# HYPERSENSITIVITY TO CELL DEATH UNDER GLUCOSE STARVATION INVOLVES OXIDATIVE STRESS AND AMPK INSTABILITY

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**DECLARATION**

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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## SUMMARY

AMP-activated protein kinase (AMPK) is an evolutionarily conserved energy sensor and regulator in mammalian cells, activated upon stress conditions including nutrient starvation, oxidative stresses, etc. It has been demonstrated that AMPK activity can be positively controlled by upstream kinases (liver kinase B1 (LKB1), calmodulin-activated protein kinase kinase 2 (CaMKKβ/CaMKK2), and transforming growth factor-beta-activated kinase 1 (TAK1)), and negatively regulated by phosphatases like protein phosphatase 2A (PP2A) and protein phosphatase 2C (PP2C). However, regulation of AMPK activity by protein stability is rarely investigated. Therefore, the main objective of this study is to investigate the involvement of protein stability in AMPK down regulation upon metabolic stress condition (glucose starvation), and further to elucidate the role of oxidative stress induced by energy deficiency in AMPK protein instability.

In this study, we first discovered that LKB1-mutant non-small cell lung cancer cell line NCI-H460 was particularly hypersensitive to glucose starvation. In response to metabolic stress induced by glucose starvation, cellular reactive oxygen species (ROS) were significantly elevated, accompanied by rapid AMPK phosphorylation and activation. However, prolonged depletion of glucose for 3 hours markedly reduced AMPK protein level, which cannot be suppressed by proteasome inhibitors and lysosome

inhibitors. Only glycolysis inhibitor 2-deoxyglucose (2DG) and antioxidant N-acetylcysteine (NAC) were able to reduce ROS level, stabilize AMPK protein and eventually protect against cell death. Further studies will focus on the molecular mechanism by which AMPK is down regulated upon glucose starvation, especially post-translational modification of AMPK.

Taken together, our data demonstrate that AMPK protein stability and activity was negatively regulated under glucose starvation, leading to rapid cell death.

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 2DG | 2-deoxyglucose |
| ACC | acetyl-CoA carboxylase |
| ADP | adenosine diphosphate |
| AICAR | 5-amino-4-imidazolecarboxamide ribonucleoside |
| AID | auto-inhibitory domain |
| AMP | adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| Apaf-1 | apoptosis protease-activating factor-1 |
| ATCC | American Type Culture Collection |
| Atg | autophagy related |
| ATM | ataxia-telangiectasia mutated |
| ATP | adenosine triphosphate |
| BAF | bafilomycin |
| BRSK1/2 | brain-specific serine/threonine-protein kinase 1/2 |
| BSA | bovine serum albumin |
| CaMKK2/CaMKKβ | Ca2+/calmodulin-activated protein kinase kinases |
| CBM | carbohydrate-binding module |
| CBS | cystathionine β-synthase |
| CD | cluster of differentiation |
| CD36 | cluster of differentiation 36 |
| CD95 | cluster of differentiation 95 |
| CD95L | cluster of differentiation 95 ligand |
| cIAP1 | cellular inhibitor of apoptosis 1 |
| cIAP2 | cellular inhibitor of apoptosis 2 |
| CMA | chaperone-mediated autophagy |
| $CO_2$ | carbon dioxide |
| CoA | coenzyme A |
| CQ | chloroquine diphosphate |
| CTD | c-terminal domain |
| cyt c | cytochrome c |
| DISC | death-inducing signaling complex |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DNA | deoxyribonucleic acid |
| DNP | dinitrophenol |
| DUBs | deubiquitinases |

| | |
|---|---|
| E1 | Ub-activating enzyme |
| E2 | Ub-conjugating enzymes |
| E3 | Ub ligases |
| EDTA | ethylenediaminetetraacetic acid |
| ER | endoplasmic reticulum |
| ERK | extracellular signal-regulated kinases |
| FADD | Fas-associated death domain |
| FAK | focal adhesion kinase |
| FAS | apoptosis stimulating fragment |
| FASL | FAS ligand |
| FBS | fetal bovine serum |
| FIP200 | scaffold focal adhesion kinase (FAK)-family-interacting protein of 200 kDa |
| GLUT1 | glucose transporter type 1 |
| GLUT4 | glucose transporter type 4 |
| $H_2O_2$ | hydrogen peroxide |
| HIF-1α | hypoxia-inducible factor-1α |
| HMG-CoA | 3-hydroxy-3-methylglutaryl CoA |
| JNK | c-Jun N-terminal kinases |
| LC3 | microtubule-associated protein light chain 3 |
| LKB1 | liver kinase B1 |
| MAP kinase | mitogen activated protein kinase, MAPK |
| MAP3K7 | mitogen-activated protein kinase kinase kinase 7, MEKK7 |
| MARK | MAP/microtubule affinity-regulating kinase |
| MEK | mitogen-activated protein kinase kinase |
| MEKK7 | mitogen-activated protein kinase kinase kinase 7, MAP3K7 |
| MG-132 | N-(benzyloxycarbonyl)leucinylleucinylleucinal |
| MO25 | mouse protein 25 |
| MOMP | mitochondria outer membrane permeabiliziation |
| mTOR | mechanistic/mammalian target of rapamycin |
| mTORC1 | mechanistic/mammalian target of rapamycin complex |
| NaCl | sodium chloride |
| $NaN_3$ | sodium azide |
| NCI | National Cancer Institute |
| NF-κB | nuclear factor kappa B |
| NP-40 | Nonidet P-40 |
| NSCLC | non-small-cell lung cancer |
| N-terminus | amino acid terminus |

| | |
|---|---|
| NuAK1/2 | novel (nua) kinase 1/2 |
| PARP | poly(ADP-ribose) polymerase |
| PAS | phagophore assembly site |
| PBS | phosphate buffered saline |
| PCD | programmed cell death |
| PGC1α | peroxisome proliferator-activated receptor-γ co-activator 1α |
| PI | propidium iodide |
| PJS | Peutz-Jeghers syndrome |
| PKB | Protein kinase B, also known as Akt |
| PKC | protein kinase C |
| poly-Ub | polyubiquitin |
| PP2A | protein phosphatase 2A |
| PP2C | protein phosphatase 2C |
| PPARγ | peroxisome proliferator-activated receptor gamma |
| PVDF | polyvinylidene difluoride |
| Raptor | regulatory associated protein of mTOR |
| RIP | receptor interacting protein |
| RIP1 | receptor interacting protein 1 |
| RIP3 | receptor interacting protein 3 |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| RPMI | Roswell Park Memorial Institute medium |
| SD | standard deviation |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | SDS polyacrylamide gel electrophoresis |
| Ser | serine |
| SIK1/2/3 | salt-inducible kinase 1/2/3 |
| SNRK | SNF (sucrose non-fermenting protein)-related serine/threonine-protein kinase |
| STE20 | Sterile 20 |
| STK11 | serine/threonine kinase 11 |
| STRAD | STE20-related adaptor protein |
| TAK1 | transforming growth factor-beta-activated kinase 1 |
| TBST | Tris Buffered Saline with Tween 20 |
| TCA cycle | mitochondrial tricarboxylic acid cycle |
| TGF | transforming growth factor |
| Thr | threonine |
| TIFIA | transcription initiation factor IA |
| TNFα | tumor necrosis factor α |

| | |
|---|---|
| TNFR1 | TNFα receptor 1 |
| TRADD | TNFR-associated death domain |
| TRAF2 | TNFR-associated factor 2 |
| TRAF5 | TNFR-associated factor 5 |
| TRAIL | tumour necrosis factor-related apoptosis-inducing ligand |
| TRAILR1 | TRAIL receptor 1 |
| TSC | tuberous sclerosis complex |
| TZD | thiazolidinedione |
| Ub | ubiquitin |
| ULK1 | Unc-51-like kinase |
| UPS | ubiquitin-proteasome system |
| ZMP | 5-amino-4-imidazolecarboxamide ribotide |
| Z-VAD | carbobenzoxy-Val-Ala-Asp-(OMe)-fluoromethylketone |

# CHAPTER 1 INTRODUCTION

## 1.1 AMPK

### 1.1.1 Overview of AMPK

AMP-activated protein kinase (AMPK) is an evolutionarily conserved energy sensor and regulator in most eukaryotic cells. As a pivotal checkpoint of metabolism, AMPK not only maintains cellular energy homeostasis, but also governs multiple cellular processes, including cell growth and proliferation, cell cycle, cell polarity, autophagy, mitochondrial biogenesis, etc. (Hardie, 2011b) Owing to its vital role in diverse aspects of physiology, AMPK stands in an essential position in both normal cells and tumor cells.

### 1.1.2 Structure of AMPK

AMPK is a heterotrimeric serine/threonine (Ser/Thr) kinase complex consisting of three subunits, a catalytic α-subunit and regulatory β-and γ-subunit. Mammalian cells have seven genes encoding AMPK complex, two isoforms of α-subunit (α1 and α2 by *prkaa1* and *prkaa2*), two of β-subunit (β1 and β2 by *prkab1* and *prkab2*), and three of γ-subunit (γ1, γ2 and γ3 by *prkag1*, *prkag2* and *prkag3*) (Chen et al., 2009; Hardie et al., 2012). This generates 12 combinations, and the expression of each isoform varies in different tissue types (Faubert et al., 2013; Hardie, 2011c).

The typical Ser/Thr kinase domain locates at the amino terminus (N-terminus) of the catalytic α-subunit. When the residue Thr172 within the

activation loop is phosphorylated by upstream kinases, AMPK will be activated (to be described in details below). The kinase domain is followed by an auto-inhibitory domain (AID), responsible for maintaining an inactive conformation of the kinase in the absence of AMP (Chen et al., 2009). The AID is connected to the α-subunit C-terminal domain (α-CTD) by a linker peptide.

The β-subunit harbors a C-terminal domain (β-CTD), which links α-CTD and γ-subunit to form the core of the complex (Xiao et al., 2007). The carbohydrate-binding module (β-CBM) is responsible for association with glycogen particles (Bendayan et al., 2009; Hudson et al., 2003). The β-subunits can be phosphorylated and myristoylated, which may affect the activation and intracellular localization of AMPK (Oakhill et al., 2010; Warden et al., 2001).

The γ-subunit contains four repeated sequences, termed as CBS (cystathionine β-synthase) repeat (Bateman, 1997; Hardie, 2011a), forming a flattened disk with four ligand-binding sites for AMP, ADP or ATP in the center (Hardie et al., 2012). Site 1 and 3 are responsible for cellular energy status sensing by competitively binding to AMP, ADP, and ATP. Site 4 is occupied by AMP independent of adenyl nucleotide concentrations (Liang and Mills, 2013), while Site 2 is always empty (Hardie et al., 2012). The binding of AMP or ADP promotes phosphorylation of α-subunit on Thr172

and activation of AMPK (Xiao et al., 2011), whereas ATP binding antagonizes the activation. A model to illustrate the subunits of the heterotrimeric complex is summarized in Figure 1-1.

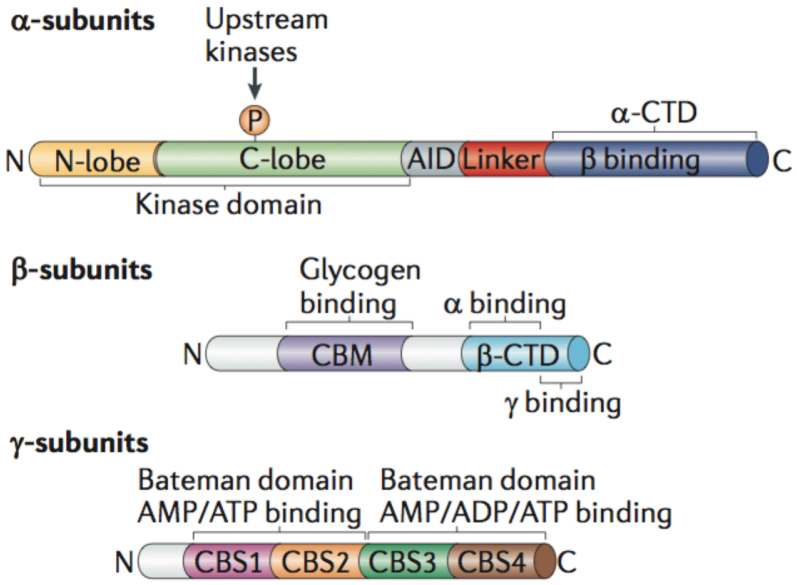



**Figure 1-1** The typical structure of AMPK subunits (Hardie et al., 2012).

### 1.1.3 Regulation of AMPK activity

*1.1.3.1 Control of AMPK activity by phosphorylation and dephosphorylation*

The kinase activity of AMPK is tightly controlled in mammalian cells. The canonical mechanisms for AMPK activation involve the increase of AMP/ATP or ADP/ATP ratios, or $Ca^{2+}$ (Hardie et al., 2012). During metabolic stresses when ATP consumption is accelerated (e.g. muscle contraction) or ATP production is inhibited (e.g. glucose starvation, hypoxia), cellular AMP/ATP and ADP/ATP ratios are increased. Binding of AMP to γ-subunits triggers conformational changes of AMPK and leads to AMPK activation via

the following three distinct mechanisms (Hardie, 2004; Kodiha and Stochaj, 2011). (1) Phosphorylation of Thr172 by upstream kinases, resulting in 50-to 100-fold activation (Gowans et al., 2013). The major upstream kinase is liver kinase B1 (LKB1)-STE20-related adaptor protein (STRAD)-mouse protein 25 (MO25) complex (Hawley et al., 2003). (2) Inhibition of Thr172 dephosphorylation by protein phosphatases (Davies et al., 1995; Gowans et al., 2013). (3) Allosteric activation of AMPK phosphorylated on Thr172 (Gowans et al., 2013; Hardie, 2004). Although AMP is the direct agonist of AMPK, recent findings revealed that ADP also has impact on phosphorylation and dephosphorylation of Thr172 (Xiao et al., 2011). It has also been reported that the initiation of Thr172 phosphorylation requires N-terminal myristoylation of the β-subunits, suggesting the critical role of the regulatory subunits in AMPK activation (Oakhill et al., 2010).

Aside from increased ADP/ATP and AMP/ATP ratios, Thr172 can be phosphorylated in response to a rise in intracellular $Ca^{2+}$ concentrations by $Ca^{2+}$/calmodulin-activated protein kinase kinase 2 (CaMKKβ, also known as CaMKK2) (Hawley et al., 1995; Woods et al., 2005). $Ca^{2+}$-dependent AMPK activation pathway does not necessarily require changes in adenine nucleotide ratios, although they can act synergistically (Fogarty et al., 2010).

An alternative mechanism for AMPK activation is through TAK1 (transforming growth factor-beta-activated kinase 1, TGF-β-activated

kinase-1, also known as MAP3K7 or MEKK7), a protein kinase activated by cytokines and upstream of JNK (MAP kinase) and nuclear factor kappa B (NF-κB) signaling. It has been reported that TAK1 phosphorylates Thr172 to switch on AMPK (Momcilovic et al., 2006; Xie et al., 2006), with detailed mechanisms remaining elusive at present.

Negative regulation of AMPK involves Thr172 dephosphorylation by phosphatases PP2A and PP2C (Moore et al., 1991). Another mechanism is the phosphorylation of Ser485 on α1-subunit (equivalent to Ser491 on α2) by PKC and possibly Akt (Kodiha and Stochaj, 2011). The regulation of AMPK by phosphorylation is summarized in Figure 1-2.

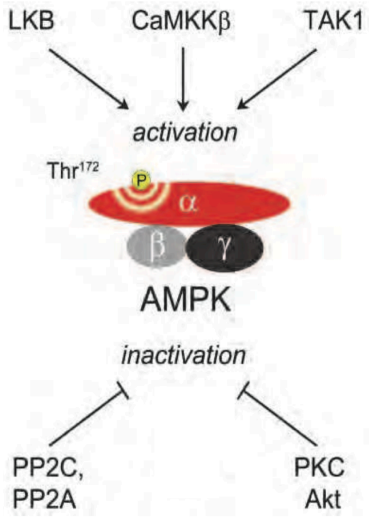



**Figure 1-2** Regulation of AMPK activity by phosphorylation (Kodiha and Stochaj, 2011).

*1.1.3.2 Pharmacological activators of AMPK*

Aside from energy stresses, a variety of pharmacological compounds also activate AMPK through AMP-dependent or AMP-independent mechanism. For example, AICAR (5-amino-4-imidazolecarboxamide (AICA) riboside), a widely used and the first discovered drug for AMPK activation, mimics the effect of AMP by generating a less potent analogue of AMP, 5-amino-4-imidazolecarboxamide ribotide (ZMP). AICAR was phosphorylated to ZMP, the mono-phosphorylated form of AICAR, by adenosine kinase (Corton et al., 1995; Sengupta et al., 2007). ZMP then binds to AMPK γ-subunit similar to AMP (Day et al., 2007). A769662 is a direct AMPK activator by mimicking the effects of AMP without binding to any of the ligand-binding sites on AMPK subunits, and carries out its function independent of AMPK upstream kinases (Göransson et al., 2007). Another AMP-independent AMPK activator is A23187, a $Ca^{2+}$ ionophore, which increases cytoplasmic $Ca^{2+}$ and subsequently activates CaMKKβ (Hawley et al., 2005).

Many pharmacological activators activate AMPK indirectly, mainly through inhibition of mitochondrial ATP production and thus altering cellular AMP/ATP ratios. Examples include classical mitochondrial inhibitors oligomycin and dinitrophenol (DNP) that are known to inhibit the mitochondrial respiratory chain (Hawley et al., 2010). Two major classes of

anti-diabetic drugs, guanidines and thiazolidinediones (TZDs) have also been reported as indirect activators of AMPK (Fryer et al., 2002). Metformin inhibits the mitochondrial electron transport chain complex I, leading to a rise in the intracellular ADP and AMP and subsequently activation of AMPK (El-Mir et al., 2000). Thiazolidinediones activate AMPK by two mechanisms, one is through inhibition of mitochondria ATP synthesis, and the other through promoting release of adiponectin from adipocytes via activation of the adipocyte transcription factor peroxisome proliferator-activated receptor gamma (PPARγ ) (Hardie, 2011c; Kubota et al., 2006; Lehmann et al., 1995). Other AMPK activators include glycolysis inhibitor 2-deoxyglucose (2DG), the barbiturate phenobarbital (Rencurel et al., 2005), nutraceuticals berberine (Lee et al., 2006), resveratrol (Baur et al., 2006), epigallocatechin-3-gallate (Hwang et al., 2007), and cytokines like leptin (Minokoshi et al., 2002), etc. The mechanisms for AMPK activation by pharmacological compounds are summarized in Figure 1-3.

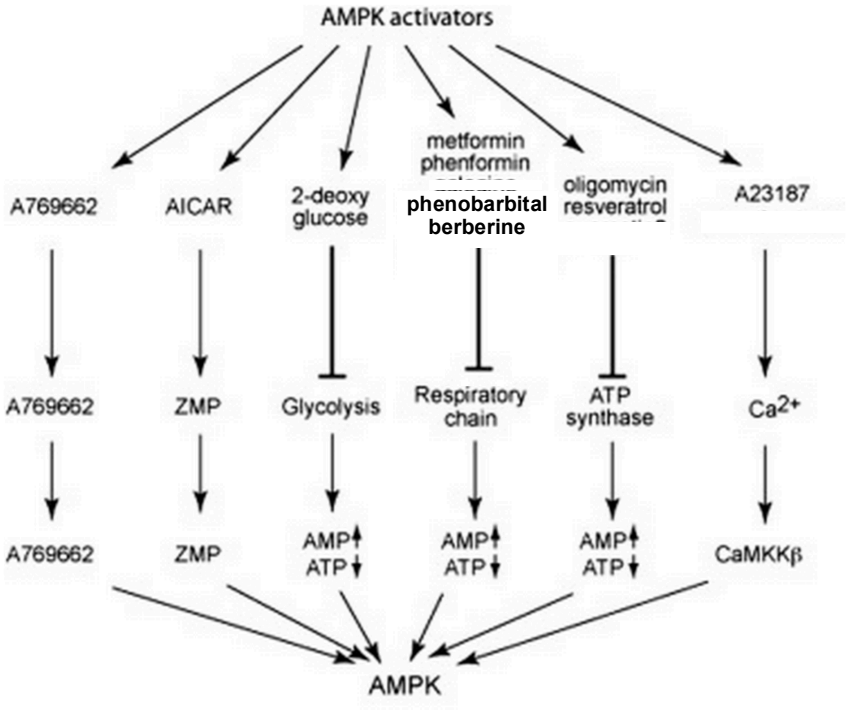



**Figure 1-3** AMPK activation by pharmacological compounds (Hawley et al., 2010).

*1.1.3.3 Activation of AMPK by oxidative stress*

Previous studies have indicated that AMPK activation can be triggered by reactive oxygen species (ROS) through decreasing cellular ATP levels (Choi et al., 2001). Some groups showed that ROS can phosphorylate LKB1 and induce AMPK phosphorylation at Thr172 (Cao et al., 2008; Han et al., 2010). Moreover, recent findings demonstrated that ROS can directly activate AMPK without altering cellular AMP/ATP or ADP/ATP ratios (Zmijewski et al., 2010). Exposure to physiologically relevant concentrations of $H_2O_2$ activates AMPK by oxidative modification, S-glutathionylation of cysteine residues of AMPK α-subunit (Zmijewski et al., 2010). Another

group identified ROS-induced ataxia-telangiectasia mutated (ATM) activation of AMPK possibly through LKB1 (Alexander et al., 2010).

However, it is controversial for the correlation between ROS accumulation and insensitivity of AMPK to various stimuli (Reznick et al., 2007; Saberi et al., 2008; Shao et al., 2014). For instance, Shao et al. showed that AMPK is oxidized by ROS stress, which prevents phosphorylation and activation of AMPK (Shao et al., 2014). Thus, the involvement of ROS and oxidation in AMPK activation remains intricate and requires further investigation.

### 1.1.4 AMPK and its diverse functions

#### *1.1.4.1 Regulation of cellular metabolism*

As a major controller of cellular metabolism, AMPK phosphorylates a variety of downstream targets in order to maintain energy homeostasis. Generally, in response to energy stress, AMPK up-regulates catabolic pathways for ATP generation while down-regulates anabolic pathways for ATP consumption. The function of AMPK is achieved by acute effects through phosphorylation of downstream metabolic enzymes, and by long-term effects through phosphorylation of transcription factors and co-activators to regulate gene expression (Hardie, 2007).

Multiple catabolic pathways are promoted by AMPK. Glucose uptake is significantly enhanced by AMPK via translocation of glucose transporter type 4 (GLUT4) from intracellular storage vesicles to the membrane (Kurth-Kraczek et al., 1999), activation of GLUT1 located at the plasma membrane (Barnes et al., 2002), or transcriptional up-regulation of *GLUT4* gene (Zheng et al., 2001). Similarly, AMPK accelerates fatty acid uptake via translocation of fatty acid transporter cluster of differentiation 36 (CD36) to cellular membrane (Habets et al., 2009). Moreover, AMPK also facilitates glucose catabolism via glycolysis pathway through phosphorylation of 6-phosphofructo-2-kinase (Hardie, 2007; Marsin et al., 2002). As for fatty acids catabolism, AMPK phosphorylates and inactivates the isoform of acetyl-CoA carboxylase (ACC2) to enhance uptake of fatty acids into mitochondria for β-oxidation (Hardie, 2004; Merrill et al., 1997). In addition, AMPK also promotes mitochondrial biogenesis via activation of peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α) to increase mitochondrial gene expression (Jäger et al., 2007; Zong et al., 2002).

On the other hand, AMPK is known to inhibit various anabolic pathways, including (i) fatty acid synthesis via ACC1 (Davies et al., 1992), (ii) cholesterol synthesis via 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (Clarke and Hardie, 1990), (iii) glycogen synthesis via glycogen synthase (Jørgensen et al., 2004), (iv) protein synthesis via mammalian target

of rapamycin (mTOR) (Gwinn et al., 2008; Inoki et al., 2003), and (v) ribosomal RNA synthesis via transcription initiation factor IA (TIFIA) (Hoppe et al., 2009).

*1.1.4.2 Regulation of autophagy and mitophagy*

Another crucial process regulated by AMPK is autophagy, a lysosomal degradation pathway involved in the breakdown and turnover of cellular organelles and macromolecules (to be discussed in detail later). In response to low energy status, activation of AMPK can stimulate autophagy through inhibition of mTOR by phosphorylation of TSC1/TSC2 (Inoki et al., 2003) or phosphorylation of a subunit of mTORC1, regulatory associated protein of mTOR (Raptor) (Gwinn et al., 2008), or direct phosphorylation of Ulk1 (Egan et al., 2011; Kim et al., 2011). Moreover, LKB1-AMPK pathway phosphorylates cyclin-dependent kinase inhibitor $p27^{Kip1}$, resulting in autophagy induction (Liang et al., 2007). AMPK activated by TAK1 is also capable of inducing cytoprotective autophagy in untransformed human epithelial cells treated with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Herrero-Martín et al., 2009).

Furthermore, AMPK has been demonstrated to induce mitophagy, a special form of autophagy targeting dysfunctional mitochondria (Egan et al., 2011). As the major site for cellular ROS generation, mitochondria are

particularly susceptible to oxidative damage. Therefore, clearance and recycling of damaged mitochondria as well as generation of new mitochondrial is important to maintain cellular ATP-generating capacity (Hardie, 2011b).

### *1.1.4.3 Other aspects of cell functions*

Apart from its best-known effects on metabolism, AMPK also has multiple functions on cellular processes, such as inhibition of cell growth and proliferation via cell cycle arrest by phosphorylation of p53 (Imamura et al., 2001) or phosphorylation of cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Liang et al., 2007) or up-regulation of cyclin-dependent kinase inhibitor $p21^{WAF1}$ (Jones et al., 2005), maintenance of cell polarity (Mirouse et al., 2007). The diverse functions of AMPK are summarized below in Figure 1-4.

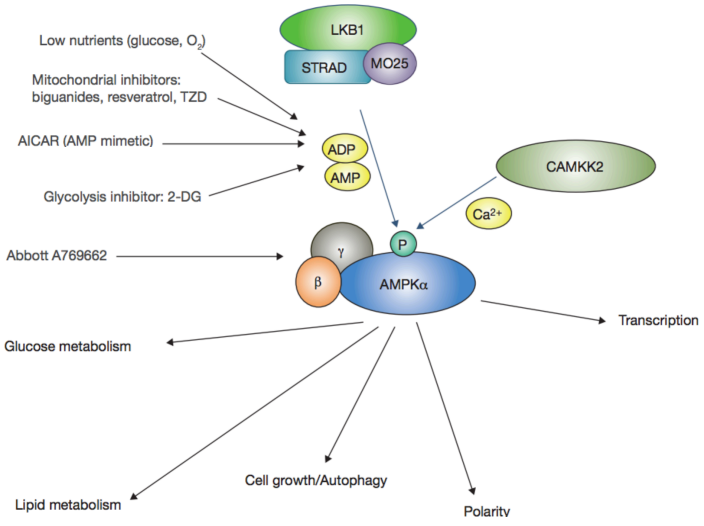



**Figure 1-4** Functions of AMPK through downstream targets (Mihaylova and Shaw, 2011).

### 1.1.5 AMPK and cancer

As a central metabolic regulator allowing cells to cope with environmental stresses, especially typical tumor microenvironment like nutrient deprivation and hypoxia, AMPK is required for cancer cell survival and oncogenic transformation (Faubert et al., 2014b). However, under metabolic stresses, AMPK inhibits cell growth and proliferation, suggesting the tumor suppressor activity of AMPK (Faubert et al., 2014b). In addition, AMPK is a crucial downstream target of a well-identified tumor suppressor LKB1, carrying out tumor suppression functions of LKB1 mainly through LKB1/AMPK/mTOR pathway. Thus, the controversial role of AMPK in tumorigenesis and metabolism remains to be elucidated.

#### *1.1.5.1 Genomic disruption of AMPK in cancer*

AMPK is rarely mutated in human cancers, with less than 3% mutation for any subunit (Liang and Mills, 2013). Instead, they are more frequently amplified in human cancers (Liang and Mills, 2013). So far, no evidence has ever been found for germline cancer predisposition syndrome involving AMPK subunits (Liang and Mills, 2013).

Complete deficiency of AMPK function is embryonically lethal in mice, and loss of the two catalytic isoforms AMPKα1 and α2 alone is insufficient to initiate tumorigenesis in mice (Liang and Mills, 2013). However, it is

reported that AMPK loss can cooperate with oncogenic drivers. For example, deletion of AMPKα1 promotes the Warburg effect and accelerates Myc-driven lymphomagenesis (Faubert et al., 2013). Genetic ablation of AMPKα2, rather than the dominant isoform AMPKα1, displays increased susceptibility to H-RasV12 transformation in murine embryonic fibroblast and tumor growth *in vivo* (Phoenix et al., 2012).

*1.1.5.2 Genetic deficiency of LKB1/AMPK signaling in cancer*

The serine-threonine kinase liver kinase B1 (LKB1, encoded by gene *STK11*), the major upstream activator of AMPK, has been reported as an important tumor suppressor (van Veelen et al., 2011). Heterozygous loss-of-function mutations in *STK11* were first discovered in inherited cancer Peutz-Jeghers syndrome (PJS) (Hemminki et al., 1998), which is associated with increased risk of malignant tumors. *STK11* is also frequently mutated in sporadic cancers, including 15-35% of non-small-cell lung cancer (NSCLC) (Ji et al., 2007; Shackelford and Shaw, 2009) and 20% of cervical carcinomas (Shackelford and Shaw, 2009; Wingo et al., 2009).

In normal conditions, inactive LKB1 locates in nucleus. Upon activation, LKB1 interacts with the STE20-related adaptor protein α (STRADα) and scaffolding mouse protein 25 (MO25). The heterotrimer is then translocated to the cytoplasm where LKB1 carries out its kinase activity

on AMPK. Upon phosphorylation and activation by LKB1, AMPK conducts multiple tumor suppression functions, especially through suppression of the tuberous sclerosis complex (TSC)/mTOR pathway, a canonical signaling pathway regulating cell metabolism and cell growth (Inoki et al., 2003). However, high level of AMPK activation is also observed independent of LKB1 in lung cancers, probably via CaMKKβ, TAK1, or other mechanisms (William et al., 2012). In addition, apart from AMPK, LKB1 also phosphorylates a family of AMPK-related kinases, like brain-specific serine/threonine-protein kinase 1/2 (BRSK1/2), novel (nua) kinase 1/2 (NuAK1/2), salt-inducible kinase 1/2/3 (SIK1/2/3), MAP/microtubule affinity-regulating kinase 1/2/3/4 (MARK1/2/3/4), SNF (sucrose non-fermenting protein)-related serine/threonine-protein kinase (SNRK) (Lizcano et al., 2004). Therefore, although AMPK and LKB1 are closely associated, they may carry out different functions during tumorigenesis.

*1.1.5.3 The complex role of AMPK in cancer*

At present, the exact role of AMPK in cancer appears to be complex and controversial. On the one hand, there is evidence suggesting the pro-cancer function of AMPK. For instance, under nutrient deprivation conditions (a common microenvironment for cancer cells), activated AMPK promotes energy homeostasis via inhibiting anabolic pathways like lipid

synthesis, mTOR-dependent protein synthesis, while stimulating catabolic pathways, like lipid oxidation and glycolysis (Hardie, 2007). Moreover, AMPK induces autophagy, a catabolic process for removal of damaged cellular components in stresses, through direct phosphorylation of ULK1 and inhibition mTOR via TSC1/2 or Raptor (discussed earlier). Thus, functional LKB1/AMPK signaling is required for cancer cells to survive metabolic stresses, whereas lacking LKB1/AMPK probably causes programmed cell death of tumor cells in energy crisis.

On the other hand, there is accumulating evidence demonstrating the anti-cancer function of AMPK. For example, AMPK negatively regulates the Warburg effect (Faubert et al., 2013), a well-characterized metabolic reprogramming when tumor cells shift to aerobic glycolysis to generate more metabolic intermediates to meet the high demands of proliferation (Vander Heiden et al., 2009; Warburg, 1926). Since glycolysis generates far less ATP per molecule of glucose compared to oxidative phosphorylation, tumor cells specifically relies on glucose metabolism with high rates of glucose uptake and lactate production (Vander Heiden et al., 2009). AMPK can reverse Warburg effect via promoting mitochondria biogenesis and mitochondrial tricarboxylic acid (TCA) cycle enzymes (discussed earlier) (Hardie, 2011a). The surprising results from Faubert et al. indicated that silence of AMPK, even LKB1, promotes the Warburg effect as observed by increased glucose

uptake, redirection of carbon flow toward lactate, and glycolytic flux. This metabolic effect requires hypoxia-inducible factor-1α (HIF-1α) (Faubert et al., 2013). Recently, this group demonstrated that similar to AMPK, loss of LKB1 also promotes HIF-1α-dependent metabolic reprogramming in cancer cells (Faubert et al., 2014b). Further, mTORC1 activation is also critical in Warburg effect as well as cell growth and cell proliferation. Loss of AMPK, an important negative regulator of mTORC1 activity, can lead to unchecked mTOR activity (Faubert et al., 2014b). Taken together, these results support the tumor suppressor role of AMPK.

*1.1.5.4. Use of AMPK agonists for cancer therapy*

The use of AMPK agonists has been proposed as an anti-cancer approach. Metformin, a widely used drug for treatment of Type II diabetes, has been found to be associated with low occurrence of cancer in diabetes patients (Decensi et al., 2010; Evans et al., 2005). Other AMPK activators, such as phenformin (El-Masry et al., 2012; Petti et al., 2012), AICAR (El-Masry et al., 2012; Petti et al., 2012; Choudhury et al., 2014), 2DG (Dong et al., 2013), and A-769662 (Huang et al., 2008; Choudhury et al., 2014) are also shown to perform anti-tumor activity *in vitro* or *in vivo*. Although most AMPK agonists do not activate AMPK directly, which will not rule out AMPK-independent mechanisms involved in anti-cancer effects,

these results provide the rationale for cancer therapy by targeting AMPK.

## 1.2 Cellular pathways controlling protein degradation

Eukaryotic cells have two major protein degradation systems to maintain protein homeostasis: the ubiquitin-proteasome system (UPS) and the lysosome system. The proteasome pathway degrades intracellular proteins primarily aberrantly folded or short-lived proteins, while the lysosome digests extracellular and membrane proteins delivered via endocytosis and cytosolic components delivered via autophagy (Shen et al., 2013b).

### 1.2.1 The ubiquitin-mediated protein degradation system

The ubiquitin-proteasome system (UPS) is a complicated and tightly regulated system responsible for degrading 80-90% of intracellular proteins (Shen et al., 2013b). The UPS system consists of several components, ubiquitin (Ub), a highly evolutionarily conserved small protein of 76 amino acids, the Ub-activating enzyme (E1), a group of Ub-conjugating enzymes (E2) or approximately 50 members, a large group of Ub ligases (E3) of more than 500 members, the 26S proteasome, and the deubiquitinases (DUBs) (Shen et al., 2013b).

The UPS protein degradation pathway involves two discrete and

successive steps: (1) ubiquitination, which tags multiple Ub molecules to targeted substrates by covalent conjugation and (2) proteasomal degradation, which degrades tagged protein by the 26S proteasome complex (Glickman and Ciechanover, 2002). During ubiquitination, Ub is first activated by E1 forming a high-energy thiol ester intermediate between Ub and E1 in an ATP-dependent manner. Then activated Ub is transferred to E2 via the formation of another high-energy thiol ester bond between Ub and E2, and finally transferred to E3-bound substrate directly or through a third thiol ester intermediate between Ub and E3. E3 catalyzes the covalent attachment of Ub to the targeted protein. Multiple cycles of ubiquitination leads to the synthesis of a polyubiquitin chain, which is recognized by 26S proteasome. The poly-Ub chain will be removed and recycled and the targeted proteins are degraded into small peptides (Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998; Komander and Rape, 2012; Naujokat and Sarić, 2007). The UPS system is summarized in Figure 1-5.

Evidence has strongly suggested that endogenous AMPK can be regulated at the level of protein stability by Cidea-mediated ubiquitin proteasome degradation in brown adipose tissue (Qi et al., 2008).

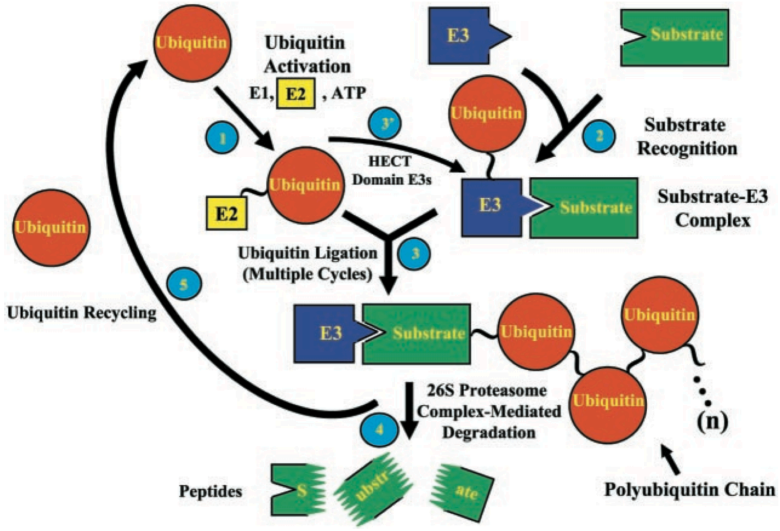



**Figure 1-5** The UPS system (Glickman and Ciechanover, 2002).

### 1.2.2 Autophagy

Autophagy is an evolutionarily conserved degradation system when intracellular components are engulfed into autophagosome and delivered to lysosome (Mathew et al., 2007). Although the proteasome system serves as the major provider of amino acids for cellular renovation under nutrient-rich conditions, autophagy is readily induced by stresses such as starvation. Autophagy is divided into three categories: macroautophagy (referred as autophagy hereafter, the major type of autophagy), microautophagy and chaperone-mediated autophagy (CMA).

As shown in Figure 1-6, autophagy is a complex cellular process proceeding through sequential steps: (1) initiation/induction, (2) nucleation at the phagophore assembly site (PAS), (3) elongation/expansion of the phagophore to form autophagosome, (4) fusion with late endosome and

lysosome to form autolysosome, and (5) degradation of cargo and recycling of resulting molecules (Yang and Klionsky, 2010a).

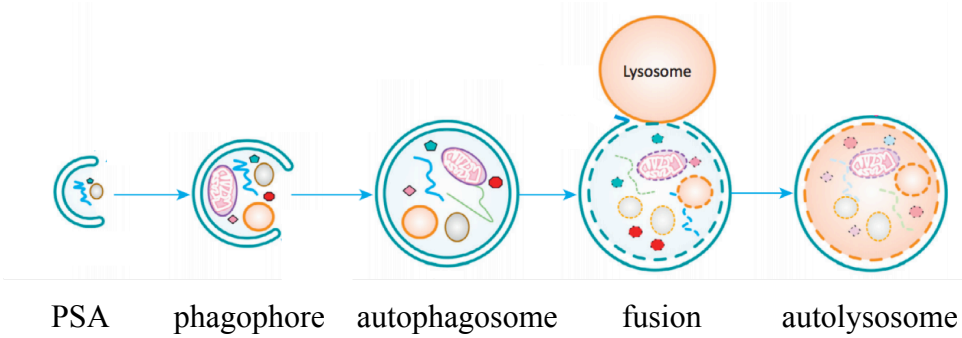



**Figure 1-6** Schematic depiction of the autophagy pathway (Shen and Mizushima, 2014).

Autophagy is tightly regulated by a complex signaling network in mammals. One of the most critical regulator of autophagy is mTOR, integrating amino acids, growth factors and energy status, forms two distinct protein complexes, mTORC1 and mTORC2 (Soulard and Hall, 2007). During amino acid starvation, mTORC1 is inactivated, leading to the activation of the Unc-51-like kinases (ULK)-Atg13-FIP200 (scaffold focal adhesion kinase (FAK)-family-interacting protein of 200 kDa)-Atg101 (an Atg13-binding protein) complex, thus initiating the autophagy machinery. Activation of growth factor receptors triggers the activation of Class I PtdIns3K-PKB/Akt-TSC1/TSC2-mTORC1 pathway and Raf-1/MEK/ERK signaling cascade, leading to autophagy activation (Yang and Klionsky, 2010b). In response to low energy status (as discussed earlier), activated AMPK inhibits mTORC1 through phosphorylation of TSC1/TSC2 (Inoki et

al., 2003), or mTORC1 subunit Raptor (Gwinn et al., 2008). AMPK can also induce autophagy via direct phosphorylation of Ulk1 (Egan et al., 2011; Kim et al., 2011). mTORC2 inhibits autophagy via phosphorylation of PKB (Sarbassov et al., 2005). Bcl-2 or Bcl-$X_L$ can inhibit autophagy via binding to Beclin 1 and disrupting the Beclin 1-associated Class III PtdIns3K complex (Yang and Klionsky, 2010a). The signaling pathways involved in autophagy regulation are summarized in Figure 1-7.

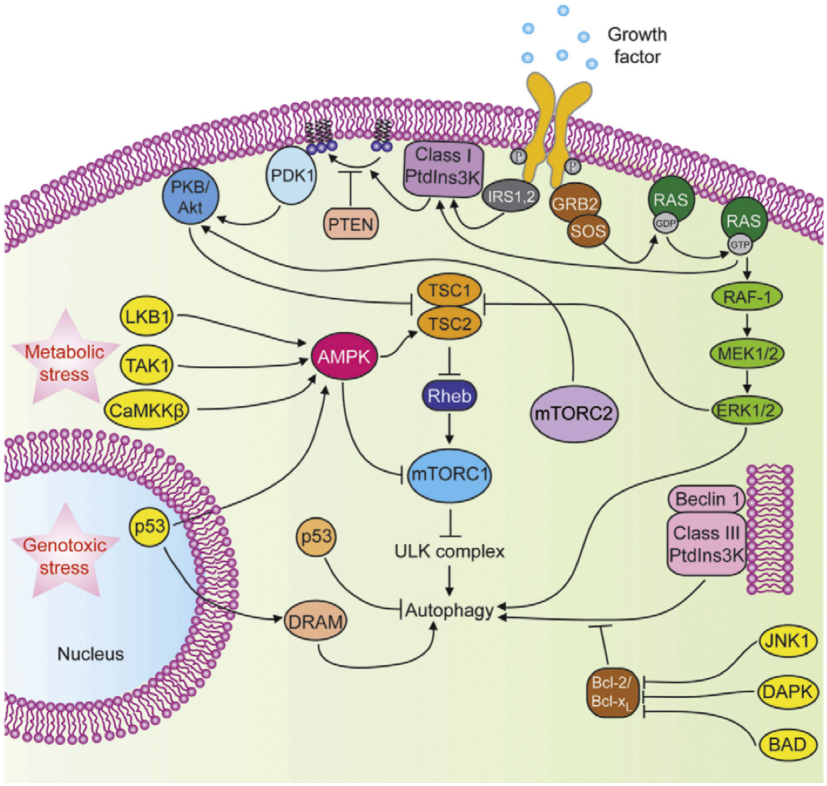



**Figure 1-7** Signaling network involved in autophagy regulation (Yang and Klionsky, 2010a).

Autophagy has multiple functions to maintain cellular homeostasis. First, autophagy eliminates unwanted organelles and macromolecules for

constitutively cellular turnover. Second, autophagy recycles energy and materials including amino acid, lipid and glycogen for cellular utilization, especially under stress conditions. However, the role of autophagy in cancer remains controversial, being regarded as a double-edged sword with both pro-survival role and pro-death role (White and DiPaola, 2009). On the one hand, autophagy functions as a tumor suppressor maintaining cellular integrity and genomic stability (Liu and Ryan, 2012; Ryan, 2011), with several related genes identified as tumor suppressors, such as beclin 1 (Liang et al., 1999) and Atg4C (Mariño et al., 2007). On the other hand, autophagy has an oncogenic role in tumor progression. Autophagy is induced in response to anti-cancer reagents for therapy resistance and metabolic stress as an adaptive mechanism (Brech et al., 2009; Mathew et al., 2007). Although autophagy plays a paradoxical and complex role in tumor initiation and progression, it has been increasingly recognized that autophagy suppresses early stage of tumor but promotes subsequent tumor development including progression (Liu and Ryan, 2012).

### 1.3 Programmed cell death

Programmed cell death (PCD) is a controlled cellular mechanism for clearance of damaged and disordered cells to maintain tissue homeostasis and normal physiological development, defending against immunological

disorders, inflammation and tumorigenesis (Fuchs and Steller, 2011). PCD has been classified into three categories: apoptosis (type I PCD), autophagic cell death (type II PCD) and programmed necrosis (necroptosis, type III PCD) (Sun and Peng, 2009).

### 1.3.1 Apoptosis

Apoptosis, an evolutionary conserved program of cell death, is characterized by morphological and biochemical hallmarks, including cell shrinkage, nuclear condensation and fragmentation, and membrane blebbing (Kerr et al., 1972; Long and Ryan, 2012). Apoptosis is executed through two pathways: the extrinsic pathway stimulated by extracellular death ligands and cell death receptors, and the intrinsic pathway triggered by intracellular stimuli, both of which converge at executioner caspases and cell death (Long and Ryan, 2012).

The extrinsic apoptotic pathway is initiated by the binding of death ligands to death receptors, such as tumor necrosis factorα (TNFα) to TNFα receptor 1 (TNFR1), and TNF-related apoptosis-inducing ligand (TRAIL) to TRAIL receptor 1 (TRAILR1) and TRAIL2, FAS/CD95 ligand (FASL/CD95L) to FAS/CD95 (Long and Ryan, 2012). The ligation of death receptors and their ligands promotes receptor trimerization and the formation of the death-inducing signaling complex (DISC), consisting of multiple

adaptor molecules such as Fas-associated death domain (FADD), TNFR-associated death domain (TRADD), and TNFR-associated factor 2 (TRAF2). Subsequently, these adaptor molecules recruit initiate pro-caspase-8 to the DISC (Fulda and Debatin, 2006; Lavrik et al., 2005; Long and Ryan, 2012). Upon DISC formation, pro-caspase-8 is activated through self-cleavage. Active caspase-8 then stimulates downstream executioner caspases such as caspases-3, 6 and/or -7, or induces mitochondrial outer membrane permeabiliziation (MOMP) (Galluzzi et al., 2012; Long and Ryan, 2012), ultimately resulting in apoptotic cell death.

The intrinsic apoptotic pathway is stimulated by intracellular stress conditions, such as oxidative stress, DNA damage, excessive cytosolic $Ca^{2+}$, endoplasmic reticulum (ER) stress, growth factor starvation, etc. (Galluzzi et al., 2012; Long and Ryan, 2012). These lethal signals activate MOMP, leading to mitochondrial proteins leakage. The release of cytochrome c (cyt c) from mitochondria promotes apoptosis protease-activating factor-1 (Apaf-1) oligomerization and formation of cyt c/Apaf-1/caspase-9 apoptosome (Cain et al., 2000), causing activation of initiator caspase-9. Caspase-9 further cleaves and activates effector caspases-3, 6 and/or -7, eventually leading to cell death.

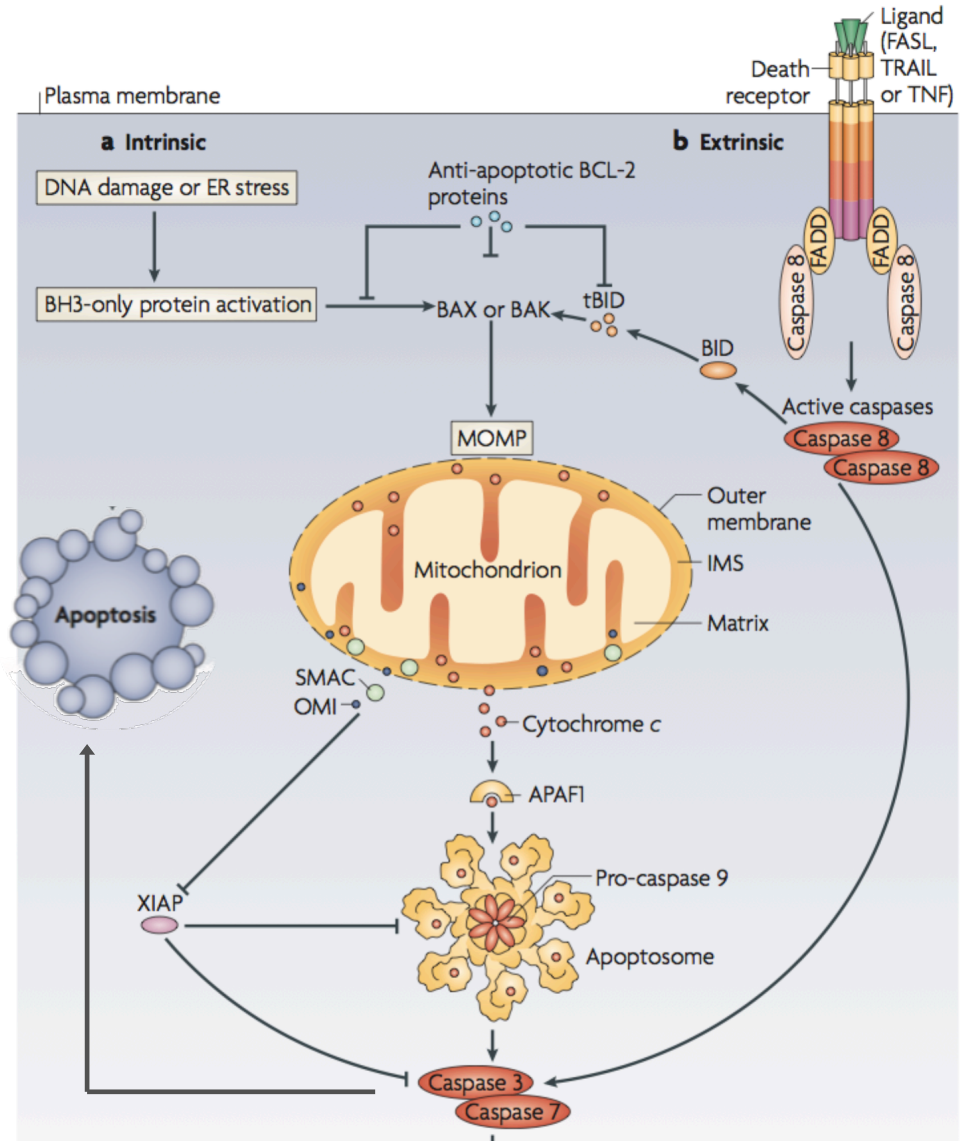



**Figure 1-8** The extrinsic and intrinsic apoptosis pathways (Tait and Green, 2010).

### 1.3.2 Necroptosis

Necrosis is morphologically characterized by an early onset plasma membrane permeabilization, organelle swelling and finally rupture of the cells, causing leakage of intracellular contents, but the nuclei remain intact (Vandenabeele et al., 2010). Necrotic cell death can be induced by multiple stimuli, like DNA damage, ROS, excitotoxins, etc. (Galluzzi et al., 2012)

Although necrosis has long been considered as an accidental cell death mechanism, it is now demonstrated a regulated form of necrosis mediated by death receptor via the receptor interacting protein (RIP) family, RIP1 and RIP3, termed as “necroptosis”.

Upon binding with TNFα, TNFR1 trimmers form a complex (referred as complex 1) by recruiting signaling molecules including RIP1, cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, TRADD, TNFR-associated factor 2 (TRAF2) and TRAF5 (Vandenabeele et al., 2010). Proteins in complex 1 are ubiquitylated by E3 ligases (cIAP1 and cIAP2) for further recruiting signaling proteins responsible for NF-κB survival (Long and Ryan, 2012; Vandenabeele, 2010). RIP1 can be deubiquitylated and form a complex II with RIP3, TRADD, FADD and caspase-8, which induces cell death signal and decides to go through apoptosis or necroptosis pathway.

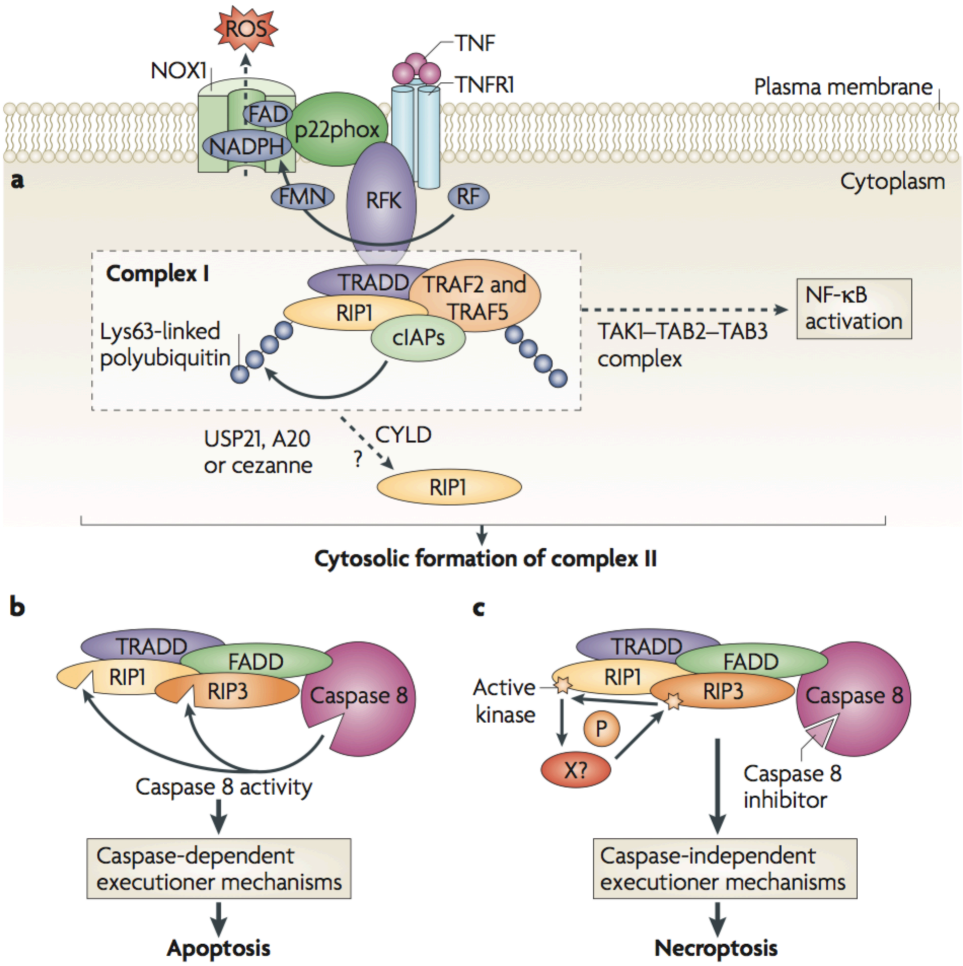



**Figure 1-9** TNFR1-elicted signaling pathways (Vandenabeele et al., 2010).

## 1.4 Objectives of the study

The main objectives of this study are as follows:

1. To study cell death in response to glucose starvation in NCI-H460 cells;
2. To investigate the role of AMPK protein stability in glucose starvation-induced cell death.

The present study discovered an LKB1-deficient non-small cell lung

cancer (NSCLC) cell line NCI-H460 hypersensitive to glucose starvation-induced cell death. In this cell line, cellular ROS was significantly elevated, and AMPK was rapidly phosphorylated and activated. However, prolonged glucose starvation for 3 hours markedly reduced AMPK protein level. 2-deoxyglucose (2DG) and antioxidant N-acetylcysteine (NAC) were able to reduce ROS level, stabilize AMPK protein and eventually protect against cell death. Further studies will focus on the molecular mechanism by which AMPK is down regulated upon glucose starvation, especially post-translational modification of AMPK.

In summary, our data demonstrate that AMPK protein stability and activity was negatively regulated under glucose starvation, leading to rapid cell death. These results provide the rationale for cancer therapy targeting AMPK protein stability as well as activity, which was important to cancer cell survival. The potential of a novel therapeutic target for cancer treatment will benefit cancer patients, especially NSCLC.

# CHAPTER 2 MATERIALS AND METHODS

## 2.1 Cell lines and cell culture

NCI-H460, NCI-H1299, A549 and HeLa cell lines were purchased from American Type Culture Collection (ATCC). NCI-H460 and A549 were cultured in DMEM-F12 Ham medium (Sigma, #D8437), HeLa cells were cultured in DMEM medium (Sigma, #D1152), and H1299 were cultured in RPMI-1640 medium (Sigma, #R8758). All types of medium were supplemented with 10% fetal bovine serum (FBS, Hyclone, #SV30160.03), 1% penicillin-streptomycin (Invitrogen, #15140-122) and maintained in an incubator with 5% $CO_2$ at 37 °C.

The following media were used for different forms of starvation: DMEM (Sigma, #D1152) without FBS, DMEM without glucose (Gibco, #11966-025) supplemented with 10% dialyzed FBS, DMEM without glutamine (Gibco, #11960-044) supplemented with 10% dialyzed FBS, and amino acid free DMEM (protocol provided by Noboru Mizushima, University of Tokyo) supplemented with or without 10% dialyzed FBS. The protocol of amino acid free DMEM is as follows:

| | |
|---|---|
| $NaHCO_3$ | 7.4 g |
| NaCl | 12.12 g |
| KCl | 0.8 g |
| $MgSO_4 \cdot 7H_2O$ | 0.4 g |
| $CaCl_2 \cdot 2H_2O$ | 0.528 g |

| | |
|---|---|
| 10 mg/mL $Fe(NO_3)_3$ | 20 μL |
| D-glucose | 2 g |
| MEM vitamin solution (×100) | 80 mL |
| 1M HEPES (pH7.5) | 30 mL |
| $NaH_2PO_4 \cdot 2H_2O$ | 0.22 g |
| Phenol red | 0.03 g |
| Add $ddH_2O$ to 2 L | |
| Adjust pH to 7.2-7.6 | |

**2.2 Reagents and antibodies**

The following reagents used in this study were purchased from Sigma-Aldrich: 2-deoxyglucose (2DG, Sigma, #D6134), AICAR (Sigma, #A9978), metformin hydrochloride (Sigma, #1396309), Compound C (Sigma, #P5499), chloroquine diphosphate (CQ, Sigma, #C6628), bafilomycin A1 (BAF, Sigma, #B1793), Rapamycin (Sigma, #R0395), MG-132 (Sigma, #7449), N-acetylcysteine (NAC, Sigma, #A9165). Other chemicals were necrostatin-1 (Merck, #480065), and Bortezomib (Santa Cruz, #sc-217785).

The following antibodies were purchased from Cell Signaling: AMPKα (Cell Signaling, #2532), phospho-AMPKα1 (Thr 172) (Cell Signaling, #2535), phospho-AMPKα1 (Ser485) (Cell Signaling, #2537), ACC (Cell

Signaling, #3662), phospho-ACC (Ser79) (Cell Signaling, #3661), LKB1 (Cell Signaling, #3050). Anti-LC3 (Sigma, #L7543), anti-α-tubulin (Sigma, #T6199) were purchased from Sigma Aldrich. Goat anti-rabbit (Thermo Fisher, #31460) or anti-mouse (Thermo Fisher, #31430) horseradish peroxidase-linked antibodies were used as secondary antibodies.

Antibodies were prepared as follows: 0.5 g of bovine serum albumin (BSA, Sigma, #A9418) was dissolved in 10 mL of 1 X Tris Buffered Saline with Tween 20 (TBST). Then $NaN_3$ was added into 5% BSA to make up to 0.01% $NaN_3$ solution to prevent bacterial contamination. All primary antibodies were diluted by 1:1000 except anti-α-tubulin (1:5000), and secondary antibodies were diluted by 1:5000. All antibodies were stored at 4 °C.

**2.3 Western blot**

After designated treatments, cells were collected and lysed in Laemmli SDS buffer (62.5 mM Tris at pH 6.8, 25% glycerol, 2% SDS, phosphatase inhibitor and proteinase inhibitor cocktail). After determination of protein concentration, an equal amount of protein was resolved by sodium SDS-PAGE and transferred onto PVDF membrane (Bio-Rad). After blocking with 5% non-fat milk for 30 min, the membrane was probed with designated first antibodies overnight at 4°C, washed by TBST and probed with second

antibodies for 1 hour at room temperature. The membrane was developed with the enhanced chemiluminescence method (Pierce and Merck) and visualised using Kodak Image Station 440CF (Kodak) and ImageQuant LAS500 (GE Healthcare).

**2.4 Propidium iodide (PI) live cell exclusion staining for cell viability**

Cells were cultured in 24-well plate overnight. After designed treatments, the medium in each well was collected and cells were harvested with trypsin. Then, cell pellets obtained were resuspended in 1× phosphate buffer saline (PBS) containing PI at a final concentration of 5 μg/mL and incubated for 10 minutes at 37°C. Ten thousand cells from each sample were analysed with FACS Calibur flow cytometry (BD Bioscience) using CellQuest software.

**2.5 CM-H2DCFDA for cellular ROS**

Chloromethyl 2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Life technologies, #C6827) was used for detection of intracellular ROS production. Cells were first cultured in 24-well plate overnight. After the designated treatments, cells were incubated with 1 μM CM-H2DCFDA in PBS for 10 min. Then the CM-H2DCFDA was removed and the cells were washed with PBS twice. The cells were harvested with trypsin and fluorescence intensity was measured by FACS Calibur flow

cytometry (BD Bioscience) using CellQuest software.

### 2.6 Microscopy image

Cell were cultured in overnight and treated with designed experiments. The morphological changes were detected under phase-contrast microscopy, and representative cells were selected and photographed.

### 2.7 Statistical analysis

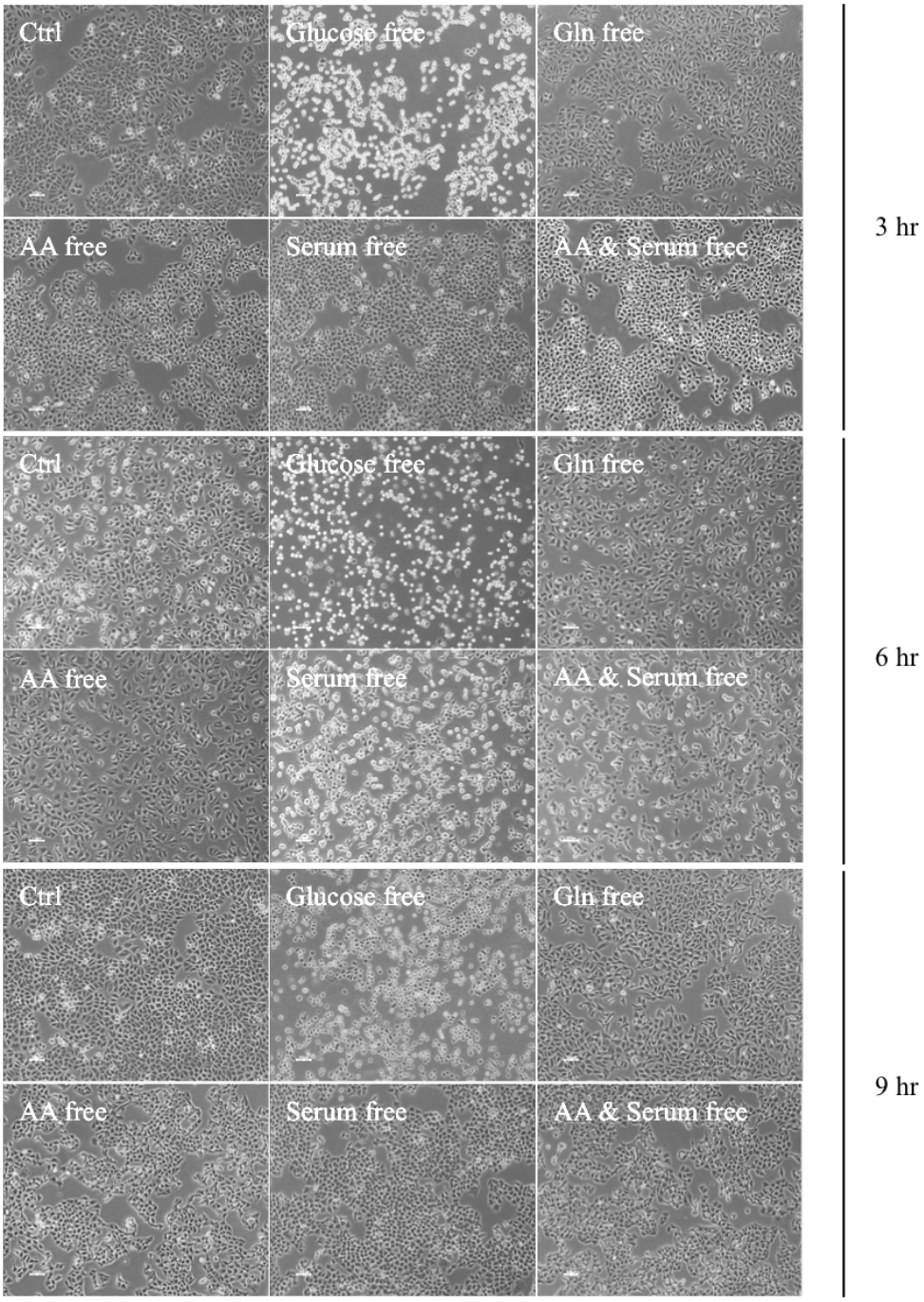
The image data were representatives from at least three repeated experiments. All numeric values were expressed as mean ± SD from at least three independent experiments. The p-value was calculated using Student's t-test with p-values<0.05 (*p<0.05) or p-values<0.01 (**p<0.01) is considered to be statistically significant.

# CHAPTER 3 RESULTS

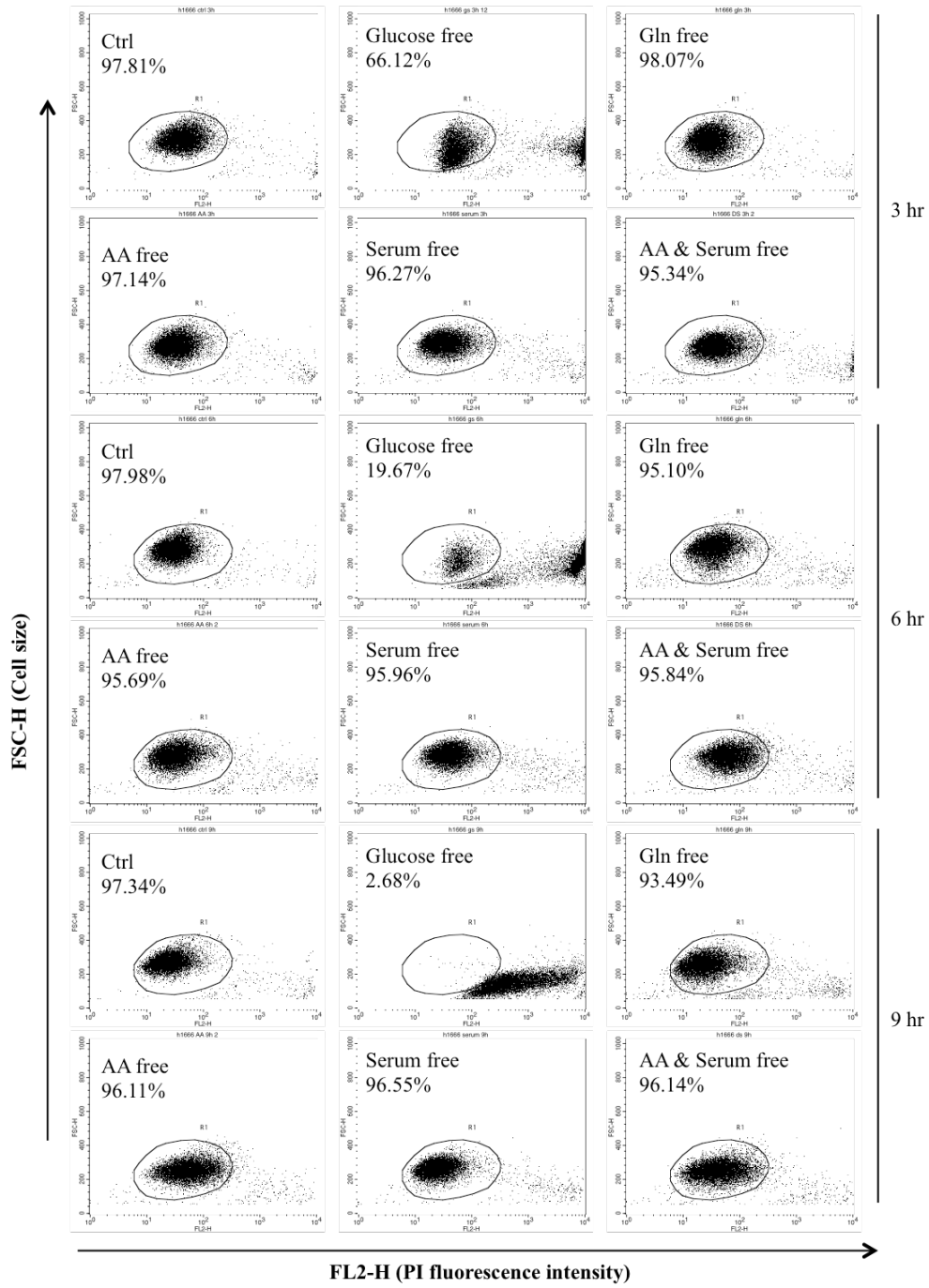
### 3.1 NCI-H460 cells are hypersensitive to cell death induced by glucose starvation

To measure the cell death sensitivity to different nutrients starvation, NCI-H460 cells were treated with various starvation conditions for 3, 6 and 9 hours, including glucose starvation, amino acid starvation, serum starvation, glutamine starvation, and double starvation (deprived of amino acid and serum). First, we examined the cellular morphology upon different starvation treatments. As shown in Figure 3-1A, dead cells with rounded shapes were floating over the medium, whereas live cells remained attached to the culture dish. Obvious cell death was observed only in cells under glucose starvation. Then we quantified the cell death through PI exclusion test coupled with flow cytometry (Figure 3-1B). Under glucose starvation, the percentage of viable cells was significantly reduced to 66.12% (3 hr), 19.67% (6 hr) 2.68% (9 hr), while the other starvation conditions showed little effects on cell survival. Therefore, data from this part of our study demonstrate the hypersensitivity of NCI-H460 cell line to glucose starvation. The statistical analysis of cell death was summarized in Figure 3-1C.

A


Ctrl
Glucose free
Gln free
AA free
Serum free
AA & Serum free
3 hr
Ctrl
Glucose free
Gln free
AA free
Serum free
AA & Serum free
6 hr
Ctrl
Glucose free
Gln free
AA free
Serum free
AA & Serum free
9 hr

B


Ctrl
97.81%
Glucose free
66.12%
Gln free
98.07%
AA free
97.14%
Serum free
96.27%
AA & Serum free
95.34%
Ctrl
97.98%
Glucose free
19.67%
Gln free
95.10%
AA free
95.69%
Serum free
95.96%
AA & Serum free
95.84%
Ctrl
97.34%
Glucose free
2.68%
Gln free
93.49%
AA free
96.11%
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3 hr
6 hr
9 hr
FSC-H (Cell size)
FL2-H (PI fluorescence intensity)

C

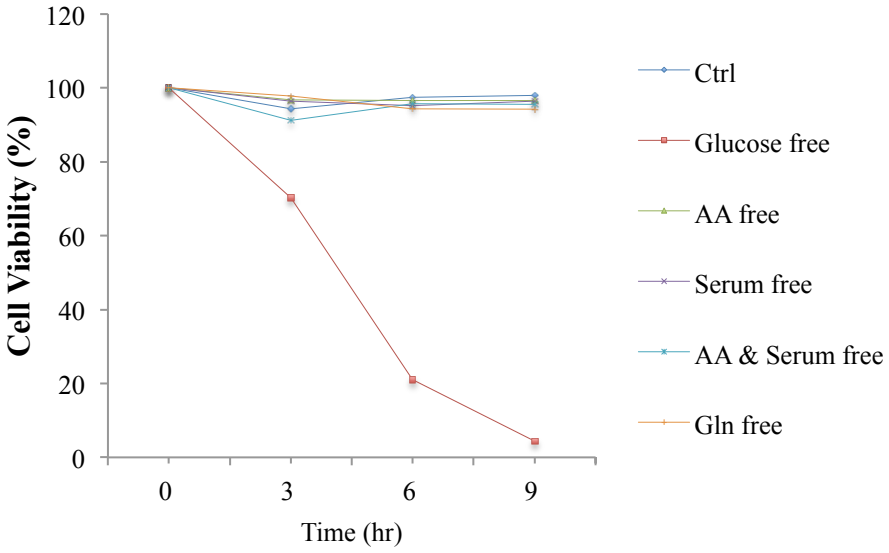
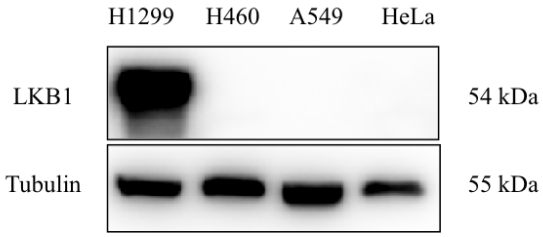


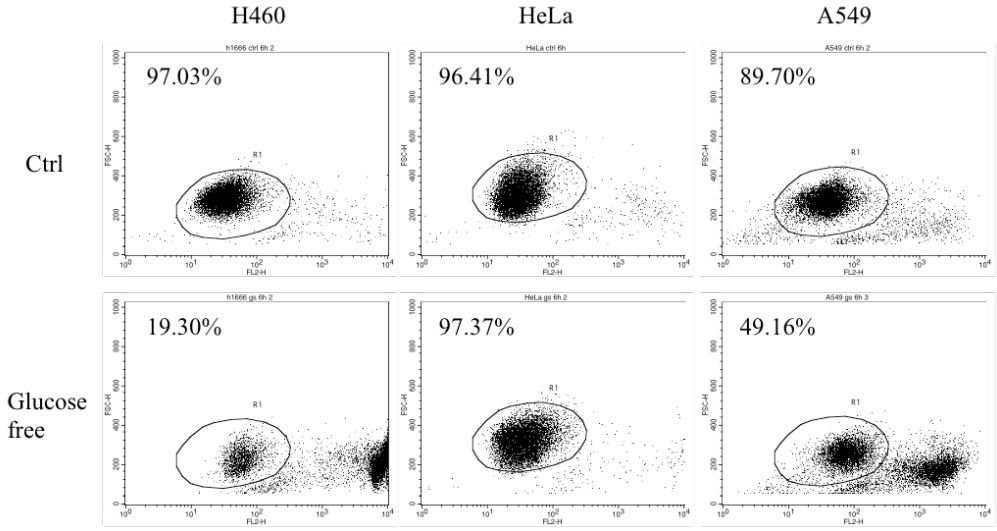

**Figure 3-1** NCI-H460 is hypersensitive to glucose starvation-induced cell death. (A) Morphological representatives of NCI-H460 cells in different starvation conditions for indicated time points. NCI-H460 cells were treated with full medium, glucose free medium, amino acid (AA) free medium, serum free medium, AA and serum free medium, or glutamine (Gln) free medium for 3, 6 and 9 hours. Cells were photographed under a light microscope. Scale bar, 200 μm. (B) Dotplot of PI live exclusion assay for quantification of cell viability. NCI-H460 cells were treated as indicated in (A) and the cell death was measured by flow cytometry using PI (5 μg/mL) staining (circle, a representative of viable cells). (C) Statistical analysis of the percentage of viable cells of three independent experiments performed as in (B) (mean ± SD).

Accumulating evidence has indicated that tumor cells lacking functional LKB1 are susceptible to metabolic stress (Dupuy et al., 2013; Faubert et al., 2013, 2014a; Shaw et al., 2004b). Shaw et al. showed that LKB1-deficent MEFs are defective in AMPK activation and sensitive to apoptosis in response to elevated AMP conditions by AICAR (Shaw et al., 2004b), suggesting that LKB1-deficient tumor cells may be sensitized to cell death when cellular AMP/ATP or ADP/ATP ratios are increased by stimuli. It has been reported that HeLa and A549 are LKB1-deficient cell lines, confirmed by examination of mRNA and protein expression (Tiainen et al., 1999; Sanchez-Cespedes et al., 2002; Hawley et al., 2003; Ma et al., 2014). Therefore, we used HeLa and A549 for comparison. As confirmed by western blot (Figure 3-2A), LKB1 was not detected in NCI-H460, HeLa and A549, while NSCLC cell line NCI-H1299 expressed high level of endogenous LKB1 as a positive control. Then NCI-H460, HeLa and A549 cell lines were treated with glucose starvation for 6 hours. As showed in Figure 3-2B and C, significant cell death upon glucose starvation for 6 hours in NCI-H460 and A549 cells was observed, but there was no significant cell death in HeLa cells. Although NCI-H460 was more susceptible to glucose starvation than A549, one possible mechanism of the hypersensitivity to cell death is the identical LKB1 nonsense mutation at codon 37 of these two cell lines (Carretero et al., 2004; Sanchez-Cespedes, 2007).

A


H1299
H460
A549
HeLa
LKB1
54 kDa
Tubulin
55 kDa

B


H460
HeLa
A549
Ctrl
Glucose free
97.03%
96.41%
89.70%
19.30%
97.37%
49.16%
R1
FSC-H
FL2-H

C

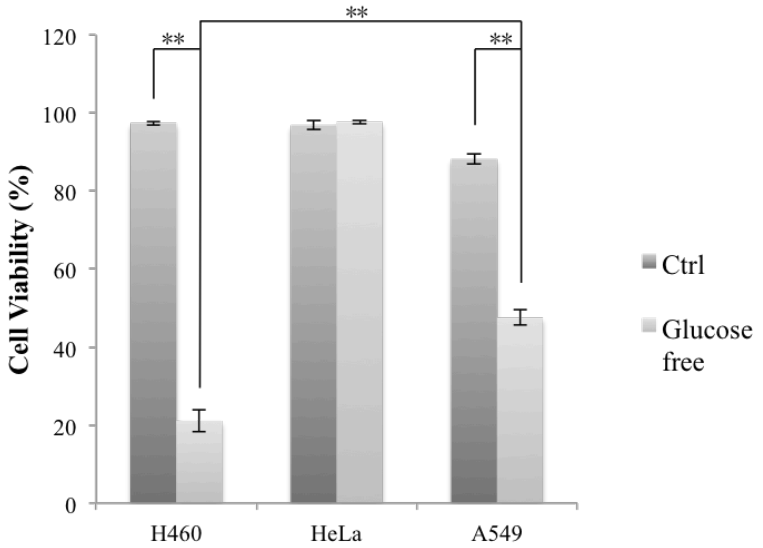
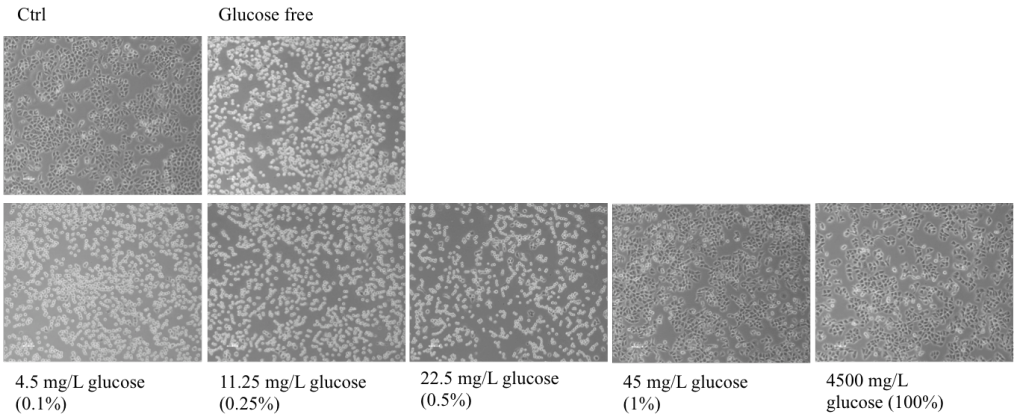



**Figure 3-2** NCI-H460 is more sensitive to cell death induced by glucose starvation than HeLa. (A) LKB1 deficient and wide type cells. NCI-H460, A549, HeLa and NCI-H1299 were collected after seeded overnight in full medium and cell lysates were used for western blotting. Tubulin was used as loading control. (B) Dotplot of PI live exclusion assay for quantification of cell viability. NCI-H460, A549 and HeLa were treated with or without glucose free medium for 6 hours and the cell death was measured by flow cytometry using PI (5 μg/mL) staining (circle, a representative of viable cells). (C) Statistical analysis of the percentage of viable cells of three independent experiments performed as in (B). (mean ± SD, *$p<0.05$, **$p<0.01$).

To investigate whether glucose supplementation can rescue cell death upon glucose starvation in NCI-H460, we added glucose for indicated concentrations to glucose free medium (4500 mg/mL (100%), 90 mg/mL (2%), 45 mg/mL (1%), 22.5 mg/mL (0.5%), 11.25 mg/mL (0.25%), and 4.5 mg/mL (0.1%)). According to the morphological changes and cell death quantification (Figure 3-3A and B), we found that glucose supplementation can potently reverse glucose starvation-induced cell death in a dose-dependent manner. It is to be noted that 1% (45 mg/L) of glucose can fully protected NCI-H460 from cell death, further confirming the importance of glucose in survival of NCI-H460.

A




B

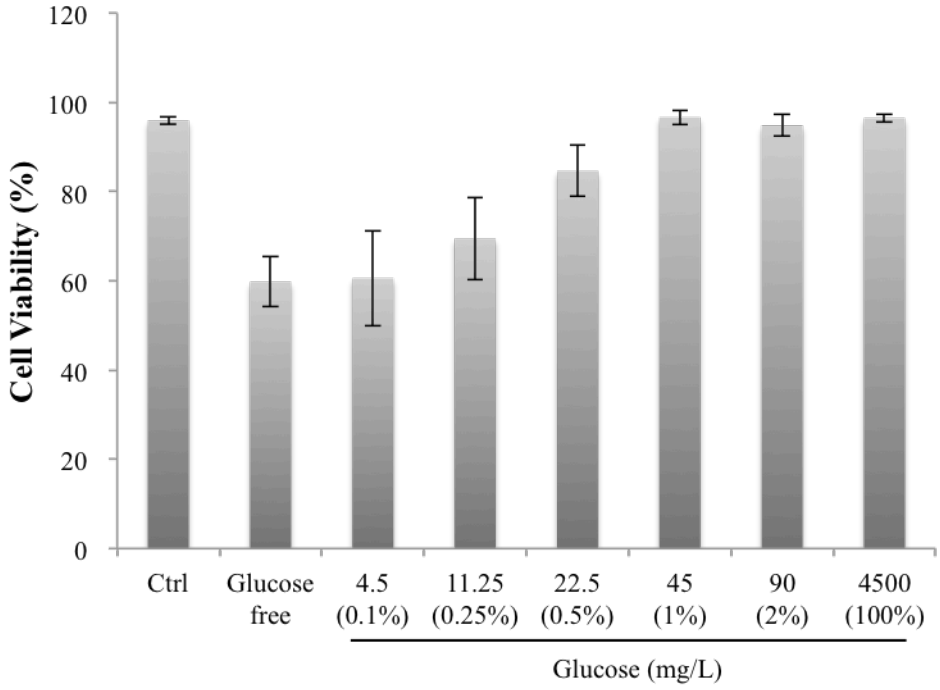



**Figure 3-3** Glucose supplement can inhibit glucose starvation-induced cell death in a dose-dependent manner. (A) Morphological representatives of NCI-H460 cells upon glucose supplementation. NCI-H460 cells were treated with full medium or glucose free medium for 3 hours. Indicated concentrations of glucose were added back in glucose free medium. Cells were photographed under a light microscope. Scale bar, 200 μm. (B) Statistical analysis of PI live exclusion assay for quantification of cell viability. Cells were treated as indicated in (A). The percentage of viable cells was measured with PI (5 μg/mL) staining by flow cytometry. (mean ± SD).

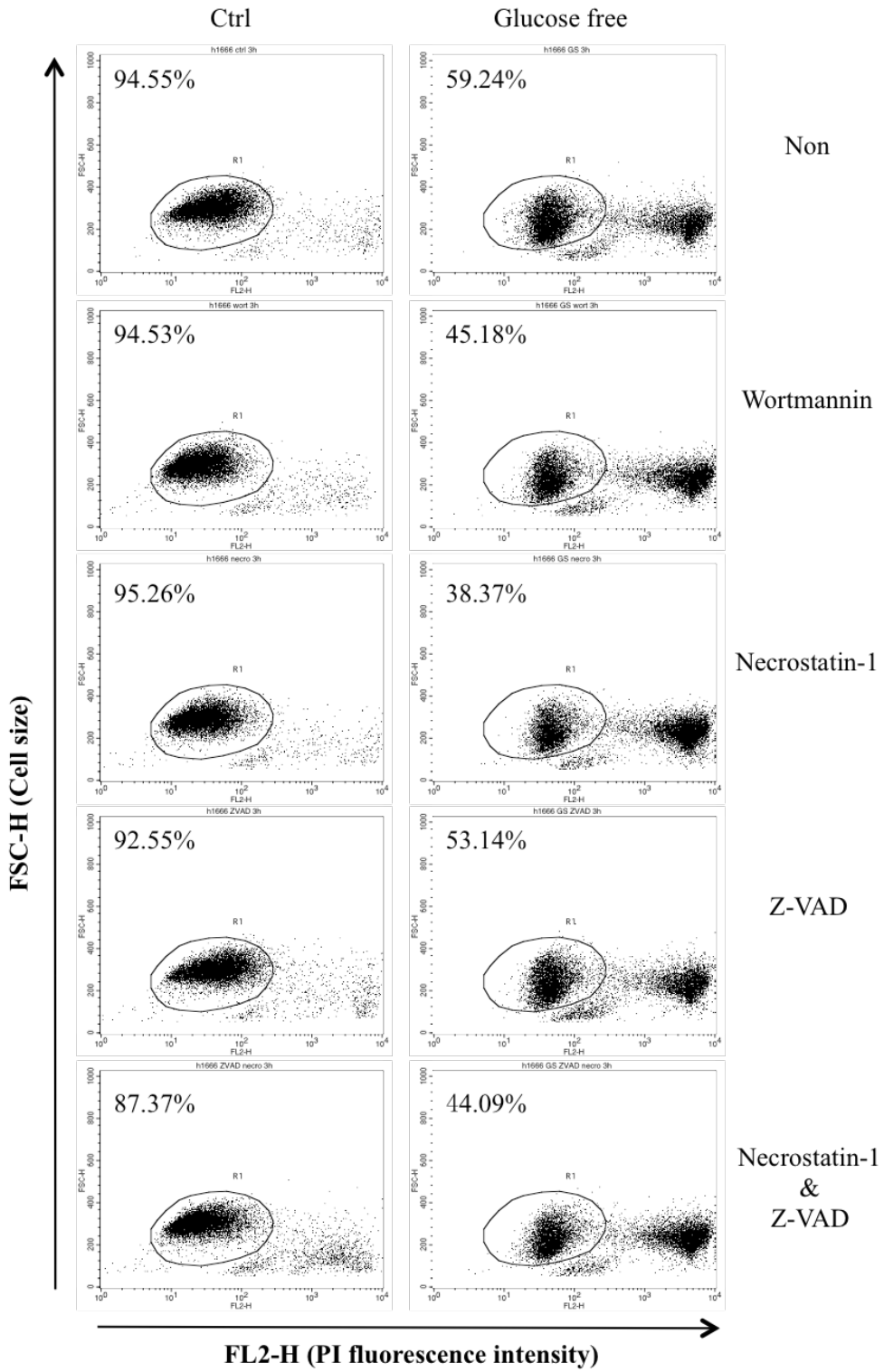
### 3.2 Apoptosis, necroptosis or autophagy is not the major cell death mechanism upon glucose starvation in NCI-H460

Previous findings have indicated the protective role of AMPK in response to poor nutrient environments, and defective AMPK signaling may render tumor cells sensitive to apoptosis under metabolic stress (Faubert et al., 2013; Svensson and Shaw, 2012). Thus, to explore the type of cell death in NCI-H460 subjected to glucose starvation, we used the pan-caspase inhibitor Z-VAD, which was not able to inhibit glucose starvation-induced cell death (Figure 3-4A), suggesting that apoptosis may not be the major form of cell death. Additionally, necrostatin-1, a specific necrosis inhibitor functioning through suppression of RIP1, was unable to protect against cell death under glucose starvation (Figure 3-4A). Moreover, the combination of Z-VAD and necrostatin-1 presented no effect on glucose starvation-triggered cell death (Figure 3-4A), implying that NCI-H460 does not go through apoptosis or necrosis when deprived with glucose.

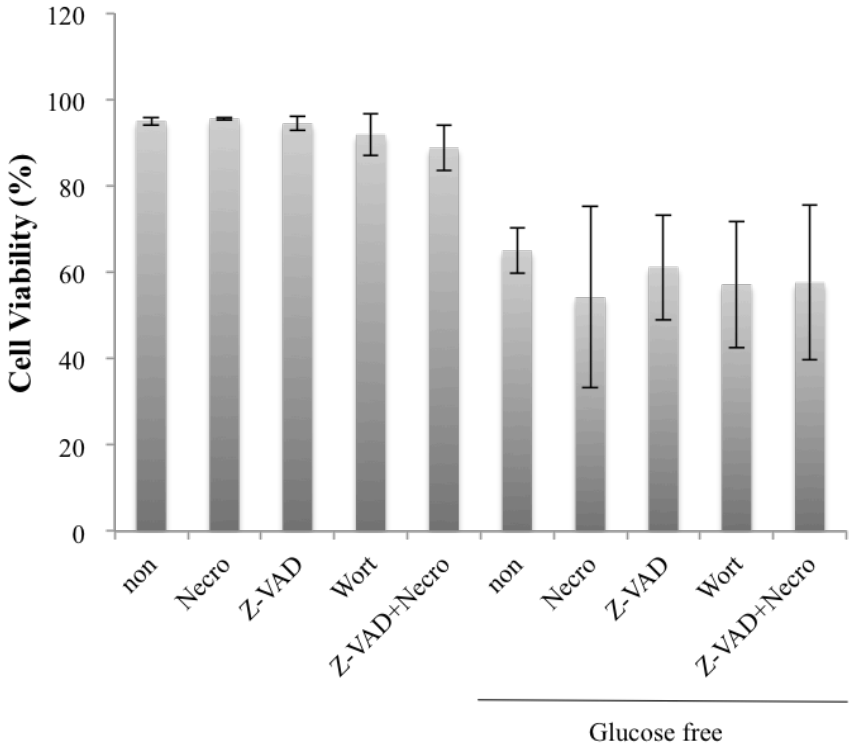
Under nutrient starvation, cells initiate a cellular protective mechanism known as autophagy to maintain energy homeostasis. The regulation of autophagy by AMPK-mTOR signaling pathway in response to glucose starvation has been well studied (Egan et al., 2011; Gwinn et al., 2008; Inoki et al., 2003; Kim et al., 2011). So we next examined changes of mTOR activity and the classical autophagy marker LC3 under glucose starvation. As

shown in Figure 3-4C, No significant changes of p-S6 were found upon 3 hours glucose starvation treatment. We then used PI3K inhibitor wortmannin to suppress autophagy. Notably, wortmannin did not protect against glucose starvation-induced cell death (Figure 3-4A). Therefore, it is believed that apoptosis, necrosis or autophagic cell death is not the major mechanism in glucose starvation-induced cell death in NCI-H460, and an alternative mechanism might be involved in this particular form of cell death.

A


Ctrl
Glucose free
94.55%
59.24%
Non
94.53%
45.18%
Wortmannin
95.26%
38.37%
Necrostatin-1
92.55%
53.14%
Z-VAD
87.37%
44.09%
Necrostatin-1
&
Z-VAD
FSC-H (Cell size)
FL2-H (PI fluorescence intensity)

B


120
100
80
60
40
20
0
Cell Viability (%)
non
Necro
Z-VAD
Wort
Z-VAD+Necro
non
Necro
Z-VAD
Wort
Z-VAD+Necro
Glucose free

C

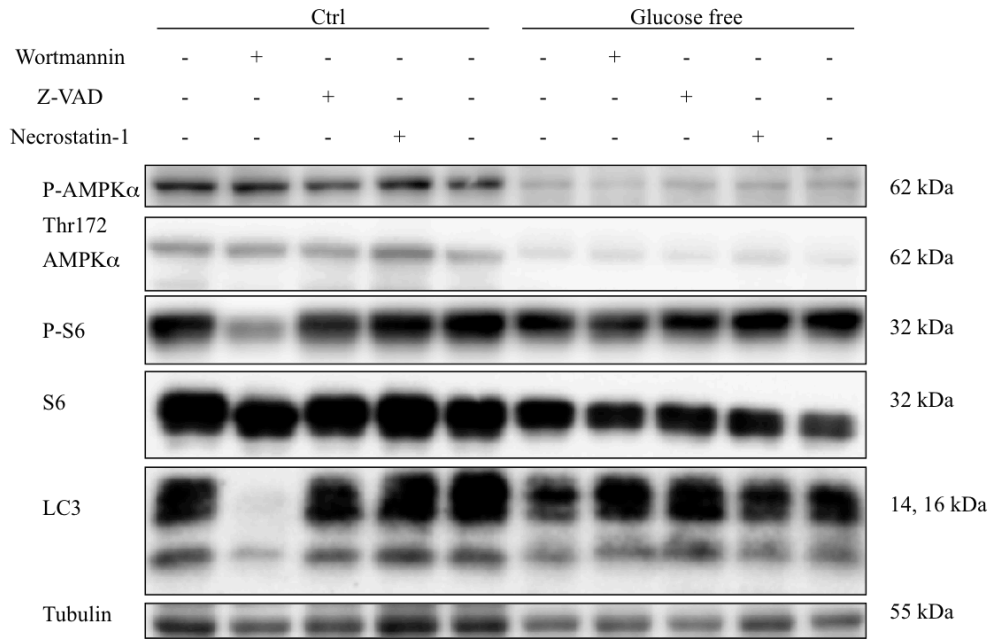

Ctrl
Glucose free
Wortmannin - + - - - - + - - -
Z-VAD - - + - - - - + - -
Necrostatin-1 - - - + - - - - + -
P-AMPKα
Thr172
62 kDa
AMPKα
62 kDa
P-S6
32 kDa
S6
32 kDa
LC3
14, 16 kDa
Tubulin
55 kDa

**Figure 3-4** Cell death inhibitors cannot protect NCI-H460 from glucose starvation-induced cell death. (A) Dotplot of PI live exclusion assay for quantification of cell viability. NCI-H460 were treated with Z-VAD (10 mM), necrostatin-1 (30 mM), wortmannin (100 mM), and combination of Z-VAD (10 mM) and necrostatin-1 (30 mM), with or without glucose free medium for 3 hours. The cell death was measured by flow cytometry using PI (5 μg/mL) staining (circle, a representative of viable cells). (B) Statistical analysis of the percentage of viable cells of three independent experiments performed as in (A) (mean ± SD). (C) Cells were treated as indicated in (A) and cell lysates were analyzed by western blotting. Tubulin was used as loading control.

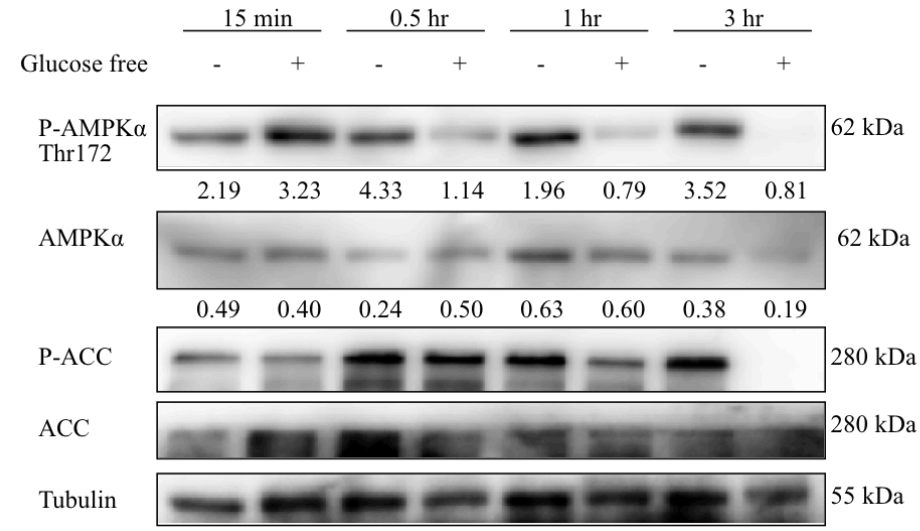
### 3.3 AMPK activity and protein stability is changed upon glucose starvation

AMPK is activated in response to declining cellular ATP level, especially when glucose supply is limited (Hardie et al., 2012). Activated AMPK promotes catabolic pathways while suppresses anabolic pathways to maintain cellular homeostasis (Hardie, 2011b). However, NCI-H460 has been identified with LKB1 nonsense mutation (Koivunen et al., 2008), suggesting the possibility of defective AMPK activation in response to nutrient starvation. To address this question, we examined AMPK activation upon glucose starvation by western blot. NCI-H460 cells were subjected to glucose free medium for different periods of time (15 min, 0.5 hr, 1 hr, and 3 hr). Interestingly, as shown in Figure 3-5A, phosphorylated AMPKα (Thr172) was first increased (15 min) then decreased upon glucose starvation, suggesting the inability of sustained AMPK activation under prolonged metabolic stress. To further determine the activation of AMPK, we examined the phosphorylation of one of its critical downstream targets, acetyl CoA carboxylase (ACC). In Fig 3-5A, in early time points (15 min and 0.5 hr), no significant increase of p-ACC was observed under glucose starvation. However, in 1 hr and 3 hr time points, the level of p-ACC was reduced, which was consistent with p-AMPKα (Thr172). The early activation of AMPK may be result of other AMPK upstream kinases such as CaMKKβ

and TAK1, which may not be sufficient to stimulate AMPK activation with persistent glucose starvation. Moreover, total AMPKα level also declined under prolonged glucose starvation treatment (for 3 hr), suggesting the protein instability of AMPKα during glucose starvation.

To further confirm the role of AMPK activity and protein stability in glucose starvation, we investigated whether glucose supplementation can reactivate AMPK and restore AMPKα protein level. As shown in Figure 3-5B, AMPKα protein level and p-AMPKα was increased by glucose supplementation dose-dependently. These results demonstrated the critical role of AMPK in cell survival under glucose starvation, and suggest that AMPK protein instability upon prolonged glucose starvation may be the cause of defective AMPK activation.

A




B

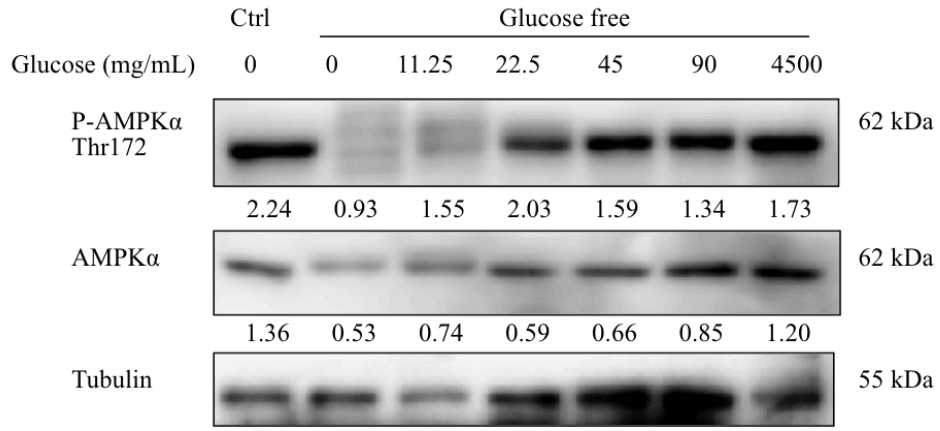
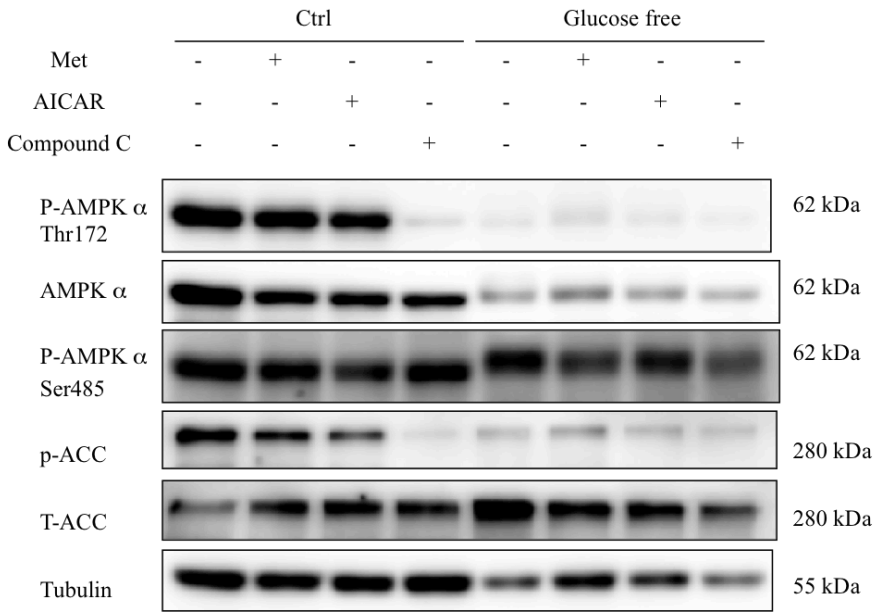



**Figure 3-5** AMPK level changes during glucose starvation. (A) NCI-H460 cells were treated with or without glucose free medium for 15 min, 0.5 hr, 1 hr, 3 hr. Cell lysates were analyzed by western blotting. Tubulin was used as loading control. AMPKα was normalized to control, and p-AMPKα was normalized to total AMPKα. (B) NCI-H460 cells were treated with or without glucose free medium for 3 hours. A gradient of concentrations of glucose were added back to glucose free medium. Cell lysates were analyzed by western blotting. Tubulin was used as loading control.

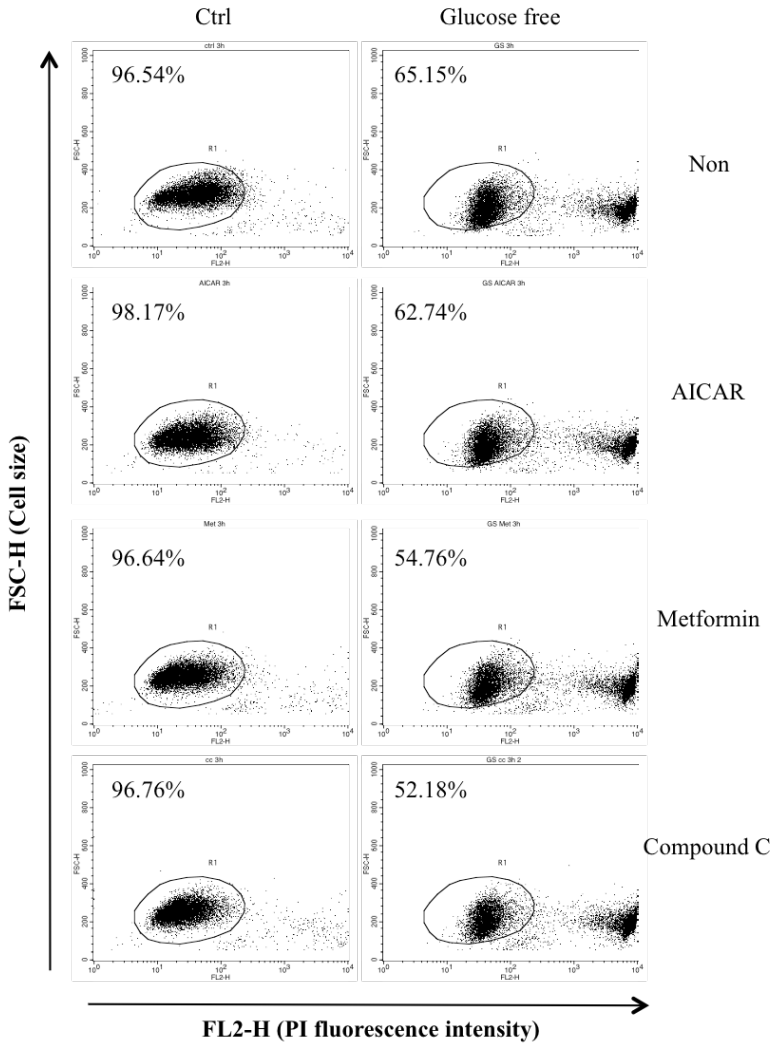
### 3.4 AMPK activator 2DG stabilizes AMPK protein level and protects against glucose starvation-induced cell death

Next, we used classical AMPK activators AICAR and metformin to investigate whether AMPK can be reactivated under glucose starvation condition, and whether cell death can be suppressed. AMPK inhibitor Compound C was used as a negative control. We found that both pharmacological activators of AMPK cannot trigger AMPK activation under glucose starvation, as evidenced by changes of p-AMPKα (Figure 3-6A). In addition, AICAR and metformin showed no effect on glucose starvation-induced cell death as quantified by flow cytometry (Figure 3-6B). One possible explanation is that both AICAR and metformin require LKB1 to fully activate AMPK. AICAR generates AMP analogue ZMP, which binds to AMPK γ-subunit in the same manner as AMP (Day et al., 2007). Metformin suppresses mitochondria ATP synthesis to increase cellular ADP/ATP and AMP/ATP rations (Hardie et al., 2012).

A


Ctrl
Glucose free
Met - + - - - + - -
AICAR - - + - - - + -
Compound C - - - + - - - +
P-AMPK α Thr172
62 kDa
AMPK α
62 kDa
P-AMPK α Ser485
62 kDa
p-ACC
280 kDa
T-ACC
280 kDa
Tubulin
55 kDa

B


Ctrl
Glucose free
96.54%
65.15%
Non
98.17%
62.74%
AICAR
96.64%
54.76%
Metformin
96.76%
52.18%
Compound C
FSC-H (Cell size)
FL2-H (PI fluorescence intensity)

C

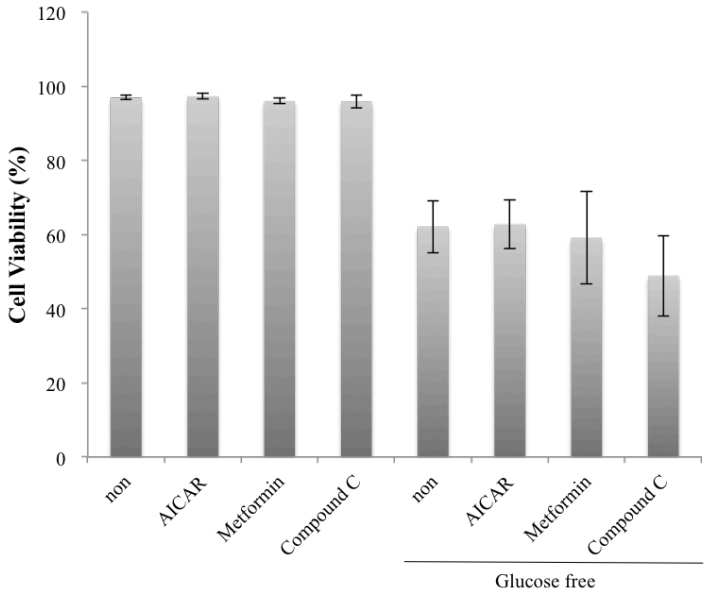
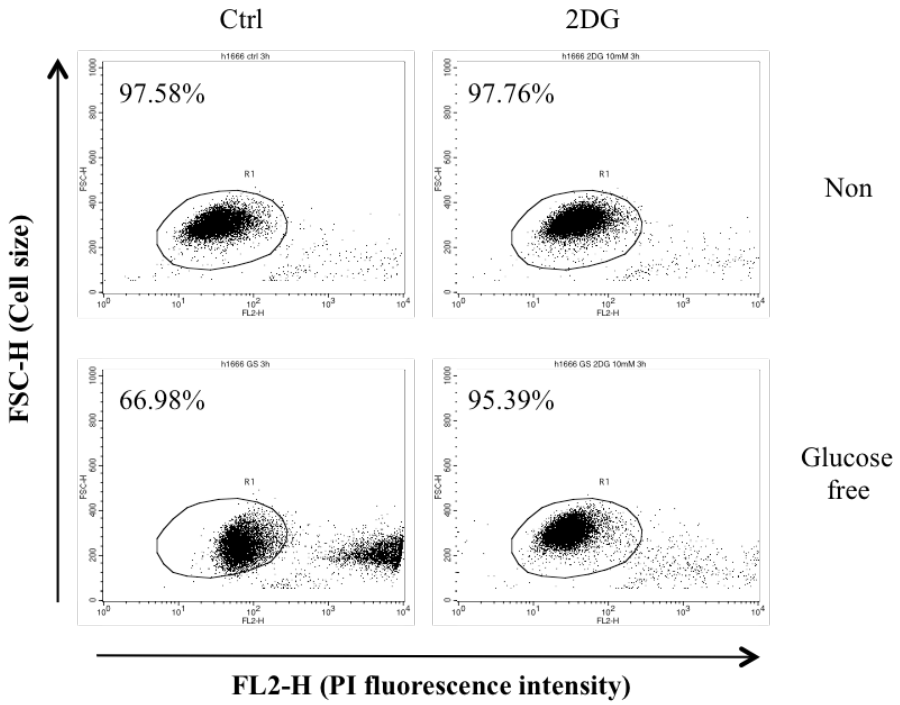


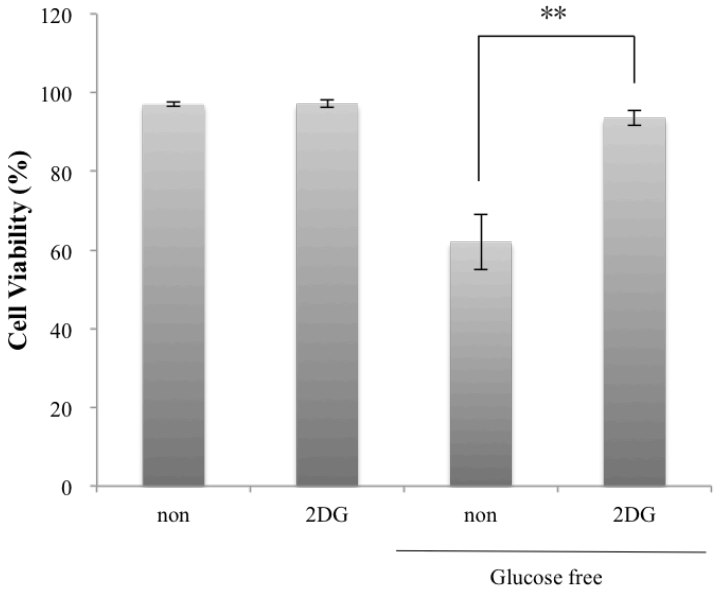

**Figure 3-6** AMPK activators cannot protect against glucose starvation-induced cell death. NCI-H460 cells were treated metformin 1 mM, AICAR 0.5 mM, Compound C 10 mM, with or without glucose free medium for 3 hours. (A) Cell lysates were analyzed by western blotting. Tubulin was used as loading control. (B) Dotplot of PI live exclusion assay for quantification of cell viability. NCI-H460 cells were treated as indicated in (A) and the cell death was measured by flow cytometry using PI (5 μg/mL) staining (circle, a representative of viable cells). (C) Statistical analysis of the percentage of viable cells of three independent experiments performed as in (B). (mean ± SD).

Further, we replaced glucose with the non-metabolizable glucose analogues 2-deoxyglucose (2DG). 2DG enters the cells by glucose transporters and is phosphorylated by hexokinase to 2DG-6-P, which cannot be further metabolized. Thus, 2DG-6-P is trapped and accumulated in cells, resulting in competitive inhibition of glycolysis at the step of phosphorylation of glucose by hexokinase (Aft et al., 2002; Pelicano et al., 2006). Glycolysis inhibition can lower cellular ATP level, leading to AMPK activation (Kodiha and Stochaj, 2011). As a glycolysis inhibitor, 2DG has been reported as a promising antitumor strategy. For example, it has been reported that 2DG increases cytotoxicity for human cancer cells (Li et al., 2014). In our study, surprisingly, 2DG showed potent ability to protect against cell death induced by glucose in LKB1-deficient NCI-H460 cell line (Figure 3-7A). Unlike AICAR and metformin, 2DG directly activated AMPK independent of LKB1 and restored total AMPKα level during glucose starvation (Figure 3-7C). These results are consistent with the previous findings that 2DG inhibits LKB1-deficient cells A549 mainly through direct activation of AMPK and partial utilization in the pentose phosphate pathway (Jeon et al., 2012).

A


Ctrl
2DG
97.58%
97.76%
66.98%
95.39%
Non
Glucose free
FSC-H (Cell size)
FL2-H (PI fluorescence intensity)

B


Cell Viability (%)
120
100
80
60
40
20
0
**
non
2DG
non
2DG
Glucose free

C

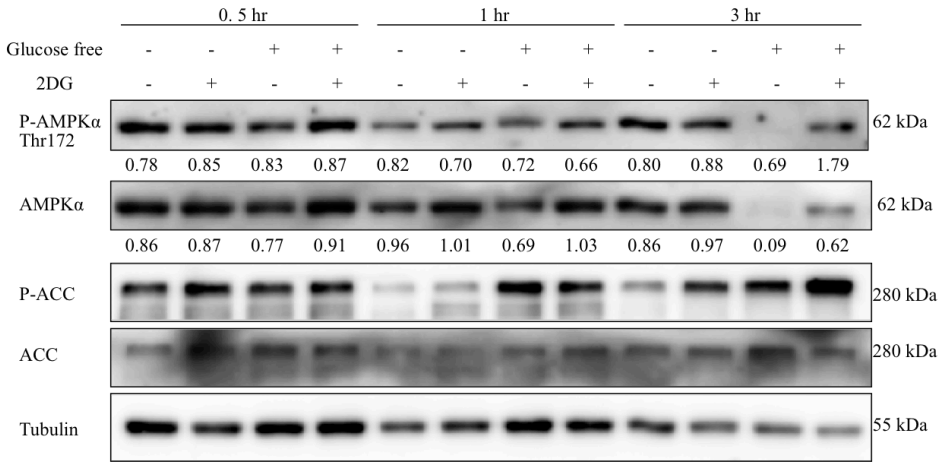
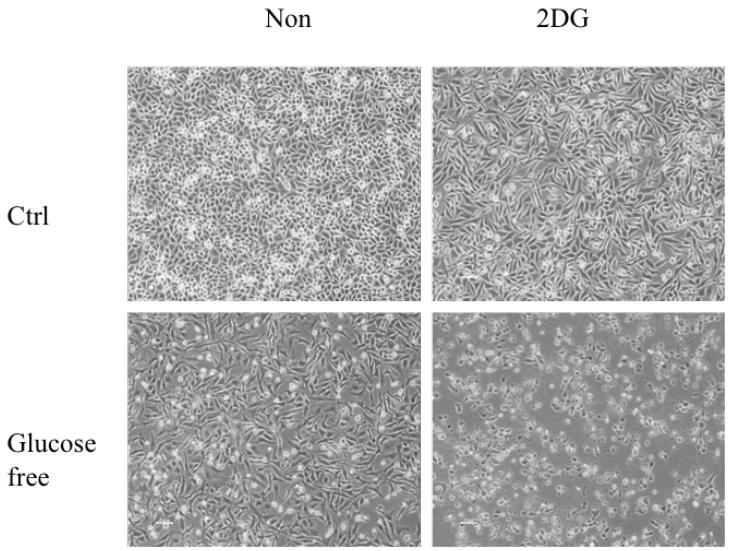


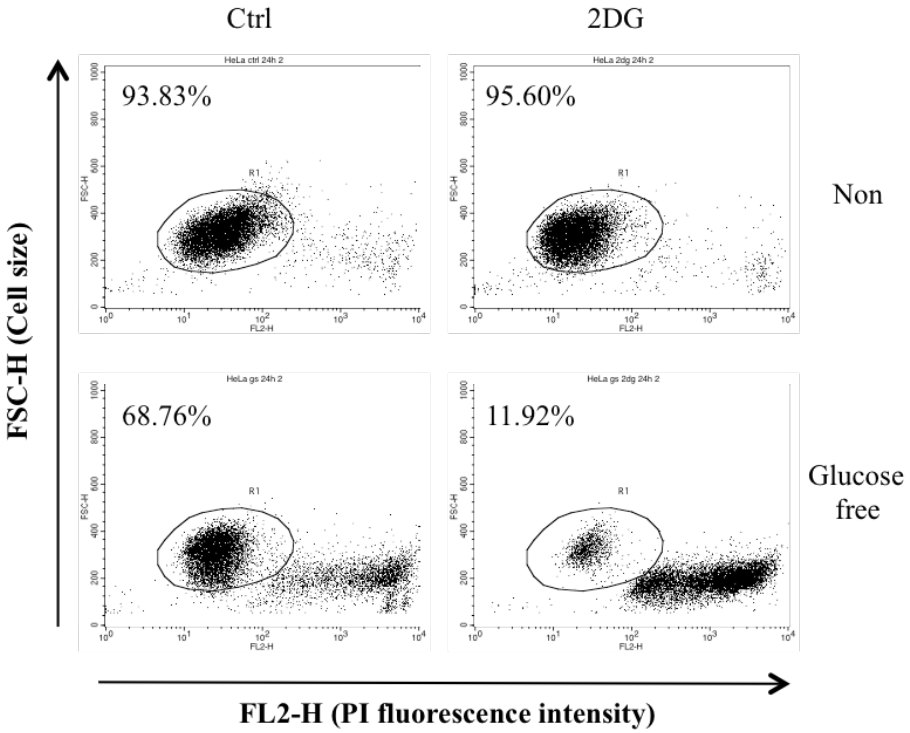

**Figure 3-7** 2DG significantly inhibit glucose starvation-induced cell death. (A) Dotplot of PI live exclusion assay for quantification of cell viability. NCI-H460 cells were treated 2DG 10 mM, with or without glucose free medium for 3 hours. The percentage of viable cells was measured with PI (5 μg/mL) staining by flow cytometry (circle, a representative of viable cells). (B) Statistical analysis of the percentage of viable cells of three independent experiments performed as in (A). (mean ± SD, *$p<0.05$, **$p<0.01$). (C) NCI-H460 cells were treated 2DG 10 mM, with or without glucose free medium for 0.5, 1, and 3 hours. Cell lysates were analyzed by western blotting. Tubulin was used as loading control. AMPKα was normalized to control, and p-AMPKα was normalized to total AMPKα.

Intriguingly, we found that 2DG promotes, instead of protecting, HeLa cell from glucose starvation-induced cell death. As shown in Figure 3-8A, B and C, 12 hours combined treatment of 10 mM 2DG and glucose starvation showed significant increase of cell death in HeLa cells. Prolonged treatment of 2DG for 24 hours presented higher level of cell death in HeLa (Figure 3-8 C). However, 2DG alone had no effect on HeLa cell survival, even in 24 hours treatment.

A


Non
2DG
Ctrl
Glucose free

B


Ctrl
2DG
93.83%
95.60%
68.76%
11.92%
Non
Glucose free
FSC-H (Cell size)
FL2-H (PI fluorescence intensity)

C

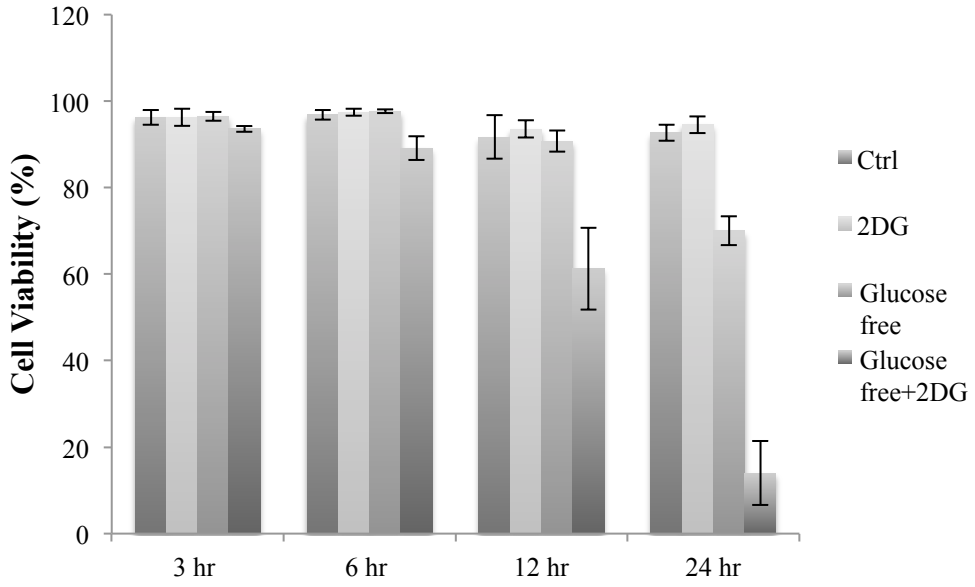



**Figure 3-8** 2DG cannot protect HeLa from glucose starvation-induced cell death. (A) Morphological representatives of HeLa cells treated with 2DG 10 mM, with or without glucose free medium for 12 hours. HeLa cells were photographed under a light microscope. Scale bar, 200 μm. (B) Dotplot of PI live exclusion assay for quantification of cell viability. HeLa cells were treated 2DG 10 mM, with or without glucose free medium for 12 hours. The percentage of viable cells was measured with PI (5 μg/mL) staining by flow cytometry (circle, a representative of viable cells). (C) Statistical analysis of the percentage of viable cells of three independent experiments for indicated periods of time. (mean ± SD).

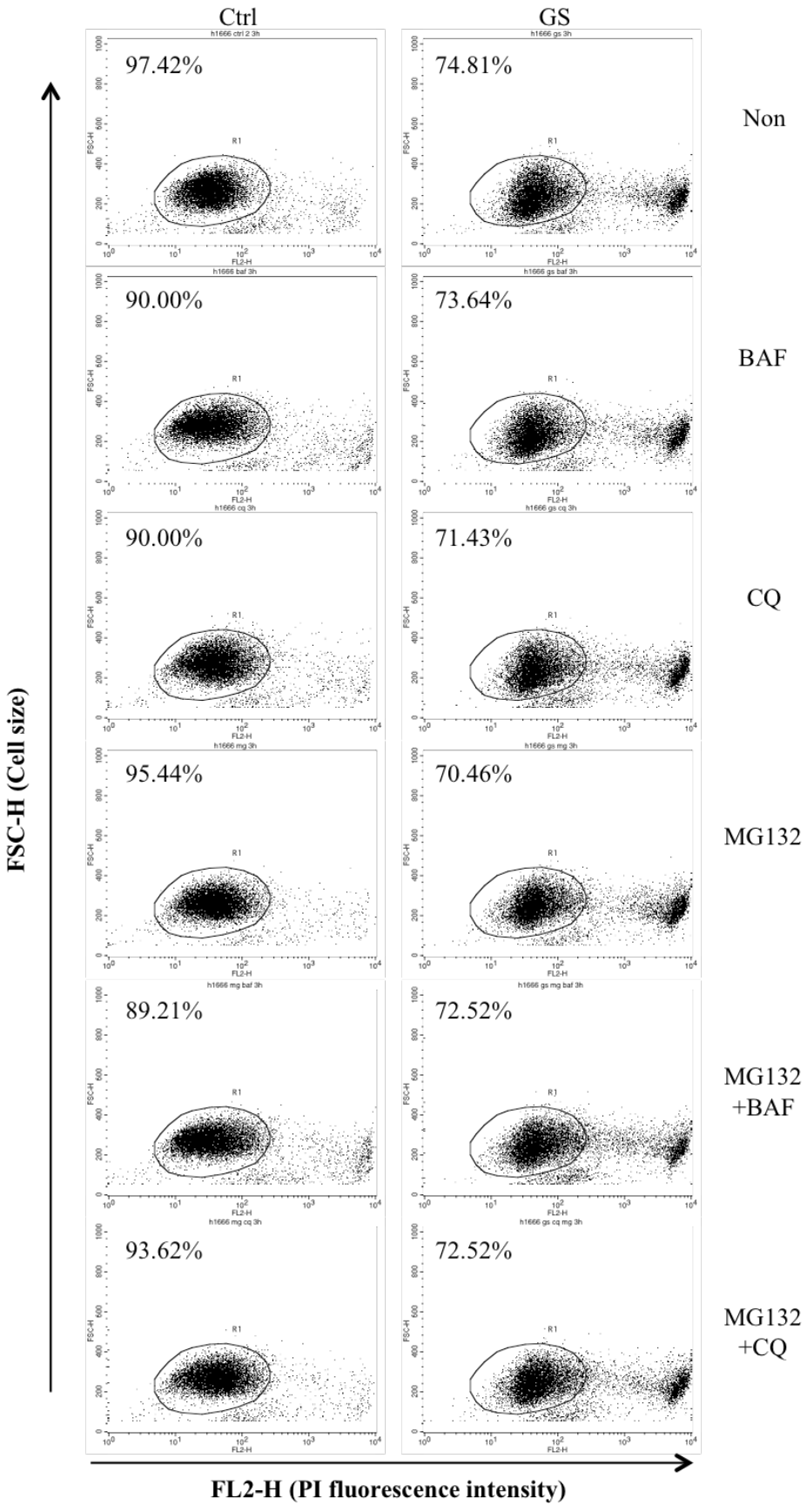
### 3.5 The down-regulation of AMPK is independent of lysosome- or proteasome-mediated pathways

Since glucose starvation-induced cell death is accompanied with the loss of AMPK, we are therefore determined to examine the molecular mechanisms controlling AMPK protein stability in this particular cell line. First, we tested whether AMPK is degraded via lysosome by using lysosome inhibitors bafilomycin A1 (BFA), a specific inhibitor for the lysosomal V-ATPase, and chloroquine (CQ) that is capable of neutralizing lysosomal pH (Zhou et al., 2013). However, both inhibitors showed no protective effect against glucose starvation-induced cell death or the decrease of AMPK protein level (Figure 3-9A, B and C), suggesting that lysosome-dependent degradation pathway is not involved in the down-regulation of AMPK under glucose starvation.

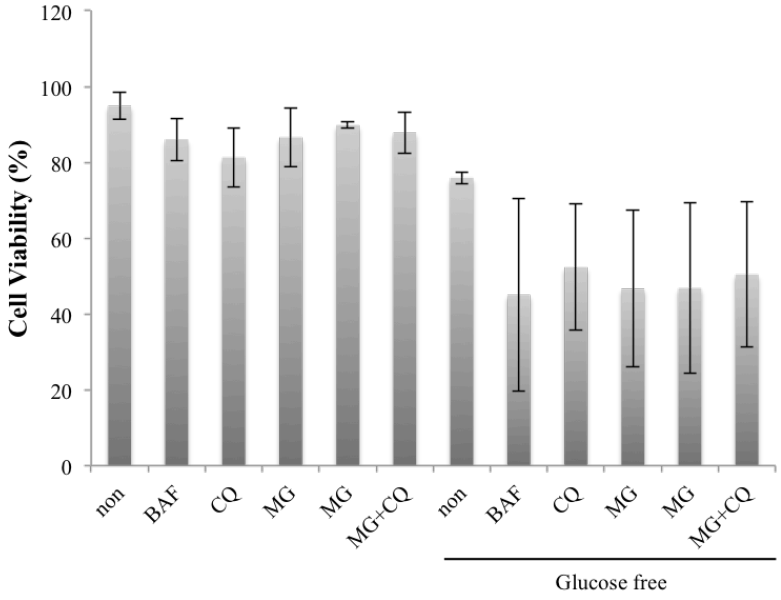
The ubiquitin-proteasome system (UPS) is an alternative protein degradation mechanism responsible for 80-90% of intracellular proteins (Shen et al., 2013b). One interesting paper from Qi et a earlier study has demonstrated the ubiquitination-mediated degradation of AMPK mediated by Cidea through interaction with the β-subunit of AMPK (Qi et al., 2008). Thus we examined the involvement of proteasomal degradation of AMPK in cell death triggered by glucose withdraw. We used MG-132 (carbobenzoxy-Leu-Leu-leucinal) and Bortezomib to block the proteolytic

activity of the 26S proteasome (Shen et al., 2013b). However, both inhibitors did not protect NCI-H460 from rapid cell death stimulated by glucose starvation (Figure 3-9A and B). Even the combination of proteasome inhibitor MG-132 and lysosome inhibitors showed no effect on cell death quantification. Importantly, proteasome inhibitors did not significantly change AMPK protein level under glucose starvation (Figure 3-9D). Therefore, these results exclude the possibility that AMPK is down regulated through lysosome-dependent or proteasome-mediated degradation pathways upon glucose starvation.

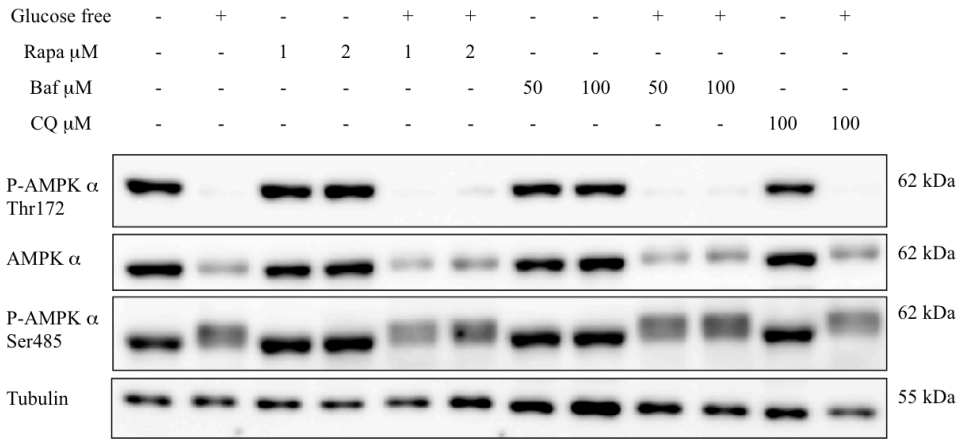
A


Ctrl
GS
97.42%
74.81%
Non
90.00%
73.64%
BAF
90.00%
71.43%
CQ
95.44%
70.46%
MG132
89.21%
72.52%
MG132
+BAF
93.62%
72.52%
MG132
+CQ
FSC-H (Cell size)
FL2-H (PI fluorescence intensity)

B


120
100
80
60
40
20
0
Cell Viability (%)
non
BAF
CQ
MG
MG
MG+CQ
non
BAF
CQ
MG
MG
MG+CQ
Glucose free

C


Glucose free - + - - + + - - + + - +
Rapa μM - - 1 2 1 2 - - - - - -
Baf μM - - - - - - 50 100 50 100 - -
CQ μM - - - - - - - - - - 100 100
P-AMPK α Thr172
62 kDa
AMPK α
62 kDa
P-AMPK α Ser485
62 kDa
Tubulin
55 kDa

D

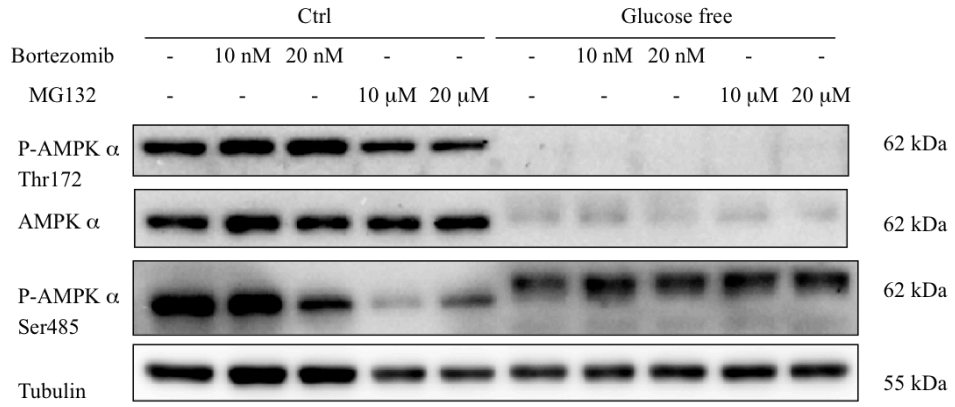


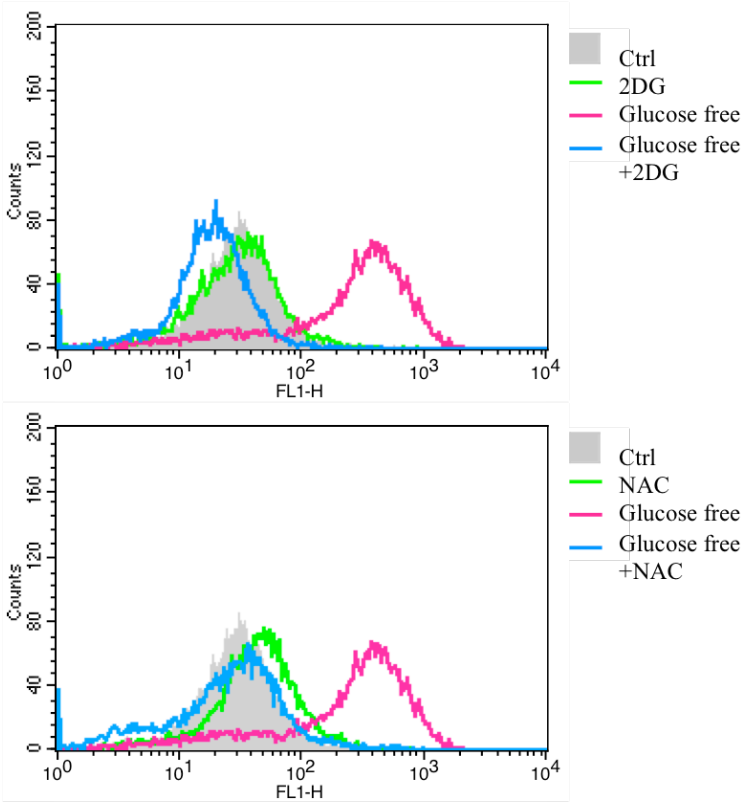

**Figure 3-9** Lysosome inhibitors or proteasome inhibitors cannot inhibit glucose starvation-induced cell death. (A) Dotplot of PI live exclusion assay for quantification of cell viability. NCI-H460 cells were treated with Bafilomycin (BAF) 50 mM, Choloroquine (CQ) 50 mM, MG-132 10 μM, combination of 50 mM BAF and 10 μM MG-132, combination of 50 mM CQ and 10 μM MG-132, with or without glucose free medium for 3 hours. The percentage of viable cells was measured with PI (5 μg/mL) staining by flow cytometry (circle, a representative of viable cells). (B) Statistical analysis of the percentage of viable cells of three independent experiments for indicated periods of time. (mean ± SD). (C) NCI-H460 cells were treated with BAF and CQ and rapamyin for indicated concentrations, with or without glucose free medium for 3 hours. Cell lysates were analyzed by western blotting. Tubulin was used as loading control. (D) NCI-H460 cells were treated MG-132 and Bortezomib for indicated concentrations, with or without glucose free medium for 3 hours. Cell lysates were analyzed by western blotting. Tubulin was used as loading control.

### 3.6 Higher level of intracellular ROS in glucose starvation-induced cell death

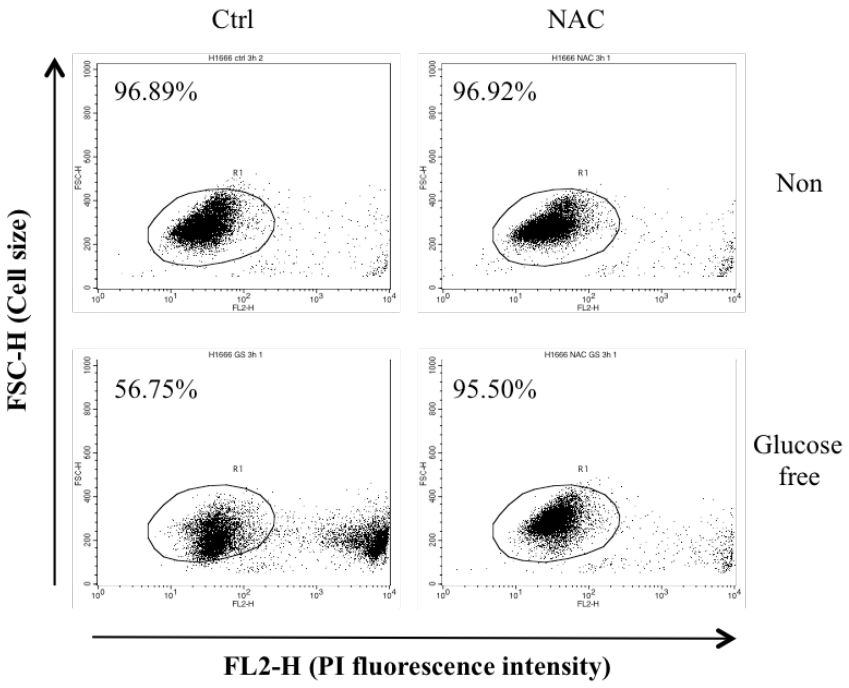
ROS refers to the chemical species generated upon incomplete oxygen reduction, including superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($HO\cdot$) (D'Autréaux and Toledano, 2007). ROS function as not only a mediator of oxygen toxicity, but also intracellular signaling molecules (D'Autréaux and Toledano, 2007). The imbalance between accumulated ROS and inadequate antioxidant defenses will lead to oxidative stress. At present, there is convincing evidence that glucose starvation is able to induce oxidative stress (Wu et al., 2013). Thus we examined cellular ROS level during glucose starvation by CM-H2DCFDA staining coupled with flow cytometry. As shown in Figure 3-10A, intracellular ROS was significantly increased when treated with glucose free medium for 1 hour. Antioxidant NAC (N-acetylcysteine) was able to reverse accelerated ROS level upon glucose starvation (Figure 3-10A). We further observed a significantly protective role in glucose starvation induced cell death by NAC, evidenced by PI staining (Figure 3-10B and C). In addition, NAC was able to stabilize AMPK protein level, as shown in Figure 3-10D, highlighting functional relevance of cellular ROS to AMPK stability in cells under glucose starvation. Since 2DG can stabilize AMPK in glucose starvation, we further investigated whether 2DG affected increased ROS level. Consistently,

we found that 2DG significantly inhibited ROS production during glucose starvation (Figure 3-10A). These results suggest the involvement of cellular ROS in AMPK stability upon glucose starvation.

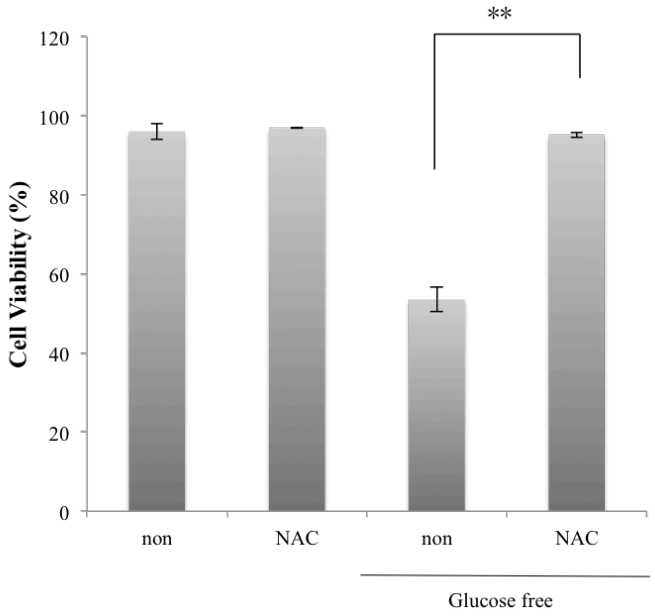
A


Counts
FL1-H
Ctrl
2DG
Glucose free
Glucose free +2DG
Ctrl
NAC
Glucose free
Glucose free +NAC

B


Ctrl
NAC
96.89%
96.92%
56.75%
95.50%
Non
Glucose free
FSC-H (Cell size)
FL2-H (PI fluorescence intensity)

C




D

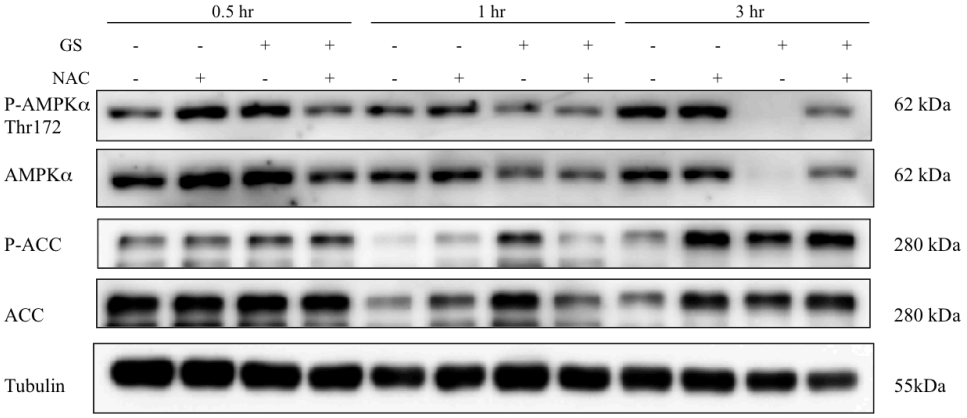



**Figure 3-10** NAC and 2DG can reverse elevated cytosolic ROS upon glucose starvation. (A) Cells were treated with 5 mM NAC and 10 mM 2DG, with or without glucose free medium for 1 hour or 3 hours. Cells were stained with DCFDA 1 mM for 15 min. (B) Dotplot of PI live exclusion assay for quantification of cell viability. Cells were treated NAC 5 mM, with or without glucose free medium for 3 hours. The percentage of viable cells was measured with PI (5 μg/mL) staining by flow cytometry (circle, a representative of viable cells). (C) Statistical analysis of the percentage of viable cells of three independent experiments performed as in (B). (mean ± SD, *$p<0.05$, **$p<0.01$). (D) Cells were treated with 5 mM NAC, with or without glucose free medium for 0.5, 1, 3 hours. Cell lysates were analyzed by western. Tubulin was used as loading control.

# CHAPTER 4 DISCUSSION

AMPK is an evolutionarily conserved energy sensor and regulator in mammalian cells, activated upon stress conditions including nutrient limitation, oxidative stresses, etc. (Hardie, 2011b) However, regulation of AMPK activity by protein stability is rarely investigated. In this study, we discovered that a non-small cell lung cancer cell line NCI-H460 is particularly susceptible to glucose starvation. In response to metabolic stress induced by glucose starvation, cellular ROS is significantly elevated, accompanied by rapid AMPK phosphorylation and activation. However, prolonged depletion of glucose (for 3 hours) induces loss of AMPK protein stability and activation, which cannot be suppressed by proteasome and lysosome inhibitors. Only glycolysis inhibitor 2-deoxyglucose and antioxidant NAC are able to stabilize AMPK, reverse ROS level and eventually protect against cell death. Further studies will focus on the molecular mechanisms by which AMPK is down-regulated upon glucose starvation, especially via post-translational modifications of AMPK.

### 4.1 NCI-H460 is heavily dependent on glucose for survival

In this study, we used five types of nutrient starvation, namely glucose, glutamine, growth factor (serum), amino acid, and double starvation (no amino acid, no serum), to determine the major nutrient requirement of NCI-H460. Cancer cells undergo metabolic reprogramming to satisfy their

energetic and synthetic requirements in the limiting circumstances, when much of the glucose enters the aerobic glycolytic pathway which is less efficient for ATP generation but supports macromolecule synthesis, instead of the citric acid cycle (Soga, 2013). In order to compensate for the metabolic changes and to sustain citric acid cycle, glutamine metabolism is elevated, providing cancer cells with glutamate hydrolyzed and subsequent production α-keto-glutarate for citric acid cycle and biosynthesis (Erickson and Cerione, 2010). Thus, cancer cells are often regarded as "glutamine addicted" due to the sensitivity to glutamine starvation, especially in low glucose/hypoxia conditions (Le et al., 2012; Teicher et al., 2012).

Additionally, deprivation of amino acids, growth factors (serum starvation) or glucose will lead to mTORC1 inactivation, and consequently repression of anabolic processes and promotion of catabolic processes, especially autophagy (Dibble and Manning, 2013; Shaw and Cantley, 2006).

To our surprise, NCI-H460 is specifically sensitive to glucose starvation, and the onset of cell death induced by glucose starvation is rapid, suggesting that NCI-H460 cells are heavily dependent on glucose for survival. In glucose-starved cells, ATP is highly required for survival and AMPK is activated by increased AMP/ATP ratio. Su et al. observed that AMPK activity was persistently increased by glucose stimulation after 24 hours glucose starvation in NCI-H460, with ATP level increased and lactate level

decreased, suggesting the inhibition of glycolysis (Su et al. 2010). These results indicated that glucose functions as a key factor for regulating energy status in NCI-H460.

### 4.2 LKB1 is frequently mutated in NSCLC

NCI-H460 is derived from large cell lung cancer, one subtype of NSCLC. NSCLC cell lines are reported to have high frequency of somatic alterations of *LKB1/STK11* tumor suppressor, mainly nonsense mutations (Sanchez-Cespedes et al., 2002; Matsumoto et al., 2007). A homozygous nonsense LKB1 mutation at codon 37 has been identified in NCI-H460, which is identical to A549 (Carretero et al., 2004; Sanchez-Cespedes, 2007). A recent report has showed that the nonsense LKB1 mutation in lung cancer cell line NCI-H460 solely produced catalytically inactive isoform of LKB1 with an N-terminal truncation (named ΔN-LKB1), which is not able to interact with LKB1 binding partners, STRADα and MO25 (Dahmani et al., 2014). ΔN-LKB1 has a dominant-positive effect on AMPK activity through binding to the AID domain of AMPKα, which requires a functional LKB1 catalytic activity (Dahmani et al., 2014).

Oncogenic *KRAS* or *BRAF* mutations often occur concurrently with *LKB1* mutation in NSCLC cell lines (Sanchez-Cespedes et al., 2002; Carretero et al. 2004; Koivunen et al., 2008; González-Sánchez et al., 2013).

It is well established that LKB1-AMPK signaling pathway functions as an essential checkpoint in cellular energy homeostasis to maintain cell survival, growth and proliferation (Zheng et al., 2009). Phosphorylation of LKB1 through RAF-MEK-ERK signaling impairs the ability of LKB1 to interact with and phosphorylate AMPK at Thr172, thereby suppressing AMPK activation even under conditions of increased AMP/ATP or ADP/ATP ratios (Zheng et al., 2009). A feed back loop between RAF-MEK-ERK and LKB1-AMPK has been identified, by which AMPK activation in response to energy stress attenuates BRAF-regulated mitogenic effects through phosphorylation of BRAF (Shen et al., 2013a). LKB1 deficiency causes hypersensitive to apoptosis in response to AICAR treatment, mimicking elevated AMP conditions by generating AMP analogue ZMP (Shaw et al., 2004b). A possible explanation is aberrant activation of mTOR due to the absence of LKB1-AMPK signaling under stresses like nutrient deprivation and low energy, resulting in apoptosis. AMPK phosphorylates upstream inhibitor of mTOR tuberous sclerosis complex TSC2 tumor suppressor (Corradetti et al., 2004) or directly phosphorylates mTOR binding partner raptor (Gwinn et al., 2008), contributing to mTORC1 suppression. The classical inhibitor of mTOR, rapamycin, is shown to potently inhibit apoptosis in LKB1-deficient MEFs under 24 hr glucose withdraw (Shaw et al., 2004a). However, mTOR inhibition by rapamycin cannot protect the

sensitivity to glucose starvation of LKB1 deficient cell line A549 (Jeon et al., 2012; Sanchez-Cespedes et al., 2002).

### 4.3 Cellular ROS is increased in glucose starvation-induced cell death

AMPK is known to regulate cellular ATP level under energy stress conditions. Nutrient deprivation can promote Warburg effect through the AMPK-dependent pathway to delay apoptosis induced by nutrient starvation (Wu et al., 2013). The absence of LKB1 or AMPK activation renders cancer cells to cell death induced by glucose starvation. However, the mechanism by which AMPK inactivation causes increased cell death remains elusive. In the present study, we observed time-dependent decrease of AMPK protein level under glucose starvation (Figure 3-7A), supporting the critical role of AMPK inactivation in cell death. However, whether reduction of AMPK protein level is the consequence or cause of cell death remains to be further investigated. Surprisingly, AMPK activators AICAR and metformin and inhibitor Compound C showed little effect on glucose starvation-induced cell death (Figure 3-6 B and C), while glycolysis inhibitor 2DG and antioxidant NAC can effectively inhibit cell death (Figure 3-7 B and C; Figure 3-10B and C). A recent study showed that in addition to regulation of ATP homeostasis, AMPK functions to maintain cellular NADPH level through inhibition of acetyl-CoA carboxylases ACC1 and ACC2 in response to

energy stress (Jeon et al., 2012). They found that LKB1-AMPK deficient cells are susceptible to glucose starvation due to impaired generation of NADPH in pentose phosphate pathway (PPP) by glucose starvation, $H_2O_2$ elevation and AMPK inactivation (Jeon et al., 2012). 2DG can maintain NADPH level by potently stimulating AMPK activation even without functional LKB1 (Jeon et al., 2012). Also, antioxidant NAC protects LKB1-deficient cells from glucose starvation-induced cell death by inhibiting oxidative stress (Jeon et al., 2012). These results are consistent with our findings and provide possible explanation why 2DG and NAC can protect LKB1-deficient cells against cell death under glucose starvation.

On the contrary, the results from Wu et al. suggest the protective role of ROS production and AMPK activation in cell death mediated by nutrient starvation, evidenced by results that antioxidants BHA, NAC and TEMPO and AMPK inhibitor Compound C accelerated nutrient deprivation (HBSS)-induced apoptosis at in HeLa cells (Wu et al., 2013). They also indicated that ROS are upstream of AMPK phosphorylation upon nutrient depletion, based on the observation that NAC inhibits starvation-induced AMPK activation. However, in the present study, we found that NAC treatment leads to stabilization of AMPK protein level and protection of NCI-H460 cells against glucose starvation-induced cell death (Figure 3-10). The discrepancy between these two findings may be attributed to different

starvation conditions and cell lines utilized, implying that although HeLa and NCI-H460 are both LKB1 deficient, there may be an alternative mechanisms involved in AMPK protein stability.

### 4.4 AMPK protein stability is impaired under glucose starvation

At present relatively little is known whether AMPK can be regulated at the level of protein stability. The significance of AMPK ubiquitination begins to attract scientists' attention recently. Qi et al. have found that Cidea (cell death-inducing DFFA-like effector a) forms a complex with AMPK through specific interaction with β-subunit, and such interaction increases ubiquitination-mediated proteasome degradation of AMPK, resulting in reduced AMPK activity (Qi et al., 2008). These results are the first evidence to show ubiquitin-dependent degradation of AMPK in brown adipose tissue, but the underlying mechanism by which Cidea controls AMPK degradation remains intricate. A recent paper from Pineda et al. described a mechanism to degrade AMPKα1 by a cancer-specific MAGE-A3/6-TRIM28 E3 ubiquitin ligase, causing hypersensitization to AMPK agonists (metformin and AICAR) (Pineda et al., 2015). However, they did not examine whether MAGE-A3/6 expressing cells are sensitive to glucose deprivation. In our study, proteasome inhibitors MG-132 and Bortezomib are not able to inhibit the decrease of total AMPK upon glucose starvation (Figure 3-9D), suggesting that AMPK may

not be degraded by proteasome system. Furthermore, two lysosome inhibitors BAF and CQ have no effects on AMPK protein level or cell death under glucose starvation (Figure 3-9A, B and C), suggesting that AMPK is unlikely to be degraded by lysosome. Moreover, since the PI3K inhibitor wortmannin, as well as BAF and CQ, is unable to suppress glucose starvation-induced cell death, suggesting that autophagy may not be the key factor in glucose starvation-induced cell death.

Interestingly, in our study we observed that AMPK displays a smear upon glucose starvation (Figure 3-5B), suggesting a post-translational modification of AMPK. Besides ubiquintination and phosphorylation, AMPK has been also reported to be a redox-sensitive kinase, which can be negatively regulated by oxidation at α-subunit (Shao et al., 2014). In response to glucose starvation, AMPK forms aggregates through intermolecular disulfide bonds at Cys130/Cys174 in α-subunit, preventing LKB1-dependent AMPK activation (Shao et al., 2014). Thioredoxin1 (Trx1) converts AMPK to the reduced form by cleaving the disulfide bonds between AMPK aggregates, thus promoting AMPK activation mediated by LKB1 (Shao et a, 2014). In addition to oxidation, AMPK activity can be regulated by acetylation/deacetylation at α-subunit. For instance, deacetylation of AMPK promotes AMPK activation through enhanced interaction with LKB1 (Lin et al., 2012). One possible explanation why in our study the total and

phosphor-AMPK become undetectable upon glucose starvation is that the post-translational modifications of AMPK render it hard to be recognized by antibody. Further investigation is required to determine whether AMPK is modified and which type of modification occurs in glucose starvation conditions, and whether this modification will eventually cause AMPK degradation.

### 4.5 Future work and summary

Since glucose metabolism and AMPK activity are essential targets in cancer therapy, the striking phenomenon discovered in this study provides insight into the critical role of AMPK in determining the susceptibility of cancer cells to metabolic stress. Although we observed some morphological characters of apoptosis, like rounded shapes and undetached cells, apoptosis inhibitor Z-VAD did not inhibit cell death, nor did necroptosis inhibitor necrostatin. Which type of the three programmed cell death plays the major role in this particular cell remains to be elucidated. Therefore, further studies will focus on the following four aspects: 1) to identify the specific type of cell death caused by glucose starvation; 2) to confirm whether AMPK protein is degraded under glucose starvation and discover the degradation mechanism; 3) to determine whether and how AMPK is post-translational modified upon glucose starvation; and 4) to check the genetic background of

NCI-H460 cell line and identify the potential upstream target which causes the hypersensitivity to glucose starvation through AMPK protein stability.

In summary, our results demonstrate that a unique NSCLC cell line NCI-H460 is heavily depend on glucose for survival. Under glucose starvation, NCI-H460 undergoes rapid cell death, which cannot be protected by apoptosis, necroptosis or autophagy inhibitors. This cell death is accompanied by increased ROS production and decreased AMPK protein level. Pharmacological activators AICAR and metformin are unable to protect cell death, but glycolysis inhibitor 2DG and antioxidant NAC can stabilize AMPK protein level and block cell death. Our findings thus provide novel approaches or strategies for human cancer therapy by targeting AMPK, especially in NSCLC.

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