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Review

# Hexokinases in cancer and other pathologies

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ABSTRACT

Glucose metabolism is indispensable for cell growth and survival. Hexokinases play pivotal roles in glucose metabolism through canonical functions of hexokinases as well as in immune response, cell stemness, autophagy, and other cellular activities through noncanonical functions. The aberrant regulation of hexokinases contributes to the development and progression of pathologies, including cancer and immune diseases.

## 1. Introduction

Glucose metabolism is an evolutionarily conserved and indispensable pathway for cell growth and survival. It produces energy and provides building blocks for the synthesis of protein, lipid, and nucleotides and is at the regulatory center of cellular activities that integrate nutrition condition and cell status. Cancer cells show reprogrammed glucose metabolism, exhibiting enhanced uptake of glucose and lactate production regardless of oxygen concentrations, known as the Warburg effect (Wang et al., 2018).

As the evolutionarily conserved enzymes that interact with imported glucose, hexokinases (HKs) phosphorylate glucose to generate glucose-6-phosphate (G-6-P). G-6-P is then metabolized into glycolytic, glycogenic, pentose phosphate and hexosamine biosynthesis pathways, thereby playing key roles in ATP production, anabolic biosynthesis, glucose storage, NADH pool enrichment, and protein glycosylation (Ciscato et al., 2021). In mammals, hexokinases have five isoforms: HK1, HK2, HK3, HK4 (also known as glucokinase, GK), and hexokinase domain-containing protein-1 (HKDC1) (Zapater et al., 2022). HK1-3, which have a high affinity for glucose (Wilson, 2003), consist of two highly similar 50-kDa N- and C-terminal lobes and can be inhibited by their product, G-6-P. HK2 has two functional catalytic lobes, whereas the N-terminal lobes of HK1 and HK3 are unable to phosphorylate glucose. HK4 has only one 50-kDa catalytic domain with a lower affinity toward glucose (Heimberg et al., 1996). *HKDC1,* which is located on chromosome 10 adjacent to *HK1*, encodes a low enzymatic activity of 100-kDa protein sharing about 70% sequence homology with HK1 (Zapater et al., 2022) (Fig. 1).

HK1-4 and HKDC1 expression varies under different physiological conditions in different tissues. HK1 is ubiquitously and stably expressed in all mammalian tissues, and its expression is unaffected by most physiological conditions or stresses. HK2 is expressed in the muscle and heart and is overexpressed in many types of cancers; furthermore, its expression can be regulated by different hormonal or metabolic changes. HK3 is also ubiquitously expressed in tissues with relatively low abundance. HK4 is primarily expressed in the liver, pancreas, small intestine, and brain (Matschinsky, 1990). HKDC1 is differentially expressed in many tissues and upregulated in expression in several types of cancers accompanying with poor prognosis (Zapater et al., 2022).

Subcellularly, HK1, HK2, and HKDC1 are localized in the outer membrane of mitochondria (OMM) through their N-terminal 15 amino acid residues (mitochondrial binding domain), which can bind to voltage-dependent anion channels (VDACs) in the mitochondria (Krasnov et al., 2013). VDACs export ATP from mitochondria to the cytosol, thus maximizing HK1- and HK2-catalyzed reactions, which use ATP as a substrate for phosphorylation and ADP production. G-6-P in high concentrations binds to HK1 and HK2, leading to conformational changes, dissociation from the outer membrane of mitochondria, and inhibition of glycolysis in a feedback manner (Robey & Hay, 2006). HK1 has a strong binding affinity to mitochondria and functions as a housekeeping protein

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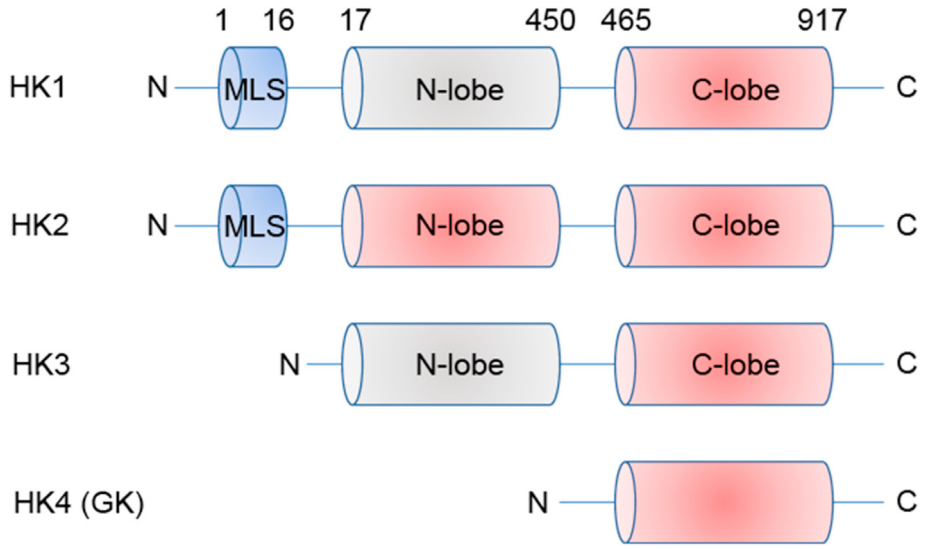


**Fig. 1. Schematic representation of the sequence and functional domains of hexokinases.** The red color tubes represent the kinase lobes that have the activity to phosphorylate glucose. The gray color lobes have no catalytic activity. MLS, mitochondrial localization sequence.

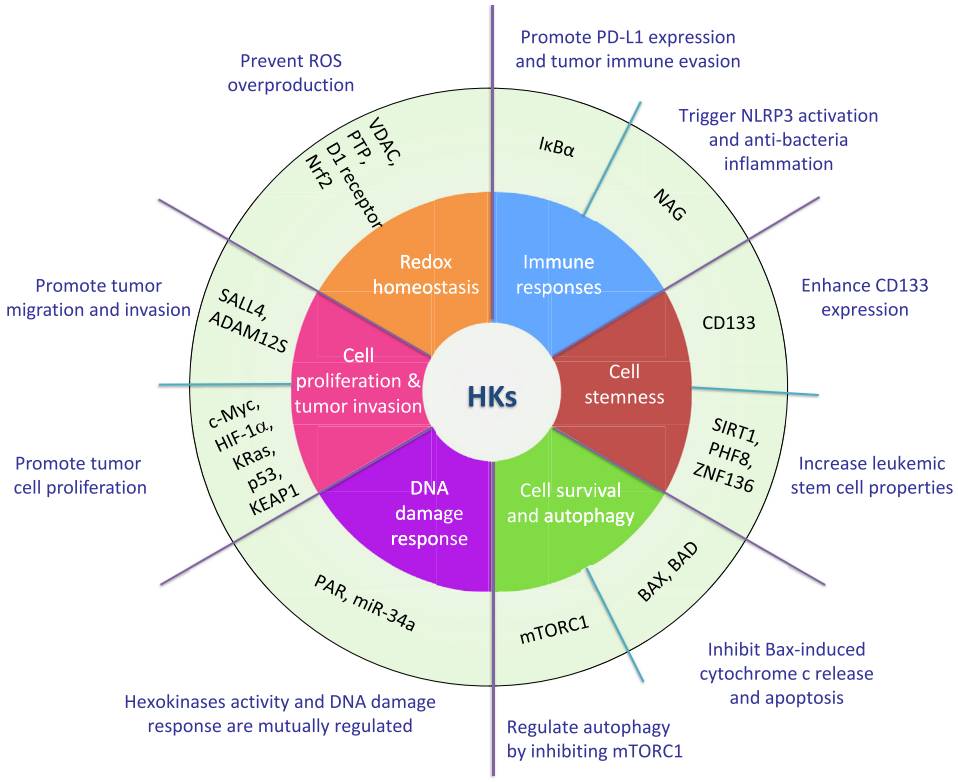


**Fig. 2. Roles of hexokinases in different cellular activities.** Hexokinases are involved in immune responses, cell stemness, cell survival and autophagy, DNA damage response, redox homeostasis, cell proliferation, and tumor invasion and migration through canonical and noncanonical functions.

of glucose metabolism, whereas HK2 binds less avidly to VDAC1 and alternates between cytoplasmic and mitochondrial-bound states in response to environmental and metabolic stress (John et al., 2011; Xu & Herschman, 2019). Unlike HK1/2, HK3 and HK4 lack an N-terminal mitochondrial binding sequence and are cytoplasmic proteins (Calmettes et al., 2015). In the pancreatic β-cells, HK4, which is localized in cytoplasm and insulin-localized granules, functions as a glucose sensor; hence, its activity allows glycaemia levels to be tightly linked to insulin release (Guzman & Gurrola-Diaz, 2021). Hexokinases were also found to localize to the nucleus (Agius, 1998; Hussain et al., 2015; Neary & Pastorino, 2010; Seiler et al., 2022; Thomas et al., 2022), nuclear periphery (Preller & Wilson, 1992), endoplasmic reticulum (Ciscato et al., 2020; Travis et al., 1999), plasma membrane (Pedley et al., 1993; Travis et al., 1999), and cytoskeleton (Balasubramanian et al., 2007; Stoddard et al., 2020).

As key metabolic enzymes in glucose metabolism, hexokinases are regulated by a variety of signaling pathways, thereby governing glucose metabolism to meet the anabolic synthesis needs of cellular activities. Importantly, hexokinases exhibit moonlighting functions that play pivotal roles in cell proliferation, cell survival and cell death, cell

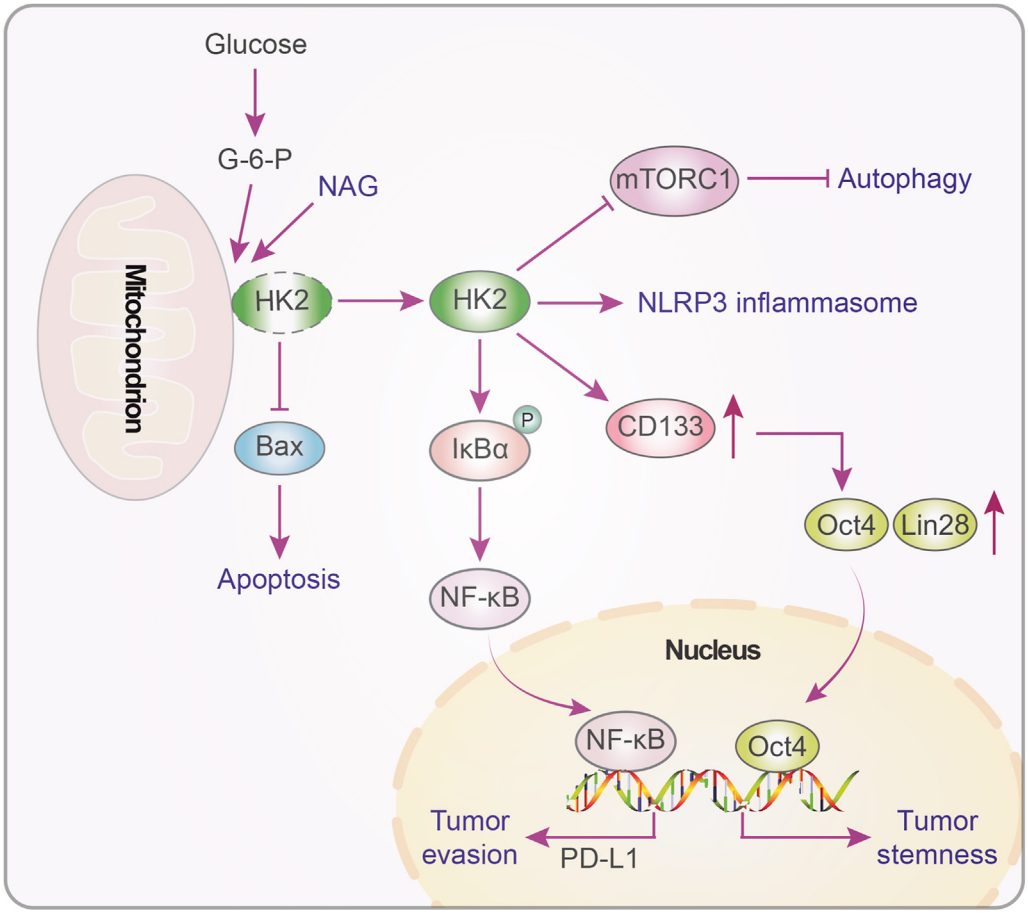


**Fig. 3. Noncanonical functions of HK2.** Mitochondria-localized HK2 inhibits apoptosis by preventing the binding of Bax to the mitochondria. High level of G-6-P releases HK2 from the mitochondria to the cytosol, where it acts as a protein kinase to phosphorylate IκBα and consequently activates NF-κB-mediated *CD274* transcription and tumor immune evasion. Cytosolic HK2 interacts with CD133 to enhance CD133 stability and promote tumor cell stemness, and interacts with and inhibits mTORC1 to induce autophagy. Bacteria-derived NAG also binds to HK2 and promotes its release from the mitochondria and activation of NLRP3 inflammasome.

stemness, cell migration, redox homeostasis, immune responses and DNA damage responses. This review summarizes the advances in our understanding of the canonical and noncanonical functions of hexokinases in cancer and other pathologies, such as immune diseases (Fig. 2).

## 2. Hexokinase expression regulation

Hexokinase expression is regulated in cancer cells at the transcriptional, mRNA, and protein levels. Under hypoxic conditions, HKs, including HK3, is transcriptionally upregulated in a hypoxia-induced-factor (HIF)-1-dependent manner (Nakajima et al., 2017; Soñanez-Organis et al., 2011). In colorectal cancer cells, c-Myc degradation reduces HK2 expression and promotes cell apoptosis (Wu et al., 2020). In B-cell lymphoma cells, NF-κB activation promotes HK2 expression (Nakajima et al., 2017). In cells with an activated Wnt/β-catenin pathway, β-catenin transactivation induces the expression of HK2, other glycolytic enzymes, and glucose transporters and suppresses mitochondrial enzyme expression, inducing a shift to glucose metabolism from oxidative phosphorylation (Chafey et al., 2009; Vallée et al., 2021). HK2 transcription can be also regulated by the intestinal microbiome. The microbially derived short-chain fatty acid (SCFA) butyrate repressed HK2 expression via inhibition of histone deacetylase 8 (HDAC8) (Hinrichsen et al., 2021). In hepatocytes, insulin increases phosphoinositide 3-kinases (PI3K)/AKT-mediated HK4 transcription through sterol regulatory-element binding proteins (SREBP)-1c and other not well-defined transcription factors (Guzman & Gurrola-Diaz, 2021). In response to inhibition of the mitochondrial respiration chain and endoplasmic reticulum (ER) stress, HKDC1 is upregulated in a manner dependent on activating transcription factor 4 (ATF4) (Evstafieva et al., 2018). In breast cancer cells, *HKDC1* transcription is co-activated by SREBP1 and the peroxisome proliferator-activated receptor-γ (PPARγ) co-activator-1β (PGC1β) (Chen et al., 2019).

It has been shown that hexokinase mRNAs can be targeted for degradation by several miRNAs, which are often downregulated in cancer cells. (Chen et al., 2019; Guo et al., 2015; Liu et al., 2021; Zhan & Ni, 2021). The long non-coding RNA (lncRNA) PVT1, which is upregulated in gallbladder cancer, binds to miR-143 thereby promoting HK2 expression and gallbladder tumor cell proliferation and metastasis (Chen et al., 2019). *HK2* mRNA contains an AU-rich element (ARE) within its 3′-untranslated region (3′-UTR). Tristetraprolin (TTP), which is an ARE-binding protein and downregulated in expression in cancer cells, binds to *HK2* 3′-UTR and enhances degradation of *HK2* mRNA (Kim et al., 2019). In addition, METTL3, a $m^6A$ methyltransferase, targets and methylates the 3′-UTR of *HK2* mRNA. This methylation recruits YTHDF1, a $m^6A$ reader, to enhance *HK2* mRNA stability and subsequent HK2 expression, resulting in an enhanced Warburg effect and cervical cancer tumorigenesis (Wang et al., 2020).

In response to inhibition of mitochondrial oxidative phosphorylation, Parkin E3 ligase-mediated ubiquitination of mitochondrial HK1 leads to its proteasomal degradation (Okatsu et al., 2012). In liver cancer cells, high levels of glycolysis with impaired autophagy are prevalent. Impaired autophagy substantially enhances glycolysis, whereas activated autophagy reduces glycolysis through the E3 ligase TRAF6-mediated K63-linked ubiquitylation of HK2 at K41 and the subsequent recognition of HK2 by the autophagy receptor protein SQSTM1/p62 for selective autophagic HK2 degradation (Jiao et al., 2018). O-GlcNAcylation was identified on HK4 and positively upregulated by high glucose levels. HK4 O-GlcNAcylation increases HK4 expression levels (Baldini et al., 2016).

Thus, HK expression is regulated at the levels of transcription by different transcription factors, mRNA by miRNAs, lncRNA, and METTL3, and protein by ubiquitylation-dependent proteasomal and autophagic degradation in a signaling- and cancer type-dependent manner.

## 3. Hexokinase activity regulation

In addition to the regulation of hexokinase activity through protein expression, posttranslational modifications of hexokinases have also been reported to regulate the activities of hexokinases. AKT phosphorylates HK2 at T473 to promote its binding to mitochondria, which increases HK2 activity by increasing the access of HK2 to the substrate ATP produced from mitochondria (Li, Lu, et al., 2019; Roberts et al., 2013). c-Src-mediated HK1 Y732 phosphorylation robustly decreases HK1 $K_m$ and increases its $V_{max}$ by disrupting dimer formation, thus increasing its catalytic activity and glycolysis (Zhang et al., 2017). Nonproteolytic K63-linked ubiquitination of HK2 by HectH9 ubiquitin ligase promotes the localization of HK2 to the outer membrane of mitochondria, glycolysis, and cancer cell stem cell expansion (Lee et al., 2019). SUMOylation at K315 and K492 of HK2 also enhance the binding of HK2 to mitochondria and glucose consumption, consequently promoting prostate cancer cell proliferation and resistance to chemotherapy (Shangguan et al., 2021). TGF-β increases HK1 palmitoylation in hepatic stellate cells and promotes the secretion of HK1, which is imported by hepatocellular carcinoma (HCC) cells to accelerate glycolysis and HCC progression (Chen et al., 2022). Nitric Oxide induces HK4 S-nitrosylation and its conformational changes and activates HK4 (Seckinger et al., 2018). Hexokinase in yeast showed autophosphorylation by an intramolecular mechanism (Fernández et al., 1988), which may inhibit hexokinase activity (Kettner et al., 2013), but whether this autophosphorylation also occurs in mammalian cells remains unknown.

The activities of hexokinases can be regulated through interactions with other proteins. In addition to being maintained at high activity levels by interaction with VDACs at the outer membrane of mitochondria, GTP- and Ras prenylation-dependent interactions between KRAS4A and HK1 on the outer membrane of mitochondria block the allosteric inhibition of HK1 and enhance the Warburg effect (Amendola et al., 2019). Polymerization of hexokinase in the actin ATPase clan inhibits hexokinase activity in yeast (Stoddard et al., 2020). HK2 was reported to bind to cytoplasmic regions of glucose transporter 4 (GLUT4) dependent on insulin (Zaid et al., 2009), suggesting that this interaction may maximize HK2 activity by increasing substrate availability. HK2 activity in liver cells was increased by its interaction with hepatitis C virus protein NS5A, resulting in enhanced aerobic glycolysis that may ensure the biomolecular supply needed for viral replication (Ramière et al., 2014). Glucokinase regulatory protein (GCKR) binds HK4 and sequesters it in the nucleus in an inactive form, and this interaction is strengthened by fructose-6-phosphate (F-6-P) but weakened by fructose-1-phosphate (F-1-P) (Guzman & Gurrola-Diaz, 2021). In addition, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (*PFK-2/FBPase-2*) interacts with and activates cytoplasmic HK4 and enhances glucose phosphorylation and glucose metabolism in insulin-producing cells (Massa et al., 2004).

These findings revealed that hexokinase activity can be regulated at multiple levels, including protein expression, posttranslational modification, subcellular localization, polymerization, and protein–protein interactions.

## 4. Hexokinases in immune responses

In general, hexokinase expression or activity is upregulated in cells and tissues undergoing immune responses, such as lipopolysaccharide (LPS)-stimulated macrophages (Tan et al., 2015), osteoarthritis synovial tissue (Bao et al., 2022), and hepatitis C virus-infected liver cells (Perrin-Cocon et al., 2022). This upregulation contributes to glycolysis enhancement, thus supporting the cells with energy and metabolic intermediates for activities related to immune responses, including cytokine production, endocytosis, and antigen presentation. Notably, HK1 and HK2 exhibit different features in regulation of immune responses. Mice lacking the HK1-mitochondrial binding domain elicit a hyperinflammatory response upon LPS challenge accompanied by decreased glycolysis and increased flux through the pentose phosphate pathway in bone-marrow-derived macrophages. Cytosolic and mitochondria-disassociated HK1 binds to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), resulting in GAPDH nitrosylation through iNOS and decreased GAPDH activity. Macrophages under conditions of low-grade inflammation, including aging and diabetes, exhibit increased cytosolic HK1 and reduced GAPDH activity (De Jesus et al., 2022), linking cytosolic and inhibited HK1 and subsequently altering glucose metabolism to inflammation. In contrast, HK2-mediated glycolysis is connected to Alzheimer's disease (AD) progression, in which HK2 expression is elevated. HK2 deletion or inhibition promotes microglial phagocytosis and reduces amyloid plaque burden and cognitive impairment in male AD mice, accompanied by increased ATP levels due to the upregulation of lipoprotein lipase expression and a subsequent increase in lipid metabolism (Leng et al., 2022). In gut epithelium, highly expressed HK2 is further upregulated during inflammation and contributes to immune responses. Mice lacking HK2 in intestinal epithelial cells are less susceptible to acute colitis, and the microbial metabolite butyrate ameliorates intestinal inflammation by downregulation of intestinal epithelial HK2 expression and suppression of cell death (Hinrichsen et al., 2021). HKDC1 was shown to play a role in glucose and lipid metabolism and progression of liver diseases, including nonalcoholic steatohepatitis (NASH). Heterozygous *HKDC1*$^{+/-}$ mice during times of metabolic stress, such as in pregnancy or aging, exhibited significantly increased glucose excursion (Ludvik et al., 2016). Additionally, HKDC1 expression is positively correlated with the degree of liver steatosis and exhibits the greatest levels in human and mouse liver samples that had progressed to NASH (Pusec et al., 2019).

In addition to regulating glucose metabolism, hexokinases can function as direct regulators or effectors of cellular immune responses. Macrophage and dendritic cell phagosomes degrade gram-positive bacterial cell wall peptidoglycan to produce N-acetylglucosamine (NAG), which releases hexokinase from the mitochondria to the cytosol. Consequent inhibition of hexokinase leads to activation of the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, a cytosolic complex that regulates the processing and secretion of interleukin (IL)-1β and IL-18 (Fig. 3). This finding demonstrated that hexokinase acts as a pattern recognition receptor that recognizes bacterial peptidoglycan and triggers the activation of inflammasomes (Wolf et al., 2016).

In the tumor microenvironment, NF-κB-inducing kinase (NIK) enhances glycolysis in $CD8^+$ T cells via prevention of reactive oxygen species (ROS)-induced autophagic degradation of HK2 in a manner dependent on glucose-6-phosphate dehydrogenase (G6PD)-mediated production of the antioxidant NADPH. HK2 deletion in T cells impairs effector T cell function and antitumor immunity (Gu et al., 2021). In non-small cell lung cancer (NSCLC) tissues, HK3 expression levels in tumor, which were possibly downregulated in tumor cells but not in immune cells, were positively correlated with the infiltration degree of immune cells and the better outcomes of patients receiving immunotherapy (Tuo et al., 2020). Similarly, in clear cell renal cell carcinoma (ccRCC) tissues, HK3 expression is highly correlated with the abundance of immune cells and stimulates the infiltration of monocytes and macrophages, thus prompting active anti-tumor immune responses in the microenvironment of ccRCC (Xu et al., 2021).

Hexokinase in tumor cells directly regulates tumor cell immune evasion. In Burkitt's lymphoma (BL), depletion of HK4 in tumor cells dampens the Warburg effect and reduces c-Myc expression and accumulation of *MYC* gene mutations. Notably, HK4 depletion reduces migration of macrophages to tumor accompanying with reduced tumor-assisting macrophage M2 phenotype and increased macrophage M1 polarization (Tandon et al., 2020). High HK2 expression in tumor was associated with immunosuppressive features, especially decreased ratio of $CD8^+$ T cells to Tregs cells, which was correlated with poor progression-free survival and overall survival in lung cancer patients and a shorter overall survival in the immunotherapy cohort (Kim et al., 2022). In glioblastoma cells, high glucose substantially increases the HK2-mediated production of G-6-P, which releases HK2 from the

mitochondria to the cytosol, where HK2 interacts with IκBα, an inhibitor protein of the NF-κB pathway. Importantly, HK2 acts as a protein kinase and phosphorylates IκBα. HK2-mediated IκBα phosphorylation promotes its binding to μ-calpain protease, leading to IκBα degradation and release of the inhibition of IκBα on NF-κB. Activated NF-κB translocates into the nucleus and induces *CD274* (encoding PD-L1) transcription, PD-L1 expression and tumor immune evasion (Fig. 3). Thus, this moonlighting function of HK2 as a glucose sensor in tumor cells intrinsically connects the Warburg effect to tumor immune evasion (Guo et al., 2022).

## 5. Hexokinases in cancer cell stemness

Upregulation of glycolysis promotes the stemness of cancer cells (Sebastian, 2014; Zhao et al., 2017; Zhu et al., 2021). Intriguingly, cancer cell stemness can also be regulated by the noncanonical functions of HK2. HK2 expression is much higher in $CD133^+$ cancer stem-like cells (CSCs) of small cell lung cancer (SCLC) than in differentiated $CD133^-$ cells. HK2 depletion inhibits CSC stemness and promotes CSC differentiation, accompanied by reduced CD133 expression. Notably, HK2 interacts with CD133 in nonmitochondrial cell fractions and promotes the binding of the deubiquitinase ubiquitin-specific protease 11 (USP11) to CD133, thereby reducing CD133 polyubiquitylation and degradation and enhancing its expression, the expression of the pluripotency transcription factor octamer-binding transcription factor 4 (Oct4) and the translation reprogramming factor Lin28, SCLC cell stemness, and tumor growth in mice (Wang et al., 2022) (Fig. 3). In addition, HK2 was found in the nucleus of leukemic and normal hematopoietic stem cells, where HK2 interacts with nuclear proteins to modulate chromatin reorganization, transcriptional regulation, and DNA damage responses. Consequently, nuclear HK2 increases leukemic stem cell properties with decreased differentiation and double-strand breaks and confers chemoresistance independent of the metabolic activity of HK2 (Thomas et al., 2022). Thus, HK2 interacts with the plasma membrane protein CD133 or nuclear proteins to substantiate cancer cell stemness.

## 6. Hexokinases in cell survival and autophagy

Mitochondria-bound hexokinases are potent prosurvival factors that counteract apoptosis. Disruption of the association between hexokinases and mitochondria potently induces cytochrome *c* release and apoptosis (Majewski et al., 2004). HK1 depletion decreases the inner mitochondrial membrane potential and accelerates tumor necrosis factor (TNF)-induced apoptosis, which is dependent on the presence of pro-apoptotic Bak and Bax and blocked by pro-survival Bcl-2 overexpression (Schindler & Foley, 2013). Mitochondrial binding of HK2 interferes with the ability of Bax to bind to mitochondria, thereby inhibiting Bax-induced cytochrome *c* release and apoptosis (Pastorino et al., 2002) (Fig. 3). HK3, which is dispensable for glycolytic activity in acute myeloid leukemia (AML) cells, promotes cell survival, possibly through direct interaction with the proapoptotic BCL-2 family member BIM during all-*trans* retinoic acid-induced neutrophil differentiation (Seiler et al., 2022). In addition, HK3 transcription is enhanced by the basic leucine zipper transcription factor CCAAT/enhancer binding protein alpha (CEBPA) during all-*trans* retinoic acid-induced neutrophil differentiation of acute promyelocytic leukemia (APL) cells (Federzoni et al., 2014). In hepatocyte mitochondria, HK4, pro-apoptotic BAD, protein kinase A, protein phosphatase 1 (PP1) catalytic units, and Wiskott-Aldrich family member WAVE-1 form a functional holoenzyme complex. BAD deficiency or non-phosphorylatable BAD mutant expression diminishes HK4 activity and blunts mitochondrial respiration in response to glucose, resulting in abnormal glucose homeostasis, including profound defects in glucose tolerance in mice. Glucose deprivation induces dephosphorylation of BAD and BAD-dependent apoptosis (Danial et al., 2003).

Hexokinases are also involved in cell autophagy in response to nutrient deprivation and other stresses (Gatica & Klionsky, 2015; Roberts et al., 2014; Xue et al., 2022; Zhang et al., 2018). In cardiomyocytes and noncardiomyocytes, HK2 binds to and inhibits the autophagy suppressor mTOR complex 1 (mTORC1), and this binding is increased by glucose deprivation, leading to potentiated autophagy. G-6-P appears to suppress the autophagic role of HK2, whereas a decrease in G-6-P promotes the transition from glycolysis to autophagy (Roberts et al., 2014) (Fig. 3). Thus, hexokinases promote cell survival by regulating mitochondria-associated prosurvival or proapoptotic factors and promotes autophagy by inhibiting mTORC1.

## 7. Hexokinases in DNA damage responses

DNA damage is a common event in eukaryotic cells and may lead to genetic mutation and even cancer. DNA damage induces cellular responses that enable the cell either to repair the damaged DNA or cope with the damage for cell survival or death. HK1 is a direct target of the activation of ADP-ribosyltransferase diphtheria toxin-like 1 (ARTD1 or PARP1), which is a key enzyme involved in DNA repair. In response to DNA damage, activated PARP1-synthesized poly (ADP-ribose) (PAR) is released from the nucleus and binds to HK1, inhibiting HK1 and subsequent glycolysis and ATP production by release of HK1 from the mitochondrial membrane into the cytosol. Prolonged PARP1 activation triggers energy collapse and cell death (Fouquerel et al., 2014). In neurons, glutamate stimulation impairs mitochondrial HK2 activity and simultaneously induces apoptosis and PAR accumulation- and nuclear translocation of apoptosis inducing factor (AIF)-induced parthanatos due to mitochondrial dysfunction. AKT activation promotes HK2 binding to mitochondria and the structural and functional integrity of mitochondria, which protects neurons from apoptosis and DNA damage (Li et al., 2019). Upon DNA damage, p53, as a tumor suppresser, induces the expression of microRNA-34a (miR-34a), which in turn represses the expression of HK1, HK2, glucose-6-phosphate isomerase, and pyruvate dehydrogenase kinase 1, resulting in repressed glycolysis and enhanced mitochondrial respiration (Kim et al., 2013). Thus, hexokinase activity and the DNA damage response are mutually regulated.

## 8. Hexokinases in redox homeostasis

Mitochondria are the main source of cellular ROS, which may be produced by the electron transport system (ETS) or by matrix dehydrogenases (Wong et al., 2017). Hexokinases participate in the cellular redox homeostasis by regulating mitochondrial respiration and maintaining the $NADP^+$/NADPH ratio (Heneberg, 2019). Mitochondria- and VDAC-associated HK1 and HK2 inhibit the opening of the mitochondrial membrane permeability transition pore (PTP), and hexokinase-produced ADP is recycled for complex V of the respiratory chain, which contributes to the sustainability of mitochondrial membrane potential and prevents ROS overproduction and apoptosis (da-Silva et al., 2004; Heneberg, 2019; Santiago et al., 2008; Waskova-Arnostova et al., 2015). Expression and activity of HKI and HK2 in the heart ventricles are increased by intermittent hypobaric hypoxia, likely contributing to an adaptation to chronic hypoxia and increasing the resistance to ischemia-reperfusion injury of the heart (Waskova-Arnostova et al., 2015). Dysregulation of dopamine signaling may play a role in the pathophysiology of psychiatric disorders, such as schizophrenia. Dopamine-activated D1 receptor decreases mitochondrial hexokinase activity and impairs ROS modulation, resulting in increased $H_2O_2$ production and decreased mitochondrial calcium uptake in neural stem cells. Mitochondrial hexokinase in neural stem cells derived from Schizophrenia patients is unable to reduce mitochondrial ROS, suggesting a critical role of hexokinase dysregulation in Schizophrenia development (Assis-de-Lemos et al., 2021). HK2 is also linked to endothelial dysfunction in diverse vascular diseases, including diabetes. Acidic fibroblast growth factor (aFGF), which has been clinically employed to facilitate wound/burn repair and ulcer regeneration in diabetes, markedly decreased mitochondrial superoxide generation in both diabetic (db/db) mice and endothelial cells through Wnt/β-catenin/c-Myc axis-upregulated HK2 expression (Sun et al., 2021).

In addition to its mitochondria-associated activity, HK2 gains non-canonical function under glucose starvation and proinflammatory cytokine IL-1β treatment, which induces its nuclear translocation. In the nucleus, HK2 functions as a transcriptional coactivator of nuclear factor erythroid 2-related factor 2 (Nrf2) to upregulate xanthine oxidoreductase (XOR) expression. The increased XOR generates reactive oxygen and nitrogen species to enhance cytotoxicity and inflammation (Sheikh et al., 2018).

## 9. Hexokinases in cell proliferation and tumor invasion and migration

The microsporidian *Nosema (*N.) *bombycis* is an obligate intracellular parasite of *Bombyx mori*. *N. bombycis* hexokinase (NbHK) can be secreted and localized to the nucleus and cytoplasm of host cells. Depletion of NbHK in the host cells inhibits the proliferation of *N. bombycis*, indicating that the parasite controls its host cells to support *N. bombycis* proliferation by secreting NbHK (Huang et al., 2018).

FGF-induced and c-Myc-upregulated HK2 is also involved in vascular development. A decrease in HK2 expression decreases glycolysis and impairs endothelial cell proliferation and migration. Panendothelial- and lymphatic-specific HK2 knockout results in blood and/or lymphatic vascular defects, whereas HK2 overexpression partly rescues the defects induced by FGF signaling suppression (Yu et al., 2017). In HCC cells, hypoxia stimulation enhances HIF-1α-dependent HK2 expression, which promotes HCC cell proliferation. HK2 inhibition suppresses HCC proliferation under hypoxic stimulation compared to normoxic conditions (Gwak et al., 2005). HK2 is also indispensable for glycolysis, proliferation, and migration of other types of cancer cells, including lung cancer cells (Roberts & Miyamoto, 2015). During oncogenic stress-induced senescence, decreased glucose uptake and metabolism were observed, and overexpression of HK2 promotes G-6-P-dependent hexosamine biosynthesis and counteracts the senescence (Gitenay et al., 2014).

Overexpression of hexokinases, especially HK2, has been shown to be important for the invasion and migration of glioblastoma cells (Wolf et al., 2011), medulloblastoma cells (Gershon et al., 2013) and breast cancer cells (Jiang et al., 2012). HK2 expression is enhanced in primary lung cancer tissue and metastatic foci, and this upregulation is reduced by depletion of KRas or overexpression of p53 or KEAP1. HK2 depletion dampens the migration and invasion of NSCLC cells, sensitizes tumors to cisplatin treatment, and decreases lung metastasis rates in mice (Zhao et al., 2020). In addition, decreased HK2 expression reduces the expression of genes in vascular endothelial growth factor (VEGF)-A signaling, a pathway important for angiogenesis and metastasis, and inhibits the growth of metastasis of mouse tumors derived from pancreatic ductal adenocarcinoma (PDAC) cells (Anderson et al., 2017). HK2 expression is associated with advanced stage and high-grade ovarian cancer. Ovarian cancer-associated fibroblast-derived IL-6 upregulates HK2, which promotes ovarian cancer cell metastasis and stemness through FAK/ERK-enhanced expression of metastasis-related MMP9 and stemness-related NANOG and SOX9 (Siu et al., 2019). In gastric cancer cells, the stem cell factor SALL4, a zinc finger DNA-binding transcription factor, increases HK2 expression and glycolysis. HK2 depletion reverses the promoting effect of SALL4 on cell proliferation, migration, and invasion (Shao et al., 2020). In SCLC cells, highly expressed A Disintegrin And Metalloproteinase 12 (ADAM12S) promotes the proliferation, migration, and invasion of SCLC cells by upregulating HK1 (Duan et al., 2019).

In colorectal cancer cells, upregulation of HK3 is associated with the expression of genes involved in epithelial-mesenchymal transition (EMT) (Pudova et al., 2018). In lung adenocarcinoma cells, overexpressed HKDC1, which increases higher glucose consumption and lactate production, enhances proliferation, migration, and invasion of the tumor cells with inhibited AMP-activated protein kinase (AMPK) and activated mTOR (Wang et al., 2020). Knockdown of HKDC1 in breast cancer cells increase ROS levels, caspase-3 activity, and apoptosis (Chen et al., 2019).

Thus, HK2 and HK1, which are upregulated in a variety of tumor cells, promote tumor cell proliferation, invasion, and metastasis.

## 10. Hexokinases as therapeutic targets for cancer treatment

In view of their critical roles in cancer, hexokinases are considered promising targets for cancer treatment. Systemic HK2 ablation in genetic mouse models inhibits tumor development (Patra et al., 2013). Although HK1 is considered to be expressed in all tissues and HK2 is overexpressed in tumors, $HK1^{-}HK2^{+}$ tumor subpopulations exist among many types of cancers. HK2 depletion in $HK1^{-}HK2^{+}$ liver cancer cells considerably reduces xenograft tumor progression, in contrast to $HK1^{+}HK2^{+}$ cells (Chen et al., 2022; Xu & Herschman, 2019), suggesting that targeting HK2 in these $HK1^{-}HK2^{+}$ tumors could be a potentially effective strategy.

The hexokinase inhibitor 2-deoxy-D-glucose (2-DG) is a synthetic glucose analog in which the 2-hydroxyl group is replaced by hydrogen, which is metabolized by HK to 2-DG-6-phosphate (Kurtoglu et al., 2007). However, 2-DG-6-phosphate cannot be further metabolized to fructose-6-phosphate due to a lack of the 2-OH group, thereby inhibiting the glycolytic pathway. 2-DG treatment inhibits glycolysis, decreases ATP production, and induces mitochondrial oxidative stress in cancer cells, ultimately resulting in growth inhibition and apoptosis (Giammarioli et al., 2012; Ramírez-Peinado et al., 2011; Sinthupibulyakit et al., 2010; Zagorodna et al., 2012). Several phase I/II clinical trials have demonstrated that the oral administration of 2-DG alone or in combination with chemotherapy/radiotherapy is well tolerated without serious or severe adverse events in advanced solid tumors (Dwarakanath et al., 2009; Raez et al., 2013; Singh et al., 2005; Stein et al., 2010). However, a phase I trial recommended a high dose of 2-DG (63 mg/kg) in combination with docetaxel for cancer treatment and suggested that the antitumor activity of 2-DG is relatively weak (Raez et al., 2013).

Through structure-based virtual ligand screening, benderizine, a dopadecarboxylase inhibitor, was identified as a hexokinase inhibitor with higher selectivity against HK2 (IC50: 5.52 μM *in vitro*) than HK1 and HK4 (IC50: 25.13 μM and 40.53 μM *in vitro*, respectively) through glucose competitive and noncompetitive binding mechanisms. Benserazide inhibits glycolysis and cell proliferation, induces apoptosis in colorectal cancer cells (CRC) and suppresses tumor growth in a CRC xenograft model (Li et al., 2017). Benitrobenrazide, an arylhydrazide derivative, exhibits potent inhibitory activity against HK2 (IC50: 530 nM) *in vitro* (Liu et al., 2020). In HK2-overexpressing CRC, benitrobenrazide blocks glycolysis and exerts antitumor activity *in vitro* and *in vivo*.

## 11. Conclusions and future perspectives

Metabolic enzymes can possess both metabolic and nonmetabolic functions (Bian et al., 2022; Li et al., 2018; Lu & Hunter, 2018; Xu et al., 2019, 2021a). Hexokinases play vital roles in both physiology and pathologies, such as cancer and immune diseases, by regulating glycolysis and its related anabolic syntheses, immune responses in immune cells and immune-target cells, DNA damage repair, redox homeostasis, cell survival, apoptosis, autophagy, cancer cell stemness, proliferation, invasion, and migration. Importantly, both canonical and moonlighting functions of hexokinases are involved in the regulation of these critical cellular activities. The demonstration that HK2 acts as a protein kinase that directly contributes to Warburg effect-promoted tumor cell immune evasion independent of HK2-mitochondrial localization and glycolytic function (Guo et al., 2022) provides critical insight into the multifaceted roles of HK2 in tumor progression. Given that protein kinases, which differ from metabolic enzymes that only conduct one metabolic reaction, can phosphorylate different protein substrates in response to diversified upstream signaling, the unexpected functions of hexokinase protein kinase activities are expected to be revealed in the future and will broaden the understanding of the physiological and pathological functions of hexokinases.

Targeting hexokinases for cancer treatment has been intensively explored. However, the developed HK2 inhibitors have not yet been successfully progressed into the advanced clinical studies. Given the indispensable role of hexokinase-meditated glycolysis in maintaining energy supply and anabolic homeostasis in normal cells and phycological functions of vital organs, such as brain and heart, inhibition of glycolytic activity of hexokinases unavoidably brings about intolerable side effects that impede the clinical application of hexokinase-canonical function inhibitors. The recent advances in discoveries of the specific roles of noncanonical functions of hexokinases, such as the protein kinase activity of HK2, in tumor progression shed light into the development of new and unique strategies that differentially intervene the tumor-specific noncanonical functions rather than canonical functions of hexokinases for cancer treatment. The future endeavor on this aspect will hopefully broaden our approaches in targeted cancer therapies.

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## Authors' contributions

Z.L. conceptualized the writing. Z.L., D.G., Y.M., and X.J. all together wrote the manuscript.

## Declaration of competing interest

The authors declare no competing interests.

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