells incubated in the presence of galactose. Western blotting of cytoplasmic extracts (Fig. 3D) revealed that 2DG increased cytoplasmic HXKII in cells incubated with glucose, but decreased cytoplasmic HXKII in cells incubated in the absence of glucose (with either fructose or galactose). Confirming the microscopy results, nuclear HXKII increased in 2DG-treated cells incubated in the absence of glucose (Fig. 3E). The addition of 2DG increased nuclear HXKII in HeLa cells incubated in the presence of glucose. In the absence of 2DG,

when fructose replaced glucose in the culture medium, there was little HXKII detected in the nucleus, while galactose-exposed cells had detectable HXKII in the nucleus, albeit at lower levels than cells incubated with glucose.

HeLa cells are avid users of glucose, deriving as much as 80% of their energy from this source [28]. 2DG mimics excess glucose by inhibiting HXKII, and could trick the cell into a response similar to yeast glucose repression. HXKII participates in glucose repression in yeast, a specific pathway in which proteins involved in glucose metabolism are down-regulated [26]. These results suggest that HXKII may participate in the nuclear response to excess glucose in mammalian cells.

Removing 2DG restores the nuclear/cytoplasmic HXKII balance

To test the reversibility of the 2DG-induced HXKII nuclear translocation, we treated cells with 2DG, in the presence of glucose, fructose, or galactose, for 4 h, then washed the cells to remove the 2DG and the sugars, and incubated them with or without LMB for another 24 h, in the presence of glucose. As expected, 2DG induced nuclear translocation of HXKII, regardless of the sugar available to the cells, although the degree of translocation differed (Fig. 4; compare first and second rows of images). Removing 2DG without adding LMB (third row of images) resulted in HXKII distribution similar to that seen in cells not exposed to 2DG. The presence of LMB, however, not only prevented translocation of HXKII back to the cytoplasm, but actually resulted in aggregation of the HXKII staining (bottom row of images).

HXKII binding to mitochondria reduces the availability of substrates for oxidative phosphorylation [29]. If fructose and galactose reduce cytosolic HXKII levels, one result may be amelioration of oxidative phosphorylation inhibition. The obvious benefit to the cell is increased oxidative phosphorylation when glucose is not available. In contrast, the 2DG data suggests that a similar effect could occur in the presence of excess glucose. If nuclear HXKII is participating in glucose repression in mammalian cells, this may be an adaptive mechanism to prevent excessive accumulation of lactic acid.

Conclusions

In this work, we have demonstrated that, in HeLa cells, HXKII is distributed almost equally between the nucleus and the cytoplasm. Inhibiting nuclear export induces an accumulation of HXKII in the nucleus, suggesting that HXKII nuclear export is controlled, at least in part, by the exportins. Furthermore, inhibiting HXKII enzymatic activity also induced HXKII accumulation in the nucleus, an effect which was reversed by the removal of 2DG. This effect was far more pronounced in cells incubated in the presence of galactose or fructose, suggesting that the presence of glucose reduces HXKII nuclear translocation. These results clearly demonstrate that HXKII nuclear translocation is dependent on HXKII function. Functional translocation of HXKII is a novel result with novel implications. Future work will focus on identifying the mechanisms by which HXKII translocation is mediated, and potential binding partners.

Acknowledgments

This work was supported by grant 5R01CA118356-04 from the NCI

References

1. Gatenby RA, Gillies RJ. Why Do Cancers Have High Aerobic Glycolysis? *Nat Rev Cancer* 2004;4:891–899. [PubMed: 15516961]

2. Burt BM, Humm JL, Kooby DA, Squire OD, Mastorides S, Larson SM, Fong Y. Using Positron Emission Tomography with [¹⁸F]FDG to Predict Tumor Behavior in Experimental Colorectal Cancer. *Neoplasia* 2001;3:189–195. [PubMed: 11494112]
3. Bos R, van der Hoeven JJM, van der Wall E, van der Groep P, van Diest PJ, Comans EFI, Joshi U, Semenza GL, Hoekstra OS, Lammertsma AA, Molthoff CFM. Biologic Correlates of ¹⁸Fluorodeoxyglucose Uptake in Human Breast Cancer Measured by Positron Emission Tomography. *J Clin Oncol* 2002;20:379–387. [PubMed: 11786564]
4. Wilson JE. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *J Exp Biol* 2003;206:2049–2057. [PubMed: 12756287]
5. Wilson JE, Felgner PL. An inverse relationship between mitochondrial hexokinase content and phosphoglucumutase activity of rat tissues. *Mol Cell Biochem* 1977;18:39–47. [PubMed: 600270]
6. Sebastian S, Horton JD, Wilson JE. Anabolic Function of the Type II Isozyme of Hexokinase in Hepatic Lipid Synthesis. *Biochem Biophys Res Commun* 2000;270:886–891. [PubMed: 10772920]
7. Kaselonis GL, McCabe ER, Gray SM. Expression of hexokinase 1 and hexokinase 2 in mammary tissue of nonlactating and lactating rats: evaluation by RT-PCR. *Mol Genet Metab* 1999;68:371–374. [PubMed: 10562464]
8. Preller A, Wilson JE. Localization of the type III isozyme of hexokinase at the nuclear periphery. *Arch Biochem Biophys* 1992;294:482–492. [PubMed: 1567204]
9. Postic C, Shiota M, Magnuson MA. Cell-specific Roles of Glucokinase in Glucose Homeostasis. *Recent Prog Horm Res* 2001;56:195–217. [PubMed: 11237213]
10. Meglasson MD, Matschinsky FM. New perspectives on pancreatic islet glucokinase. *Am J Physiol* 1984;246:E1–13. [PubMed: 6364828]
11. Girard J, Ferre P, Foufelle F. Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu Rev Nutr* 1997;17:325–352. [PubMed: 9240931]
12. de la Iglesia N, Veiga-da-Cunha M, Van Schaftingen E, Guinovart JJ, Ferrer JC. Glucokinase regulatory protein is essential for the proper subcellular localisation of liver glucokinase. *FEBS Lett* 1999;456:332–338. [PubMed: 10456334]
13. Shiota C, Coffey J, Grimsby J, Grippo JF, Magnuson MA. Nuclear Import of Hepatic Glucokinase Depends upon Glucokinase Regulatory Protein, whereas Export Is Due to a Nuclear Export Signal Sequence in Glucokinase. *J Biol Chem* 1999;274:37125–37130. [PubMed: 10601273]
14. Bednenko J, Cingolani G, Gerace L. Nucleocytoplasmic Transport: Navigating the Channel. *Traffic* 2003;4:127–135. [PubMed: 12656985]
15. Gorlich D, Kutay U. Transport Between the Cell Nucleus and the Cytoplasm. *Annu Rev Cell Dev Biol* 1999;15:607–660. [PubMed: 10611974]
16. Fukuda M, Asano S, Nakamura T, Adachi M, Yoshida M, Yanagida M, Nishida E. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* 1997;390:308–311. [PubMed: 9384386]
17. Mathupala SP, Rempel A, Pedersen PL. Aberrant glycolytic metabolism of cancer cells: a remarkable coordination of genetic, transcriptional, post-translational, and mutational events that lead to a critical role for type II hexokinase. *J Bioenerg Biomembr* 1997;29:339–343. [PubMed: 9387094]
18. Pastorino JG, Hoek JB. Regulation of hexokinase binding to VDAC. *J Bioenerg Biomembr* 2008;40:171–182. [PubMed: 18683036]
19. Pastorino JG, Shulga N, Hoek JB. Mitochondrial Binding of Hexokinase II Inhibits Bax-induced Cytochrome c Release and Apoptosis. *J Biol Chem* 2002;277:7610–7618. [PubMed: 11751859]
20. Pastorino JG, Hoek JB, Shulga N. Activation of Glycogen Synthase Kinase 3 β Disrupts the Binding of Hexokinase II to Mitochondria by Phosphorylating Voltage-Dependent Anion Channel and Potentiates Chemotherapy-Induced Cytotoxicity. *Cancer Res* 2005;65:10546.
21. Niederacher D, Entian KD. Characterization of Hex2 protein, a negative regulatory element necessary for glucose repression in yeast. *Eur J Biochem* 1991;200:311–319. [PubMed: 1889400]
22. Ahuatzi D, Herrero P, de la Cera T, Moreno F. The Glucose-regulated Nuclear Localization of Hexokinase 2 in *Saccharomyces cerevisiae* Is Mig1-dependent. *J Biol Chem* 2004;279:14440–14446. [PubMed: 14715653]

23. Ahuatzi D, Riera A, Pelaez R, Herrero P, Moreno F. Hxk2 Regulates the Phosphorylation State of Mig1 and Therefore Its Nucleocytoplasmic Distribution. *J Biol Chem* 2007;282:4485–4493. [PubMed: 17178716]
24. Pelaez R, Herrero P, Moreno F. Nuclear Export of the Yeast Hexokinase 2 Protein Requires the Xpo1 (Crm1)-Dependent Pathway. *J Biol Chem* 2009;284:20548–20555. [PubMed: 19525230]
25. Kudo N, Wolff B, Sekimoto T, Schreiner EP, Yoneda Y, Yanagida M, Horinouchi S, Yoshida M. Leptomycin B Inhibition of Signal-Mediated Nuclear Export by Direct Binding to CRM1. *Exp Cell Res* 1998;242:540–547. [PubMed: 9683540]
26. Herrero P, Martinez-Campa C, Moreno F. The hexokinase 2 protein participates in regulatory DNA-protein complexes necessary for glucose repression of the *SUC2* gene in *Saccharomyces cerevisiae*. *FEBS Lett* 1998;434:71–76. [PubMed: 9738454]
27. Deeb SS, Malkki M, Laakso M. Human Hexokinase II: Sequence and Homology to Other Hexokinases. *Biochem Biophys Res Commun* 1993;197:68–74. [PubMed: 8250948]
28. Reitzer L, Wice B, Kennel D. Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. *J Biol Chem* 1979;254:2669–2676. [PubMed: 429309]
29. Pedersen PL, Mathupala S, Rempel A, Geschwind JF, Ko YH. Mitochondrial bound type II hexokinase: a key player in the growth and survival of many cancers and an ideal prospect for therapeutic intervention. *Biochim Biophys Acta* 2002;1555:14–20. [PubMed: 12206885]

Figure 1. HXXKII is found in the nucleus of untreated HeLa cells

HXXKII was detected by immunofluorescence (A), nuclear extraction (B) and immunoprecipitation (C) in HeLa cells, grown under normal culture conditions, as described in the Materials and Methods section. In (A), the left image shows HXXKII staining, the center image is Hoechst staining of the nuclei, and the right image is an overlay done in Photoshop. Scale bars: 10 μ m. In (B), lane 1 is the HeLa nuclear extract purchased from Active Motif, lane 2 is a HeLa whole cell extract, lane 3 is the nuclear fraction, and lane 4 is the cytoplasmic fraction. In (C), lane 1 is the nuclear extract, lane 2 is the precleared nuclear extract, lane 3 is the sample immunoprecipitated with anti-HXXKII antibody, and lane 4 is the control immunoprecipitation sample, to which no antibody was added.

Figure 2. LMB exposure induces nuclear accumulation of HXKII

HeLa cells were exposed to 20 ng/mL LMB for different times (4, 16, or 24 h) prior to immunofluorescent staining (A) or nuclear extraction (B) as described in the Materials and Methods section. Scale bars: 10 μ m. The percent of cellular staining in the nucleus was quantified and plotted as a distribution for each time point (B). In (C), the lanes are labeled with the treatment; lanes labeled C are the cytoplasmic samples, and N, the nuclear samples.

Figure 3. Inhibition of HXKII by 2DG induces nuclear translocation

HeLa cells were exposed to 10 mM 2DG for 4 h after incubation in medium containing either glucose, fructose or galactose for 24 h, prior to immunofluorescent staining (A) or nuclear extraction (C–E) as described in the Materials and Methods section. In (A), the cells were exposed to 2DG (right column) in the presence of glucose (top), fructose (middle), or galactose (bottom). The left column was not exposed to 2DG. Scale bars: 10 μ m. The percent of cellular staining in the nucleus was quantified and plotted as a distribution for each time point (B). In (C–E), lane 1 is glucose – 2DG, lane 2 is glucose + 2DG, lane 3 is fructose – 2DG, lane 4 is fructose + 2DG, lane 5 is galactose – 2DG, and lane 6 is galactose + 2DG. Whole cell lysates are in (C), cytoplasmic extracts are in (D), and nuclear extracts are in (E).

Figure 4. Removing 2DG reverses the induced nuclear translocation

HeLa cells grown on coverslips were incubated in the presence of glucose (left column), fructose (center column), or galactose (right column) were left untreated (top row) or treated with 10 mM 2DG for 4 h (2nd row from top). The 2DG was washed from the cells, and the medium replaced with glucose-containing medium (3rd row from the top). LMB was added (bottom row) and the cells were incubated for a further 24 h prior to immunofluorescent staining performed as described in the Materials and Methods section. Scale bars: 10 μ m.