

## Review

# Hexokinase II integrates energy metabolism and cellular protection: Acting on mitochondria and TORCing to autophagy

This article has been corrected since Advance Online Publication and corrigendum is also printed in this issue

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Accumulating evidence reveals that metabolic and cell survival pathways are closely related, sharing common signaling molecules. Hexokinase catalyzes the phosphorylation of glucose, the rate-limiting first step of glycolysis. Hexokinase II (HK-II) is a predominant isoform in insulin-sensitive tissues such as heart, skeletal muscle, and adipose tissues. It is also upregulated in many types of tumors associated with enhanced aerobic glycolysis in tumor cells, the Warburg effect. In addition to the fundamental role in glycolysis, HK-II is increasingly recognized as a component of a survival signaling nexus. This review summarizes recent advances in understanding the protective role of HK-II, controlling cellular growth, preventing mitochondrial death pathway and enhancing autophagy, with a particular focus on the interaction between HK-II and Akt/mTOR pathway to integrate metabolic status with the control of cell survival.

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## Facts

- Hexokinase II phosphorylates glucose to regulate glucose metabolism but also functions as a protective signaling molecule.
- Hexokinase II is the predominant isoform in insulin-sensitive tissues and upregulated in tumors.
- Hexokinase II expression is regulated by the Akt/mTOR pathway.
- Hexokinase II is phosphorylated by Akt leading to increased mitochondrial binding and mitochondrial protection.
- Hexokinase II binds and inhibits TORC1 to facilitate autophagy in response to glucose deprivation.

## Open Questions

- What is the molecular mechanism by which Akt-mediated phosphorylation of HK-II increases mitochondrial association?
- How is HK-II/TORC1 binding regulated by the level of glucose-6-phosphate?
- How significant is the physiological/pathophysiological regulation of autophagy mediated by HK-II *in vivo*?

- It remains unclear how mitochondrial HK-II prevents opening of the mitochondrial permeability transition pore.

## Hexokinase II in Metabolism

Hexokinases (HKs) catalyze the first committed step of glucose metabolism. Glucose transported through glucose transporters (GLUTs) on the plasma membrane is phosphorylated by HKs to produce glucose-6-phosphate (G-6P). The activity of HKs is inhibited by G-6P providing a feedback inhibition mechanism.<sup>1–5</sup> G-6P serves as a precursor for glycolysis (ATP) as well as the pentose phosphate pathway (NADPH and ribulose-5-P), glycogenesis (glycogen) and hexosamine biosynthetic pathway (UDP-GlcNAc)<sup>2–5</sup> (Figure 1a). HK therefore plays important roles in the regulation of anabolic and catabolic processes.

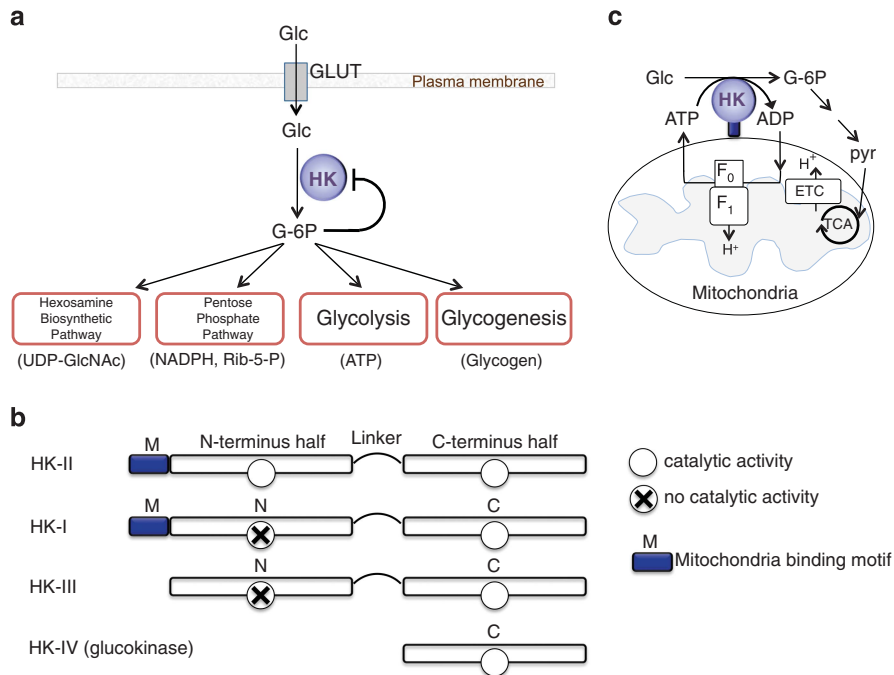
There are four isoforms of HK: I, II, III and IV (aka glucokinase) in mammalian tissues (Figure 1b). Glucokinase has a molecular mass of ~50 kDa while HK-I, II and III have a molecular weight of ~100 kDa resulting from the evolutionary duplication and fusion of a single domain HK molecule.<sup>2,4–6</sup> The catalytic activity of both domains is preserved in HK-II

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**Abbreviations:** 2-DG, 2-deoxy-D-glucose; Akt, murine thymoma viral (v-akt) oncogene homolog; AMPK, AMP-activated protein kinase; ANT, adenine nucleotide translocase; Bak, Bcl-2-antagonist/killer; Bax, Bcl-2-associated x protein; Bcl-2, B-cell leukemia/lymphoma-2; Bid, BH3-interacting domain death agonist; CyPD, Cyclophilin D; DMPK, myotonic dystrophy protein kinase; Fru-2,6-BP, fructose-2,6-bisphosphatase; G-6P, glucose-6-phosphate; GLUT, glucose transporter; GPI, glucose-6-phosphate isomerase; GSK-3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HK, Hexokinase; HIF-1, hypoxia-inducible factor 1; KO mice, knockout mice; miRNA, microRNA; mitoHK, mitochondrially associated hexokinase; MOM, mitochondrial outer membrane; mRNA, messenger RNA; mTOR, mammalian (mechanistic) target of rapamycin; mTORC1 and mTORC2, mTOR complex 1 and 2, respectively; mPTP, mitochondrial permeability transition pore; p53, tumor protein p53; PI3K, phosphatidylinositol 3-kinase; PHLPP, PH domain leucine-rich repeat protein phosphatase; ROS, reactive oxygen species; Thr, threonine; TIGAR, Tp53-induced glycolysis and apoptosis regulator; TG mice, transgenic mice; TOS motif, mTOR signaling motif; UCP, Uncoupling protein; UDP-GlcNAc, Uridine diphosphate *N*-acetylglucosamine; ULK1, unc-51-like autophagy-activating kinase 1; VDAC1, voltage-dependent anion channel 1

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**Figure 1** Hexokinase overview. (a) Metabolic roles of hexokinase. (b) Hexokinase subtypes. (c) Tight coupling of mitoHK to ATP generated by mitochondria provides energetic advantage

while activity is restricted to the carboxyl terminal domain of HK-I and HK-III. HK-I, II and III have higher affinity for glucose compared to HK-IV.<sup>2,4,5,7</sup> HK-I is the main isoform in the brain, but also ubiquitously expressed.<sup>4,5</sup> HK-II is the predominant isoform in insulin-sensitive tissues such as adipose, skeletal and cardiac muscle.<sup>8</sup> HK-III is also ubiquitously expressed but not predominant in any tissue while the expression of HK-IV, glucokinase, is restricted primarily to the liver and pancreas.<sup>2-5</sup>

Studies in the 1960s identified that significant quantities of HK-I and HK-II bind to the outer mitochondrial membrane<sup>9,10</sup> via a mitochondrial binding motif at the N-terminal.<sup>11,12</sup> HK-II binds to voltage-dependent anion channel 1 (VDAC1), the outer mitochondrial membrane protein, which interacts with the adenine nucleotide translocase (ANT), forming a contact site between the outer and inner membranes.<sup>3,4,13-16</sup> The mitochondrially bound HKs provide facilitation of coupling between glycolysis and oxidative phosphorylation through privileged/preferential access of HKs to ATP generated by mitochondria. The ADP generated by mitochondrial HK catalytic activity is shuttled back into mitochondria for rephosphorylation conferring metabolic advantage<sup>4,17,18</sup> (Figure 1c). The binding to mitochondria also undergoes feedback inhibition by G-6P.<sup>9,19-22</sup>

In the last decade, many seminal studies have emphasized the importance of metabolic re-programming in cancer biology. HK-II upregulation has been suggested to be a major contributor to the elevated glycolysis, even in the presence of oxygen, in cancer as first reported by Dr. Otto Warburg<sup>23</sup> in 1930 (Warburg effect). During tumor development in tissues normally expressing HK-IV, gene expression of HK-II is induced whereas HK-IV is silenced.<sup>24-28</sup> This switch from a lower to a higher affinity HK subtype can provide a means to

meet the high energy demand in tumors. Indeed, HK-II expression levels are closely associated with tumor grade and mortality in hepatocellular carcinoma.<sup>29</sup> Although in normal brain and low-grade gliomas HK-I is the predominant isoform, HK-II is highly upregulated in human glioblastoma multiforme<sup>30</sup> and poor prognosis is associated with upregulation of HK-II in human brain metastases of breast cancer.<sup>31</sup> Thus HK-II upregulation is considered a consequence of metabolic re-programming in cancer.

Akt is a serine/threonine kinase often upregulated in tumor cells. Akt is activated by a wide range of receptor stimuli to regulate diverse functions including glucose metabolism, cellular growth and survival through phosphorylation of many different target molecules at different cellular compartments.<sup>32-36</sup> One of the most established downstream effectors of Akt is the mammalian (mechanistic) target of rapamycin (mTOR), a serine/threonine kinase. mTOR forms two distinct functional complexes termed mTOR complex 1 and 2 (mTORC1 and mTORC2). Raptor and rictor are the defining component of mTORC1 and mTORC2, respectively.<sup>37-40</sup> In addition to its fundamental role in metabolism, it is becoming increasingly recognized that HK-II functions as a protective molecule, exerting antioxidant effects, direct protection of mitochondria against stress, and facilitation of autophagy under starvation. The direct molecular-molecular or functional interactions between HK-II and the Akt/mTOR pathways that have been identified act to integrate metabolism and cell survival, providing adaptive mechanisms in response to changes in the cellular environment. Below we summarize recent advances in our understanding of the transcriptional- and post-transcriptional interaction of these protective signaling molecules.

## Regulation of HK-II Expression by Akt/mTORC1 Pathway

HK-II is a constitutively active kinase and the changes in the expression level of HK-II directly impact cellular glucose metabolism. HK-II expression undergoes dynamic changes in various diseases. As mentioned earlier, the expression of HK-II is increased in many tumors while it is remarkably decreased in type-I diabetes (insulin-dependent diabetes).<sup>41–45</sup> Concomitantly, Akt activity is upregulated in tumors and insulin treatment recovers HK-II levels in diabetes.<sup>41–44</sup> The correlation between Akt activity and HK-II expression suggest the transcriptional regulation of HK-II by Akt, which has been supported by many studies. Insulin treatment increases HK-II mRNA and protein in various cell types and the increase is blocked by inhibition of PI3K, an upstream kinase of Akt, as well as inhibition of mTORC1, suggesting Akt/mTORC1 contribution<sup>44,46–51</sup> (Figure 2). Hyperactivity of mTORC1 is sufficient to increase HK-II expression<sup>52</sup> and a recent comprehensive and unbiased analysis also supports mTORC1-mediated HK-II upregulation and further demonstrated that mTORC1 signaling activates the genes encoding nearly every step of glycolysis.<sup>53</sup>

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor. The  $\alpha$ -subunit of HIF-1 (HIF-1 $\alpha$ ) is stabilized under hypoxic conditions, leading to the activation of a transcriptional program to adapt to the lack of oxygen. The HK-II promoter has a consensus motif for HIF-1<sup>54,55</sup> and HK-II expression is enhanced by hypoxia,<sup>54–57</sup> providing cellular protection<sup>52,56</sup> as well as a mechanism for elevated glycolysis in tumors.<sup>58–60,61</sup> HIF-1 $\alpha$  expression is also under the control of the PI3K/Akt/mTORC1 pathway,<sup>53,62–65</sup> linking Akt/mTOR activation and HK-II upregulation. mTORC1 increases HIF-1 $\alpha$  expression through regulation of transcription and translation of HIF-1 $\alpha$ ,<sup>53,59</sup> a mechanism distinct from the control of HIF-1 $\alpha$  protein stability. Indeed HIF-1 $\alpha$  expression is demonstrated to be responsible for mTORC1-induced HK-II upregulation.<sup>52</sup>

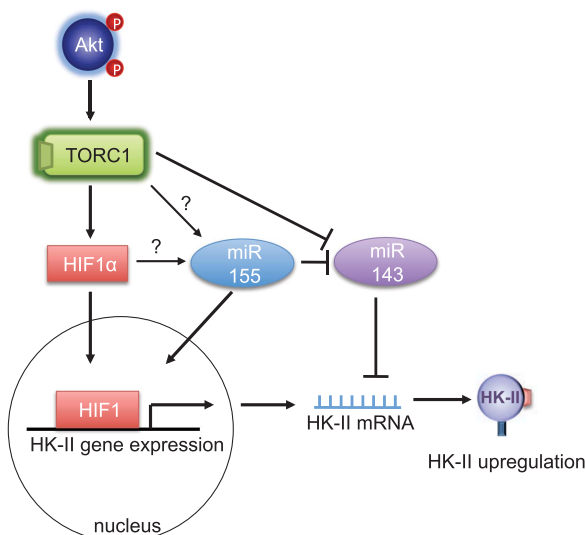
microRNAs (miRNAs) are small, non-coding RNAs and post-transcriptional inhibitory regulators of gene expression. miR-143, a tumor suppressor miRNA, which is downregulated

in many tumors,<sup>66</sup> targets HK-II mRNA.<sup>67–72</sup> miR-143 is also expressed in the heart where forced expression decreases the expression of HK-II.<sup>73</sup> Conversely, oncogenic miR-155 upregulates HK-II gene expression in tumor cells by downregulation of miR-143 as well as upregulating HK-II transcription.<sup>74</sup> In the context of regulation of miR-143 expression, it is worth pointing out that inhibition of mTOR is reported to increase the level of miR-143, and thus decrease HK-II expression, suggesting that mTORC1 suppresses miR-143 expression to increase HK-II.<sup>67,72</sup> The regulation of miR-143 expression could therefore be one of the mechanisms by which the Akt/mTORC1 pathway upregulates HK-II expression. The mechanism by which mTORC1 could inhibit miR-143 expression, that is mTORC1- and/or HIF-1-mediated upregulation of miR-155, has not been elucidated (Figure 2).

## Pro-survival Effect of HK-II

Protective effect of ectopic expression of HK-II have been established in many different cell types.<sup>75–82</sup> Conversely, decreased HK-II levels sensitize cells to apoptotic or necrotic stimuli. Overexpression of HK-II confers cellular protection against H<sub>2</sub>O<sub>2</sub> in cardiomyocytes<sup>79,80,82</sup> and also prevents maladaptive hypertrophy *in vivo* heart.<sup>82</sup> Heterozygotic HK-II knockout hearts are more susceptible to ischemia/reperfusion injury as well as pressure overload.<sup>83,84</sup> Overexpression of HK-II is proliferative and anti-apoptotic while reduction of HK-II expression in glioblastoma multiforme is anti-tumorigenic *in vivo*.<sup>30</sup> An earlier study revealed that increased glucose phosphorylation by co-expression of GLUT1 and HK-I in the hematopoietic cell line increases cytosolic NADPH levels and elicits an anti-apoptotic effect.<sup>85</sup> Thus the protective effect of HK-II could be due to its cytosolic activity to increase NADPH levels through the pentose phosphate pathway producing antioxidant effects.<sup>80</sup> This hypothesis is supported by a recent paper using cardiac-specific HK-II transgenic (TG) mice.<sup>82</sup> Cardiac hypertrophy induced by isoproterenol infusion is attenuated in the TG mice and this is accompanied by reduced reactive oxygen species (ROS) levels in cardiomyocytes overexpressing HK-II. The anti-hypertrophic and antioxidant effects of overexpression of HK-II are reversed by inhibition of glucose-6-phosphate dehydrogenase, an enzyme responsible for reducing NADP to NADPH, suggesting that HK-II attenuates cardiac hypertrophy by decreasing ROS via increased pentose phosphate pathway flux.<sup>82</sup>

Further, mitochondrially associated HKs (mitoHKs) can exert protective effects on mitochondria to prevent mitochondrial death pathways. A major mitochondrial death pathway is elicited by apoptotic Bcl-2 family proteins such as Bax and Bak and/or the mitochondrial permeability transition pore (mPTP), a mega channel formed at the mitochondrial inner membrane.<sup>86–90</sup> Activated Bax/Bak forms a pore at the mitochondrial outer membrane (MOM) resulting in a release of apoptotic factors from the intra-membrane space.<sup>86,91</sup> MitoHK-II antagonizes apoptotic Bcl-2 family proteins and thereby protects cells against apoptotic stimuli.<sup>15,78,92–95</sup> MitoHK-II competitively inhibits Bax binding to mitochondria<sup>15,78</sup> and also antagonizes truncated Bid (tBid) induced Bax/Bak-mediated apoptosis.<sup>94</sup> MitoHK-II also provides protection against the mPTP. Mitochondrial Ca<sup>2+</sup>



**Figure 2** Regulation of HK-II expression mediated by Akt/mTORC1 pathway

overload and/or ROS induce opening of the mPTP, resulting in a large amplitude permeability of the inner membrane, consequent rupture of the outer mitochondrial membrane and resultant necrotic (and somewhat apoptotic) cell death.<sup>86–90</sup> Majewski *et al.*<sup>93</sup> demonstrated that mitoHK-II provides cellular protection against Ca<sup>2+</sup> overload even in Bax and Bak double-knockout cells. We and others have also reported that an increase in mitoHK-II has an inhibitory effect on Ca<sup>2+</sup>- and ROS-induced mPTP opening and that a large dissociation of mitoHK-II sensitizes mPTP opening and cell death.<sup>77,80,82,83,96–98</sup>

A series of studies have demonstrated that mitoHK-II has an ability to decrease ROS generation at mitochondria to prevent opening of the mPTP.<sup>77,80,82,84,99</sup> As mentioned earlier, mitoHK ensures tight coupling of glucose phosphorylation and mitochondrial ATP generation. This increases electron flow in the mitochondria and thus decreases mitochondrial membrane potential, which can reduce mitochondrial ROS emission.<sup>77,99</sup> It has also been demonstrated that mitoHK-II has a direct inhibitory effect on the mPTP. The mechanism for the protection is not known, largely because the molecular composition of mPTP is not fully identified. However, cyclophilin D (CyPD) is established to be a critical positive regulator since its gene deletion results in remarkable reduction in the sensitivity of opening of the mPTP and confers strong protection.<sup>100,101</sup> Interestingly, CyPD activity stabilizes mitoHK-II binding and forced dissociation of mitoHK-II leads to disruption of CyPD binding to ANT, suggesting a functional link between a stimulatory (CyPD) and an inhibitory (HK-II) molecule of the mPTP.<sup>96,102–105</sup> It seems likely that the interaction between HK-II and CyPD is mediated by the previously mentioned VDAC/ANT interaction.<sup>106,107</sup> However, genetic evidence revealed that neither VDAC nor ANT is the main pore-forming component<sup>108–111</sup> and thus it is still not clear whether the indirect interaction of HK-II with CyPD plays a direct role in regulation of the mPTP. A recent seminal study proposed that the dimer of F<sub>0</sub>F<sub>1</sub> ATP synthase could be the pore-forming core components of the mPTP and the sensitivity of the pore formation is positively regulated by CyPD binding to the synthase.<sup>112</sup> It will be of considerable interest to determine whether mitoHK-II binding has a regulatory role on CyPD binding to F<sub>0</sub>F<sub>1</sub> ATP synthase to inhibit pore formation.

It has been disputed whether Bax/Bak regulates mPTP opening. Recent genetic studies revealed that deletion of Bax/Bak inhibits mPTP opening and necrotic cell death induced by Ca<sup>2+</sup> overload or ROS, suggesting the ability of Bax/Bak to facilitate mPTP-induced cell death.<sup>113,114</sup> Thus the aforementioned mitoHK-II-mediated inhibition of Bax binding to mitochondria could contribute to the anti-mPTP effect of mitoHK-II. Further studies will be required to delineate the interaction of these molecules to determine the mechanism by which HK-II prevents mPTP opening.

Although HK-I has similar pro-survival properties to HK-II, HK-II is preferentially upregulated by cancers. What benefits does HK-II therefore provide over HK-I? (1) The expression of HK-II is regulated by pro-survival/stress response pathways, which are necessarily activated for cancer survival and growth. (2) The catalytic activity of both N- and C-terminus is preserved in HK-II, but not in HK-I nor in -III, and these two domains in HK-II are different in their affinities to

G-6P-dependent catalytic inhibition. The C-terminal half has ~30-fold higher *K<sub>i</sub>* value than the N-terminal half (which is similar to HK-I), and HK-II could retain its catalytic activity under conditions in which HK-I catalytic activity is inhibited.<sup>1,5,115</sup> (3) Adaptability. As discussed below, Akt phosphorylates HK-II leading to increase in mitoHK-II. Although HK-I binds to mitochondria and provides protection, it lacks an Akt phosphorylation site and thus is not regulated by Akt.<sup>20</sup> Akt-mediated regulation of mitoHK-II may render HK-II more regulatory and versatile to allow tumor cells adapt to changes in the metabolic condition. Glycogen synthesis and the pentose phosphate pathway are upregulated in tumor cells subjected to hypoxia.<sup>116</sup> Thus, in response to ischemia, in which Akt activity and mitoHK-II are decreased (resulting in increased cytosolic HK-II), HK-II would stimulate these cytosolic events to preserve cellular homeostasis. (4) As discussed later, non-mitochondrial HK-II facilitates autophagy induced by glucose withdrawal,<sup>117</sup> suggesting regulation of energy conservation by HK-II.

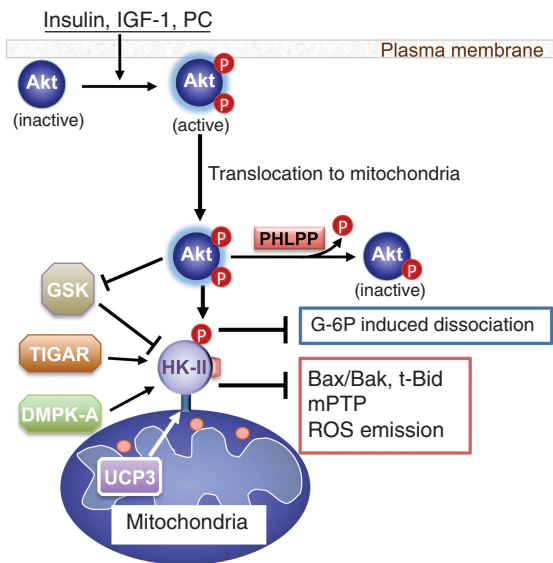
### Akt and Mitochondrial HK-II in Protection

Akt stimulates glycolysis and also elicits strong cell survival in many organs.<sup>32–36</sup> Earlier studies found that expression of active Akt increases mitochondrial HK activity and the anti-apoptotic effect of Akt required mitoHK.<sup>118</sup> The aforementioned inhibitory effect of mitoHK-II on tBid is also downstream of Akt, contributing to Akt-mediated protection.<sup>94</sup> Further, mitoHK-II increased by Akt overexpression confers protection in Bax/Bak null cells, suggesting inhibition of mPTP<sup>93</sup> and our previous work in cardiomyocytes also showed that Akt activation increases the level of mitoHK-II leading to inhibition of mPTP opening and cell death.<sup>79,97</sup> In the heart, in which HK-II is the predominant isoform, mitoHK-II activity is known to be increased by many cardioprotective interventions such as insulin, morphine and ischemic preconditioning.<sup>119–121</sup>

Given that Akt leads to increased mitoHK-II, we explored the possibility that Akt directly phosphorylates HK-II. Our previous study revealed that HK-II, but not HK-I or HK-III, has an Akt consensus sequence at position Thr473 (RARQKT\*), which is preserved in mouse, rat and human.<sup>97</sup> We found that Akt, activated by receptor agonists, translocates to mitochondria and phosphorylates HK-II at Thr473, a critical step in Akt-mediated mitoHK-II increase and protection in cardiomyocytes<sup>79,97</sup> (Figure 3). Other studies also support that Akt increases mitoHK-II in cardiac and non-cardiac cells.<sup>20,122–126</sup>

PHLPP (PH domain leucine-rich repeat protein phosphatase) is a protein phosphatase 2C family member which has been discovered to dephosphorylate and inhibit Akt.<sup>126–130</sup> Expression of PHLPP is repressed in cancer cells resulting in elevated Akt activation.<sup>128,130</sup> Interestingly, our study using PHLPP1 knockout (KO) mice suggest that PHLPP1, in addition to cytosol, localizes at mitochondria and negatively regulates mitochondrial Akt activity, decreasing mitoHK-II.<sup>124</sup> These findings have been confirmed in non-cardiac cells.<sup>126</sup> Thus the levels of mitoHK-II are regulated through a dynamic balance between kinase and phosphatase.

We demonstrated that phosphorylation of HK-II at Thr473 acted to decrease the sensitivity of HK-II to G-6P induced



**Figure 3** MitoHK-II regulation and mitochondrial protection

mitochondrial dissociation thereby increasing the net binding.<sup>79</sup> The kinetics of G-6P product inhibition of HK-II catalytic activity was not affected by phosphorylation, indicating that this modification does not reduce the affinity of G-6P binding to the kinase.<sup>79</sup> Although it is unclear how phosphorylation decreases the sensitivity of the kinase to G-6P-mediated dissociation, Thr473 in HK-II is located at the 'linker' region connecting the two kinase halves, thus phosphorylation could induce conformational change of the kinase to increase binding to its mitochondrial protein partner. HK-I has been suggested to form a tetramer preferentially at mitochondria, which becomes more resistant to product inhibition relative to the cytosolic monomeric kinase.<sup>3,4,131,132</sup> Although the possibility of tetramerization of HK-II has not been studied, it is notable that the region in HK-I analogous to the HK-II Thr473 region contains negative charges compared to the more positive HK-II. By adding back a negative charge to this region, Akt-mediated phosphorylation may therefore facilitate HK-II tetramer formation at mitochondria, suppressing G-6P-dependent mitoHK-II release.

### Other Modulation of mitoHK-II

Emerging evidence has revealed other mechanisms to regulate the level of mitoHK-II (Figure 3). (1) In addition to direct modification of HK-II, Akt has been demonstrated to increase mitoHK-II through inhibition of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ). GSK-3 $\beta$  is a constitutively active kinase and phosphorylates VDAC, inhibiting HK-II binding.<sup>87,92,133,134</sup> Akt phosphorylates and inhibits GSK-3 $\beta$  resulting in decreased suppression of mitoHK-II. (2) A recent study revealed another kinase-dependent regulation of mitoHK-II. Mutation in the myotonic dystrophy protein kinase (DMPK) gene, coding a serine/threonine kinase, causes myotonic dystrophy type 1. DMPK-A is a subtype which preferentially associates with mitochondria. DMPK-A forms a molecular complex with Src and HK-II at the MOM in skeletal muscle cells subjected to oxidative stress and stabilizes mitoHK-II

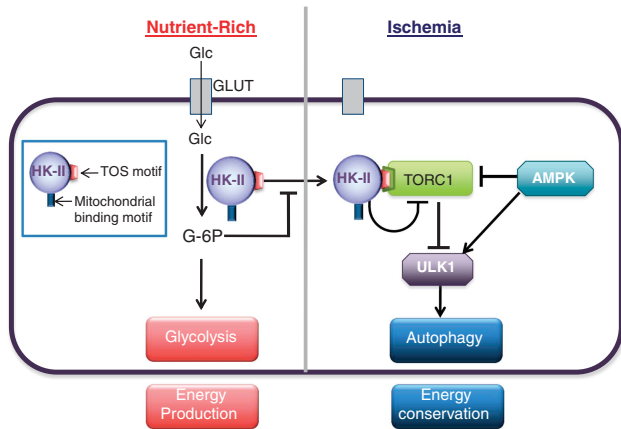
binding to confer antioxidant protection.<sup>135</sup> (3) Uncoupling proteins (UCPs) are mitochondrial anion carrier proteins that mitigate mitochondrial ROS emission, probably by regulating the mitochondrial membrane potential.<sup>136</sup> A recent study using UCP3 KO mice showed that UCP3 enhanced mitoHK-II binding in skeletal muscle, participating in a UCP3-mediated decrease in ROS emission.<sup>99</sup> (4) Another intriguing regulatory mechanism of mitoHK-II was obtained from studies of p53, a key tumor suppressor. In addition to the established role of p53 in cell cycle arrest and apoptosis, recent evidence has demonstrated that p53 reduces glycolytic flux and increases oxidative phosphorylation, halting the Warburg effect.<sup>137–143</sup> TIGAR (Tp53-induced glycolysis and apoptosis regulator) was identified to function as a fructose-2,6-bisphosphatase (Fru-2,6-BP), antagonizing the third step of glycolysis and thereby redirecting metabolite flux to the pentose phosphate pathway.<sup>138–140</sup> Interestingly, basal or low levels of p53 expression have been suggested to provide antioxidant effects and thus elicit cellular protection rather than cell death.<sup>138–140</sup> A recent study has discovered an unexpected mechanism of TIGAR to decrease ROS generation at mitochondria.<sup>144</sup> TIGAR, upon hypoxia, translocates to mitochondria and interacts with mitoHK-II, stabilizing mitoHK-II and thereby decreasing mitochondrial ROS generation and providing cellular protection. This effect was independent of its Fru-2,6-BP activity. Thus TIGAR inhibits oxidative stress through two distinct mechanisms in cytosol and at mitochondria. Taken together this new evidence reveals that mitoHK-II is a convergence of various protective signals to adapt to stress conditions.

### HK-II-mediated Regulation of mTORC1 and Autophagy

In the face of limited energy availability, cells initiate a process of catabolic self-digestion termed macroautophagy (hereafter referred to as autophagy) to ensure cellular energy homeostasis and survival. Autophagy means self-eating in Greek and can function as an intracellular recycling system by which intracellular contents and damaged organelles undergo degradation through their sequestration within autophagosomes and lysosomal degradation to supply energy substrates.<sup>145–153</sup>

mTORC1 is a critical negative regulator of autophagy.<sup>40,59,154</sup> mTORC1 phosphorylates and inhibits the autophagy-activating kinase ULK1.<sup>155,156</sup> Upon nutrient deprivation, the activity of mTORC1 is suppressed and AMPK is activated. Consequently, ULK1 is activated and initiates membrane nucleation, which is followed by sequestration and degradation.<sup>155–160</sup> Although it has been widely accepted that autophagy is induced in response to metabolic suppression, the molecular links between metabolic and autophagic pathways have not been fully elucidated. Recently, we made the surprising discovery that HK-II facilitates autophagy in response to glucose deprivation to protect cells<sup>117</sup> (Figure 4).

The initial observation for this unexpected role of HK-II was derived from experiments using 2-deoxy-D-glucose (2-DG). 2-DG is an analog of glucose, which is phosphorylated by HKs but not metabolized further, thus it is used as a HK inhibitor by many studies and in clinical trials for cancer therapy.<sup>161–163</sup> We observed that a low concentration of 2-DG (500  $\mu$ M compared



**Figure 4** HK-II binds to and inhibits mTORC1 to facilitate autophagy in response to glucose deprivation

with 5-25 mM commonly used<sup>164–166</sup>) inhibited autophagy development in the absence of glucose in cardiomyocytes. This observation implied that HK-II acts to stimulate autophagy in the absence of its substrate. Autophagy induced by glucose deprivation is attenuated by HK-II knockdown but potentiated by its overexpression. By comparison, HK-I knockdown or overexpression does not affect the development of autophagy. These observations suggest that HK-II can switch between energy production and energy conservation pathways dependent on the availability of glucose. Remarkably this autophagic effect of HK-II is independent of its mitochondrial binding as well as its kinase-activity, suggesting a previously unrecognized, non-mitochondrial scaffold function to confer cellular protection. A series of experiments demonstrated that HK-II binds to mTORC1 in the absence of glucose, decreasing mTORC1 activity.<sup>117</sup> This interaction was mediated by an mTOR signaling motif (TOS motif), identified at positions 199-203 (FDIDI) in HK-II but absent in HK-I. The sequence is found in various species, including mouse, rat, horse, boar and human HK-II, suggesting that the TOS motif in HK-II is well conserved. The TOS motif is critical for mTORC1 substrate binding to raptor and subsequent phosphorylation by mTOR.<sup>167,168</sup> Notably, expression of HK-II with a mutated TOS motif fails to bind to or inhibit mTORC1 and does not increase autophagy induced by glucose deprivation. Thus, in the absence of glucose, HK-II binds mTORC1 via its TOS motif, acting as a decoy substrate to facilitate autophagy.<sup>117</sup> As discussed earlier, mTORC1 activation stimulates HK-II expression under growth conditions conferring metabolic support while HK-II provides negative feedback to mTORC1 under starvation to stimulate autophagy. This dual regulation for HK-II and mTOR provides an adaptive mechanism to preserve cellular homeostasis dependent on metabolic status.

What regulates the switch between the glycolytic and autophagic roles of HK-II? We found that, in contrast to 2-DG, the glucose analog 5-thio-glucose, which binds to HK-II but cannot be phosphorylated, does not inhibit autophagy induced by glucose deprivation.<sup>117</sup> This suggests that phosphorylated glucose, but not glucose binding, is the key factor responsible for suppressing the autophagic effect of HK-II. To support this hypothesis, it was demonstrated that expression of a kinase-dead HK-II mutant (in the absence of

endogenous HK-II) stimulated autophagy that was insensitive to inhibition by glucose, while expression of WT HK-II retained glucose dependent regulation.<sup>117</sup> These observations lead us to conclude that accumulation of G-6P results in inhibition of the autophagic effect of HK-II in glucose-rich conditions.

It remains to be determined how G-6P inhibits the binding of HK-II to mTORC1. We speculate that G-6P changes the intracellular localization of HK-II to regulate the association of HK-II with mTORC1. As mentioned above, mitochondria are not the location where the interaction occurs, thus it will be critical to identify the intracellular compartment for the interaction triggered by decreased level of G-6P. Recent studies have identified lysosomes as an activation site for TORC1.<sup>169–171</sup> In response to a decrease in G-6P levels, HK-II may translocate to the lysosome to inhibit mTORC1. Although much additional study will be required to determine the precise mechanism by which G-6P inhibits the binding of HK-II to mTORC1, nonetheless, it provides a molecular basis for previous observations showing that mTOR activation requires glucose in cardiomyocytes<sup>172</sup> and that an increase in the work load in the heart is associated with G-6P accumulation and mTOR activation.<sup>173</sup> This suggests that HK-II acts as a glucose/G-6P sensor, allowing it to monitor and react to changes in status of glucose metabolism, to adjust the balance between glycolysis-mTORC1-mediated growth and autophagy-mediated preservation of energy homeostasis. This signaling axis could shed light on the mechanism underlying some metabolic disorder diseases, since mTORC1 has been demonstrated to control biosynthesis of protein, nucleotides and lipids.<sup>174</sup> For example, glucose-6-phosphate isomerase (GPI) deficiency is the second most frequent cause of inherited glycolytic erythroenzymopathy in humans.<sup>175</sup> GPI catalyzes the second step of glycolysis, reversible interconversion of G-6P and fructose-6-phosphate. It is reported that GPI deficiency results in an accumulation of G-6P and mTOR activation. Activated mTORC1 directly phosphorylates lipin-1 and inhibits its localization at ER as well as to nucleus.<sup>176</sup> Lipin-1 positively regulates triacylglycerol production at ER.<sup>176</sup> Glycerolipid deficiency is suggested to be associated with non-spherocytic hemolytic anemia in GPI-deficient patients.<sup>177</sup> In addition, nuclear lipin-1 interacts with transcription factors to regulate genes, thereby regulating fatty acid oxidation and lipogenesis.<sup>176</sup> These complex transcriptional events mediated by mTORC1/lipin-1 would also relate to the pathologies in GPI-deficient patients. Taken together it will be of considerable importance to determine the physiological and pathophysiological roles of HK-II-mediated control of mTORC1 in various *in vivo* settings.

#### Overall Summary of Interaction Between HK-II and Akt/TORC1 Pathway

It is intriguing that HK-II, the predominant isoform in insulin-sensitive tissues and often upregulated tumors, closely interplays with Akt/mTOR pathway. Akt mediated increases in HK-II expression and mitochondrial association support the growth response through efficient energy production<sup>4,17,18</sup> as well as preserving mitochondrial integrity.<sup>3,15,28,76,77,79,80,93,96,97,118,178</sup> In contrast, regulation of the pentose phosphate pathway or glycogenesis by HKs is considered as a cytosolic event. Thus

Akt-mediated phosphorylation endows HK-II with diverse and complex roles to regulate energy production *versus* storage, and cell growth and survival by changing its intracellular localization. This is in sharp contrast to HK-I, which is unregulated by Akt.<sup>20,49,179,180</sup> HK-I more tightly binds to mitochondria with less sensitivity to G-6P-dependent dissociation than HK-II, rendering it primarily a glycolytic enzyme<sup>4,5</sup> which provides logical support for its predominant expression in the brain, a tissue highly dependent on glucose for energy generation. MitoHK-I displays similar anti-apoptotic characteristics to mitoHK-II thus HK-I appears to be a more specialized isoform geared primarily to facilitate glycolysis and mitochondrial protection. During ischemia, limited energy supplies and delivery of growth factors to cells could reverse the regulation, from Akt/mTORC1-mediated regulation of HK-II to HK-II-mediated inhibition of the mTORC1 pathway stimulating autophagy to prevent metabolic collapse.<sup>117</sup> From the perspective of mammalian HK evolution, it is interesting that HK-II, but not other isoforms, contains the Akt consensus sequence and TOS motif and also retains two active kinase domains since it is postulated that HK-II mostly closely resembles the ancestral 100 kDa HK.<sup>7</sup> Thus through evolutionary changes and resultant generation of four isoforms, HKs acquire isoform-specific diversity in their properties; two intact kinase domains (HK-II), Akt-mediated phosphorylation (HK-II), TOS motif to regulate mTORC1 (HK-II), mitochondrial binding (HK-I and HK-II) and two domains *versus* one domain (HK-I, II, III *versus* IV) to fulfill distinct, physiologically important functions in different tissues.

## Conclusion

Accumulating evidence reveals that metabolic and cell survival pathways are closely connected and functionally interdependent. Direct molecular signals from metabolic to cell survival pathways allow prompt detection of metabolic status to rapidly activate adaptive responses to ensure preservation of cellular homeostasis. HK-II, the first molecule in glycolysis, serves as a critical nexus of integration among energy production, preservation of mitochondrial integrity and cell viability, as well as energy conservation (Figure 5). These

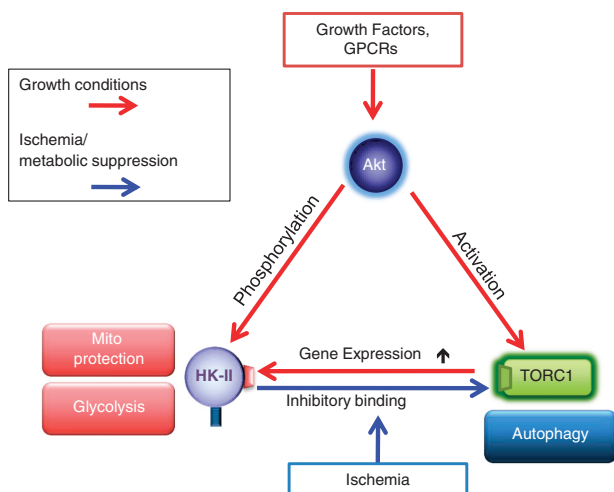
diverse effects of HK-II are achieved by changes in its expression levels, intracellular distribution and molecular binding through an interplay with the Akt/mTORC1-signaling pathways. These findings set the stage for further exploration of the potential importance of HK-II in physiological, as well as pathophysiological settings such as cancer, diabetes, inflammation and heart disease, which would lead to new insights for therapeutic interventions.

## Conflict of Interest

The authors declare no conflict of interest.

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**Figure 5** Interaction between HK-II and Akt/mTORC1 pathway

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