THE ROLE OF AMP-ACTIVATED PROTEIN KINASE (AMPK) IN TUMORIGENESIS

Fei Han

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THE ROLE OF AMP-ACTIVATED PROTEIN KINASE (AMPK) IN TUMORIGENESIS

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THE ROLE OF AMP-ACTIVATED PROTEIN KINASE (AMPK) IN TUMORIGENESIS

A DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Fei Han

Houston, Texas

May 2016
This Ph.D. thesis work is dedicated to

My parents, husband and daughter.
Acknowledgements

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girl Mia, who bring me to a new world that I can never imagine. She is like an
angel, and I am so grateful to have such an amazing and supportive family to
help me fulfill my dream.
The Role of AMP-Activated Protein Kinase (AMPK) In Tumorigenesis

Fei Han

Advisory Professor: Hui-Kuan Lin, Ph.D.

AMPK plays a central role in controlling cellular and whole body energy level. Increasing studies have also discovered the diverse function of AMPK in cancer, such as autophagy and mitochondria biogenesis. However, how AMPK promotes cancer progression is still not clear. Here, we show that AMPK is essential for EGF-induced Akt activation, Glut1 expression, and glucose uptake. AMPK is also required for various stresses induced Akt activation and promote cell survival, including hypoxia and glucose deprivation. In addition, we found glucose deprivation-induced VEGF expression and secretion is also depend on AMPK, which may contribute to angiogenesis of surrounding endothelial cell in the tumor microenvironment. We also discovered that AMPK directly phosphorylates Skp2 at S256 residue and this phosphorylation is essential for Skp2 SCF complex integrity and E3 ligase activity to ubiquitinate Akt, which is critical for Akt activation. Also, Skp2 S256 phosphorylation can be induced by many stress and EGF. The phosphorylation status is necessary for breast cancer tumor progression and correlated with poor survival of breast cancer patient.
# TABLE OF CONTENTS

APPROVAL FORM ........................................................................................................ i
TITLE PAGE .................................................................................................................... ii
DEDICATION ................................................................................................................... iii
ACKNOWLEDGEMENTS ................................................................................................. iv
ABSTRACT ..................................................................................................................... vi
TABLE OF CONTENTS ................................................................................................... vii
LIST OF FIGURES .......................................................................................................... x
LIST OF TABLES ........................................................................................................... xiii
LIST OF ABBREVIATIONS ........................................................................................... xiv

## CHAPTER 1 INTRODUCTION ......................................................................................... 1

1.1 Ubiquitination and Skp2-SCF complex ................................................................. 2
1.2. Akt ubiquitination in growth factor-induced Akt activation .............................. 5
1.3 PI3K/Akt pathway in hypoxia ............................................................................ 7
1.4. The structure of AMPK. .................................................................................... 10
1.5. AMPK activation and hypoxia ........................................................................ 14
1.6 Downstream substrates and potential oncogenic function of AMPK............ 17
1.7 Rationale ............................................................................................................. 19

## CHAPTER 2 MATERIAL AND METHODS ................................................................. 21

2.1. Cell culture and reagents ............................................................................... 22
2.2. Immunoprecipitation and immunoblotting .................................................... 24
2.3. In vitro kinase assay ................................................................. 24
2.4. Viral infection ........................................................................ 25
2.5. In Vivo Ubiquitination assay ..................................................... 27
2.6. Cell Survival Assay ................................................................. 27
2.7. Recombinant Protein Purification ............................................ 28
2.8. In vitro cell migration Assay .................................................... 28
2.9. Glucose Uptake and Lactate Production Assay ......................... 29
2.10. Tube formation Assay ............................................................. 29
2.11. In Vivo Tumorigenesis Assay .................................................. 30
2.12. Patients, human materials and immunohistochemistry ................. 30
2.13. Statistical analysis .................................................................. 31

CHAPTER 3 RESULTS ....................................................................... 32

3.1 AMPK is required for Akt activation under various stress ............. 33
3.2. AMPK is involved in EGF-induced Akt activation and activated by CaMKKβ. ................................................................. 40
3.3. Akt ubiquitination is induced under hypoxia and AMPK/Skp2 are involved in this process ................................................................. 45
3.4. AMPK directly phosphorylates Skp2 at S256 ................................ 50
3.5. S256 phosphorylation stabilizes Skp2 SCF E3 ligase complex integrity and is important for its E3 ligase activity ........................................ 58
3.6. AMPK is important for EGF-induced glucose uptake, survival under stress as well as glucose deprivation induced VEGF secretion through phosphorylating Skp2 and activating Akt. ......................................................63

3.7. Xenograft and clinical relevance of Skp2 S256 phosphorylation. ............75

CHAPTER 4 DISCUSSION AND FUTURE DIRECTIONS ...........................................85

BIBLIOGRAPHY ...............................................................................................96

VITA .................................................................................................................108
LIST OF FIGURES

Figure 1-1 Diagram of substrate ubiquitination triggered by Skp2-SCF complex. 4
Figure 1-2 Akt ubiquitination is a common mode for IGF-1, EGF and HRG-induced Akt membrane recruitment and activation. ................................................. 6
Figure 1-3 Diagram shows the hypoxic tumor microenvironment. ....................... 9
Figure 1-4 Schematic representation of AMPK α β γ subunits ......................... 13
Figure 3-1-1 Screening of kinases in the hypoxia-induced Akt phosphorylation using kinase inhibitor library .................................................................................. 35
Figure 3-1-2 Compound C inhibits both AMPK and Akt phosphorylation ......... 37
Figure 3-1-3 AMPK is required for hypoxia-induced Akt phosphorylation and activation .............................................................................................................. 38
Figure 3-1-4 AMPK is also required for glucose deprivation and H2O2-induced Akt activation ........................................................................................................... 39
Figure 3-2-1 AMPK is involved in EGF but not PDGF or IGF-induced Akt activation .......................................................................................................................... 41
Figure 3-2-2 CaMKKβ is required for EGF-induced AMPK/Akt activation ....... 42
Figure 3-2-3 LKB1 is not required for EGF-induced AMPK/Akt activation....... 43
Figure 3-2-4 EGF-induced changes in intercellular Ca2+ ................................. 44
Figure 3-3-1 Skp2 is also involved in hypoxia and glucose deprivation induced Akt ubiquitination ........................................................................................................... 47
Figure 3-3-2 Skp2 E3 ligase is important for Hypoxia-induced Akt ubiquitination ......................................................................................................................... 48
Figure 3-3-3 Akt ubiquitination is induced under hypoxia and AMPK is required for Akt ubiquitination .....................................................................................................................49
Figure 3-4-1 AMPK phosphorylates Skp2 in vitro ..........................................................................................52
Figure 3-4-2 AMPK phosphorylates Skp2 at residues after 200 ...........................................................53
Figure 3-4-3 Screening of Skp2 Serine residue phosphorylated by AMPK ..............................................54
Figure 3-4-4 AMPK phosphorylates Skp2 at S256 in vivo using S256 phosphor-specific antibody ........................................................................................................................................56
Figure 3-4-5 AMPK also phosphorylates Skp2 at S256 in vitro using S256 phosphor-specific antibody .................................................................................................................................57
Figure 3-5-1 Skp2 S256 phosphorylation is important for its E3 ligase function .........................................................60
Figure 3-5-2 Skp2 S256 phosphorylation stabilizes Skp2 SCF complex integrity .............................................................61
Figure 3-5-3 Skp2 S256 interacts with Cullin1 K221 to promote SCF complex stability .............................................................62
Figure 3-6-1 Skp2 S256 phosphorylation is induced by EGF, hypoxia, glucose deprivation and H2O2 treatment ........................................................................................................................................65
Figure 3-6-2 EGF-induced glucose uptake is impaired in AMPK knockdown cells and can be restored by activated Akt ........................................................................................................................................66
Figure 3-6-3 AMPK regulates EGF-induced Glut1 expression through Akt .................................................................................67
Figure 3-6-4 EGF-induced glucose uptake impairs in AMPK knockdown cells and can be restored by Skp2 S256 phosphorylation ........................................................................................................................................68
Figure 3-6-5 AMPK promotes cell survival under hypoxia and glucose deprivation through Akt ........................................................................................................................................69
Figure 3-6-6 AMPK promotes cell survival under hypoxia and glucose deprivation through Skp2 S256 phosphorylation ..............................................70

Figure 3-6-7 AMPK-Skp2-Akt upregulates glucose deprivation induced VEGF transcription .............................................................................................................71

Figure 3-6-8 AMPK-Skp2-Akt upregulates glucose deprivation induced VEGF secretion .............................................................................................................73

Figure 3-6-9 AMPK / Skp2 regulates glucose deprivation induced VEGF secretion in MDA231 cells, which affect the endothelial cell tube formation ability .............................................................................................................74

Figure 3-7-1 Knockdown of AMPKα1 suppresses tumor growth of breast cancer in vivo.............................................................................................................77

Figure 3-7-2 Overexpression of Skp2 WT and S256D promotes tumor growth of breast cancer in vivo .............................................................................................................78

Figure 3-7-3 pSkp2 (S256) and pAMPK expression are upregulated in breast cancers .............................................................................................................79

Figure 3-7-4 Overexpression of pSkp2 (S256), pAMPK and in an advanced stage of breast cancer patient .............................................................................................................80

Figure 3-7-5 pAMPK and pAkt expression both correlate with pSkp2 (S256) in breast cancer patients .............................................................................................................81

Figure 3-7-6 Overexpression of pSkp2 or pAMPK predicts poor survival outcome of Breast cancer patients .............................................................................................................82

Figure 4-1 Crystal structure of Skp2-SCF complex .........................................................92
Figure 4-2 Hypothetic model of the role of pAMPK in Stress and EGF-induced Akt activation and tumorigenesis.
LIST OF TABLES

Table 1-1 List of compounds and mode of AMPK activation/inhibition .......... 16
Table 2-1 Primers for site direct mutagenesis ..................................... 23
Table 2-2 Sequence of shRNAs. .......................................................... 26
Table 3-1 List of kinase inhibitors and their targets.............................. 34
Table 3-2 Associations between pSkp2 expressions with important clinic pathologic variables and pAkt, p27, pAMPK expression.......................... 83
Table 3-3 Univariate survival analysis .................................................. 84
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>Acetyl Co-A carboxylase</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>AID</td>
<td>Autoinhibitory domain</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAMKKβ</td>
<td>Calmodulin-dependent protein kinase kinaseβ</td>
</tr>
<tr>
<td>CBM</td>
<td>Carbohydrate binding motif</td>
</tr>
<tr>
<td>Cul1</td>
<td>Cullin 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's medium</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>GSK3-β</td>
<td>Glycogen synthase kinase-β</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>KD</td>
<td>Kinase-dead</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LKB1</td>
<td>Live Kinase B1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/Erk kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF-R</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PDK-1</td>
<td>Phosphoinositide-dependent kinase-1</td>
</tr>
<tr>
<td>RHO</td>
<td>REL-homology domain</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1-Cul1-F-box-protein</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>Skp1</td>
<td>S-phase kinase-associated protein 1</td>
</tr>
<tr>
<td>Skp2</td>
<td>S-phase kinase-associated protein 2</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1 Ubiquitination and Skp2-SCF complex

Ubiquitination is a post-translational modification that marks a protein with ubiquitin, a widely distributed small protein in cells. The process is conducted by three main enzymes: E1, E2, and E3. E1 is an ubiquitin activating enzyme that links ubiquitin with a thio-easter bond. The ubiquitin is then transferred by E2 to a substrate on the lysine residue. The different linkage of ubiquitin usually contributes to the diverse outcome. K48-linked ubiquitination will mark protein for ubiquitination-dependent proteasome degradation, while K63-linked ubiquitination is usually activating signaling. The substrate specificity is determined by E3. E3 can be either a single protein or have multiple subunits, such as the SCF complex. The main biological function of the SCF complex (Skp1, Cullins, F-box proteins) is to target protein for degradation by ubiquitination. It controls DNA replication, cell cycle progression, as well as signal transduction. The complex consists of a scaffold protein Cullin, an RING-containing enzyme Rbx1/2, an adaptor F-box protein and Skp1 (Berndsen and Wolberger, 2014). Cul1 acts as a scaffold protein to link Skp1 and Rbx1. C-terminal of Skp1 also bridges N-terminal of F-box protein, which determines the substrate specificity. There are 69 F-box proteins in human (Lee and Diehl, 2014), but only a few F-box proteins had been studied in detail. The most studied F-box proteins are β-TrCP and Skp2. The ubiquitination triggers by Skp2-SCF complex is displayed in Figure 1-1. The ubiquitination-dependent degradation of p27 was triggered mainly by Skp2. Skp2 null mice have small body size and weight but mice with double knockout of p27 and Skp2 could rescue MEFs cell growth.
which is much slower in Skp2 null mice. Moreover, Skp2 and p27 expression were inversely correlated in multiple cancer patients with poor prognosis (Chan et al., 2010; Frescas and Pagano, 2008). Skp2 also has a non-proteolytic function. Our group previously found that Skp2 co-activated RhoA transcription and promote metastasis. Skp2 also mediates K63-linked polyubiquitination of Nbs1, which is important for DNA damage induced by ATM activation. Moreover, Skp2 null mice were reported to have reduced the chance to develop obesity when fed in long-term high-fat diet, associated with insulin resistance and glucose intolerance (Sakai T 2007). Mechanisms are believed to be both p27 dependent or independent.
Figure 1 Diagram of substrate ubiquitination triggered by Skp2-SCF complex.
1.2. Akt ubiquitination in growth factor-induced Akt activation

PI3K/Akt signaling plays an important role in tumor initiation and progression. It controls cell proliferation, metabolism, apoptosis and migration (E. Gonzalez 2009). It can be activated by a variety of extracellular signals like growth factors and cytokines. The activation of membrane receptors thus triggers numerous signal events which finally contribute to cell proliferation. After PI3K is activated, it catalyzes the phosphorylation of PI(4,5)P2 to form PI(3,4,5) P3, which then recruits Akt to the plasma membrane (LC. 2001). Akt binds to PI(3,4,5)P3 phospholipid via its N-terminal PH domain, which is required for being recruited to the cell plasma membrane (S. R. Datta 1999, LC. 2001). After membrane recruitment, Akt is then phosphorylated by PDK1 at Thr308 in its activation loop of the kinase domain. The mTORC2 will also phosphorylate Akt at Ser473 located in the regulatory domain in the cytoplasm. Our group discovered Traf6 E3 ligase ubiquitinated Akt in response to IGF-1 treatment and Skp2 SCF complex is responsible for EGF-induced ubiquitination of Akt (Wei-Lei Yang 2009, Chia-Hsin Chan 2012). Both Traf6 and Skp2 trigger K63-linked polyubiquitination and facilitate Akt membrane localization and activation as showed in Figure 1-2. Consistently, loss of Traf6 or Skp2 has tumor suppression effect in xenograft mouse model. However, except for IGF and EGF treatment, whether Akt polyubiquitination is a prerequisite for Akt phosphorylation in other contexts is still not fully addressed.
Figure 2 Akt ubiquitination is a common mode for IGF-1, EGF and HRG-induced Akt membrane recruitment and activation.
1.3 PI3K/Akt pathway in hypoxia

During solid tumor progression, there is a portion of cells will be hypoxic since they are away from blood vessels and have limited oxygen supply. As showed in Figure 1-3, right in the center of the tumor, severe hypoxia usually leads to necrosis. However, moderate hypoxia near the center of the tumor will survive and promote angiogenesis and metastasis. Hypoxic tumor cells are also less sensitive to radio- and chemotherapy, since they are not actively cycling and the chemotherapy drug fails to reach to the hypoxic region. HIF1a is the major downstream transcription factor to mediate adaptive response under hypoxia. Limited oxygen will protect HIF1a from the von Hippel-Lindau protein (VHL)-mediated ubiquitination and proteasome degradation, thereby stabilize HIF1a. HIF1a then binds to hypoxia responsive element (HRE) and mediates transcription of many downstream effectors, such as glucose transporter and VEGF. HIF1a is overexpressed in a variety of malignant cancers and acts as a prognostic parameter for aggressive tumor. Hypoxia response can be HIF1a dependent or independent. Many studies have suggested that PI3K/Akt pathway is enhanced by hypoxia. In rat phaeochromocytoma (PC12) cells, hypoxia induces Akt phosphorylation and increases its downstream GSK3b phosphorylation at the peak of 6 hours and can prolong up to 24 hours (Dana Beitner-Johnson 2001). A similar phenomenon is found in six head and neck squamous cell carcinoma (HNSCC) cell lines (Hanneke Stegeman 2012). Inhibition of Akt under hypoxia induces apoptosis. These evidence suggested that hypoxia may protect the cell from apoptosis through activating Akt pathway y.
However, the mechanism of how hypoxia activates Akt signaling is largely unknown.
Figure 3 Diagram shows the hypoxic tumor microenvironment.

Figure is adopt and modified from
http://mail.mc.pref.osaka.jp/omc2/eng/biochemistry.html
1.4. The structure of AMPK.

AMPK is an evolutionally conserved serine/threonine kinase. It consists of three subunits: α, β, and γ (Davies SP 1994, K.I. Mitchelhill 1994). The catalytic α subunit has two isoforms: α1 and α2, which encoded by PRKAA1 and PRKAA2. Their expression varies in different cell types. The α1 isoform is expressed widely in heart, liver, lung, brain, spleen, etc. while the α2 isoform is more tissue-restricted, and mainly expressed in neuron and muscle cells (B. E. Crute 1998). The kinase domain is located in N-terminal of α subunit; allowing phosphorylation by upstream kinases. Five phosphorylation sites have been reported in the past few years: Thr172, Ser173, Thr258 and Ser485/491 (Woods, Vertommen et al. 2003, Djouder, Tuerk et al. 2009). Threonine 172 is the major phosphorylation site induced under various situations and is required to fully activate downstream substrates, while Ser173, Ser485 on α1 subunit and Ser491 on α2 subunit are autophosphorylated by PKA or Akt, which is believed to inhibit AMPK activity (Djouder, Tuerk et al. 2009). After the kinase domain, there is an autoinhibitory domain (AID) which binds and inhibits the kinase domain, followed by the β/γ interaction domain on the C-terminal. The reported constitutively activated form of AMPKα is the best evidence for the autoinhibitory domain (AID) function since it is a truncated form without C-terminal AID and β/γ interaction domain (Jaleel 2006).
The regulatory β subunits also have two isoforms: β1 and β2, which are encoded by PRKAB1 and PRKAB2. Similar to α subunit, the β1 isoform is also expressed ubiquitously but the β2 isoform is only expressed in skeletal muscle and heart (Chen 1999). The β subunits consist of a carbohydrate binding module (CBM), previously called glycogen binding domain (GBD) and a C-terminal sequence that bridges α/γ-subunits. It is also reported that a myristoylation site is located within the β subunits, which might contribute the membrane location of AMPK and increased ability of AMP to activate AMPK α subunit allosterically (Mitchelhill 1997, Oakhill JS 2010). The central carbohydrate binding motif binds the α1-6 linked branch glycogen instead of α1-4 linear glycogen and inhibits α subunit kinase domain allosterically. It provides an additional sensing mechanism of glycogen store besides ATP level (Koay 2007, McBride 2009).

The γ subunit is important to AMPK activation while cells encounter changes in ATP level. It has 3 transcriptional variants: γ1, γ2, and γ3. The γ1 isoform has 331 residues and is most widely expressed. The 569 - residues γ2 subunit is majority expressed in the brain, heart, placenta and skeletal muscle. The isoform γ3 which composed of 489 residues is only expressed in skeletal muscle. The three isoforms are different in N-terminal sequence but what is common is that they all have a sequence to interact with α/β subunits and two Bateman domains. The two Bateman domains consist of four symmetric cystathionine β-synthetase (CBS) motifs, allowing four possible adenyl-binding sites (Mahlapuu 2004). The hydroxyl groups of AMP ribose interact with an aspartic acid residue on the α-helix adjacent to the site. Binding of AMP instead of ATP to the CBS site will lead
to a conformational change in the kinase domain of α subunit, allowing upstream kinases interact with and phosphorylate AMPK Thr172. Except for AMP: ATP ratio sensing, cells can also sense ADP: ATP ratio by binding of ADP to γCBS 1 and 3 and stimulating Thr172 phosphorylation (Oakhill, Steel et al. 2011). A schematic picture of the heterotrimeric AMPK subunits is showed in Figure 1-4.
Figure 1-4 Schematic representation of AMPK α β γ subunits

Various α, β, and γ AMPK subunits and isoforms with important domains highlighted:

- Kinase domain
- Autoinhibitory domain (AID)
- β subunit interaction domain (β-SID)
- Carbohydrate binding module (CBM)
- αγ subunits binding sequence (αγ SBS)
- Cystathionine beta sheet (CBS) regions.
1.5. AMPK activation and hypoxia

There are two major upstream kinases for AMPK Thr172 phosphorylation: LKB1 and CaMKKβ. LKB1 was initially identified as a tumor-suppressor gene due to its inherited germline mutations to cause cancer-prone Peutz-Jeghers syndrome (Hemminki A 1998, Jenne DE 1998). LKB1 associates with STRAD, MO25 and functions as a complex. LKB1 activity is much weaker without other components in the complex (Hawley SA 2003). LKB1 tumor suppressor is commonly inactivated in lung and cervix cancers and thought to promote cancer progression. There is more than 20% of cervical cancer that harbors LKB1 somatic mutations (Shana N. Wingo 2009). LKB1 as a master kinase for AMPK can also phosphorylate thirteen members in the AMPK superfamily, while the other 12 members cannot response to AMP (Lizcano JM 2004, Sakamoto K 2004). Increasing evidence favors that LKB1 constitutively activates AMPK to maintain a basal AMPK function in normal physiological conditions. Muscle-specific LKB1 KO mouse is reported to have impaired glucose uptake and AMPKα2 phosphorylation induced by AICAR or contraction (Sakamoto K 2005). A similar phenotype can be observed in liver LKB1 KO mice. Metformin-induced AMPK phosphorylation and glucose uptake is also impaired without LKB1 expression in liver (Shaw RJ 2005). These LKB1 animal models suggest that LKB1 is the major kinase for AMPK in skeletal muscle and liver.

Ca2+/calmodulin dependent kinase kinases (CaM KK) is found by two groups at the similar time as an alternative AMPK upstream kinase in the brain as well as in LKB1 deficient cells (Hawley SA 2005, Woods A 2005). However, CaM KK
is reported to share more similar sequence to yeast snf1/AMPK activating kinase than LKB1. It suggests CaMKKβ might be more specific to AMPK but not the other members of AMPK superfamily. There are three snf1-activating kinases in yeast: Pak1, Tos3, and Elm1. CaMKK overexpression can restore snf1 activity and phenotype in the deletion of Pak1, Tos3, and Elm1 cells in yeast. It can also phosphorylate yeast snf1 in vitro (Seung-Pyo Hong 2005). All these evidence strengthened that CaMKK is the also a major AMPK kinase, which might function in other cell types. Unlike LKB1 which activates AMPK through lower AMP: ATP ratio, CaMKKβ activates AMPK through changed intercellular Ca2+ levels independently of ATP levels. Both of LKB1 and CaMKK may act synergistically or independently in different contexts.

There are many physiological processes, stressor, and compounds that can alter AMPK activity. The compound that can activate or inhibit AMPK activation is listed in Table 1-1. The activation mode is either through changes in AMP/ATP ratio or intercellular Ca2+ level. Hypoxia is also reported to activate AMPK through ROS-mediated ER calcium release and calcium release-activated calcium (CRAC) channels activation, which all contributes to intercellular Ca2+ increase and CaMKKβ activation. Knockdown of CaMKKβ abolished AMPK activation under hypoxia (Brooke M. Emerling 2009, Mungai PT 2011).
### Table 1-1 List of compounds and mode of AMPK activation/inhibition

<table>
<thead>
<tr>
<th>Compounds that activate AMPK</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>AICAR</td>
<td>Generation of ZMP, an analog of AMP</td>
</tr>
<tr>
<td>Antimycin A</td>
<td>Inhibition of respiratory chain</td>
</tr>
<tr>
<td>Arsenite</td>
<td>Inhibition of TCA cycle</td>
</tr>
<tr>
<td>A23187</td>
<td>Activation by increase in cytosolic calcium ions</td>
</tr>
<tr>
<td>A769662</td>
<td>Direct AMPK activator</td>
</tr>
<tr>
<td>β-guanadinopropionic acid</td>
<td>Creatine analog; increases AMP/ATP ratio</td>
</tr>
<tr>
<td>2-Deoxyglucose</td>
<td>Inhibition of glycolysis</td>
</tr>
<tr>
<td>Dinitrophenol</td>
<td>Uncoupler of electron transfer/ATP synthesis</td>
</tr>
<tr>
<td>NO</td>
<td>Inhibition of respiratory chain</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>Inhibition of ATP synthase</td>
</tr>
<tr>
<td>Phenformin</td>
<td>Inhibition of respiratory chain</td>
</tr>
<tr>
<td>Resveratrol</td>
<td>Change in ATP synthase activity; prevents acetylation of LKB1 via modulation of SIRT1; upregulation of adiponectin synthesis and multimerization</td>
</tr>
<tr>
<td>TZD</td>
<td>Stimulate the expression and secretion of adiponectin; increase in AMP concentration; activation of PPARy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compounds that inhibit AMPK</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound C (dorsomorphin)</td>
<td>Reversible, ATP-competitive inhibitor</td>
</tr>
</tbody>
</table>
1.6 Downstream substrates and potential oncogenic function of AMPK

AMPK mediates numerous cellular functions through diverse downstream substrates. As described above, AMPK is a master regulator of metabolism. The first identified downstream substrate is ACC. AMPK directly phosphorylates ACC and inhibits its function in lipogenesis. AMPK increases energy levels by stimulating fatty acid oxidation. Specifically, AMPK phosphorylates ACC1 at residue Ser79 and ACC2 at Ser212 (Hardie DG 2002). ACC converts acetyl CoA to malonyl CoA and promotes lipid synthesis (Hardie DG 2002). Malonyl coA inhibits CPT1b to block fatty acid transport to mitochondria for β-oxidation (Mills SE 1983). AMPK phosphorylates ACC to keep it inactive, thereby decreasing the rate of fatty acid synthesis and promoting fatty acid oxidation to generate more ATP and fulfill the cellular requirement of energy.

For roles in the glucose metabolism, AMPK is reported to increase glucose uptake and affect Glut4 membrane translocation in skeletal muscle (Kurth-Kraczek EJ 1999). It directly phosphorylates TBC1D1/4, a Rab GTPase activating protein believed to associate and attenuate Glut4 translocation (Taylor EB 2008). However, there are still many unaddressed questions about how AMPK regulates glucose uptake. AMPK also reduces glycogen synthesis through phosphorylating and inhibiting glycogen synthase at Ser7 and promoting glycolysis by phosphorylating PFK2 on Ser466 (Marsin AS 2000). PFK2 is responsible for converting fructose 6 phosphate to fructose 2, 6-biophosphate in the glycolysis pathway. Therefore, AMPK increases ATP production by activating glucose uptake and glycolysis in the muscle cell.
AMPK also phosphorylates TSC2 and raptor to block the mTOR pathway and reduce protein synthesis (Inoki K 2003, Gwinn DM 2008). AMPK indirectly inhibits the mTOR pathway and also leads to changes in transcription process thus affecting many cellular functions, for example, autophagy. Also, AMPK directly phosphorylates ULK to promote autophagy when cells have limited nutrient (Egan DF 2011).

Except for the cytoplasm, AMPK is also located in the nucleus and phosphorylates HDAC at Ser259 and Ser498. Phosphorylation of HDAC dissociates it from MEF2 and 14-3-3 protein, which in turn lead to HDAC nuclear export (McGee SL 2008). The release of HDAC5 from the nucleus increased histone acetylation and recruited other transcription factors like PGC-1α to Glut4 promoter (Michael LF 2001). An HDAC5 phosphorylation-deficient mutant has a less Glut4 expression, which might be another mechanism of how AMPK regulates glucose uptake. Recently, AMPK is reported to phosphorylate YAP at Ser94 and inactivate YAP function by disassociating it with 14-3-3 and promoting its cytoplasm sequestration. Therefore, AMPK negatively regulates YAP-Hippo pathway under energy stress (Jung-Soon Mo 2015).

Tumor cells need to adjust their metabolism to generate energy to support constant cell division in stressed conditions, such as hypoxia and nutrient deprivation (DeBerardinis RJ 2008). AMPK promotes metabolic adaptation during tumor growth. During energy stress, the generation of NADPH by the pentose phosphate pathway is impaired. AMPK phosphorylates ACC and inhibits fatty acid synthesis. The NADPH level will be stabilized because of the
decreased consumption in the fatty-acid synthesis and increased NADPH synthesis rate by fatty acid oxidation. At low intracellular ATP levels, AMPK activation induces alternative routes for NADPH production in order to balance NADPH homeostasis (Jeon SM 2012). In addition, AMPK also activates the eukaryotic elongation factor 2 kinase (eEF2K), which promotes cell survival under nutrient depletion by blocking translation elongation (Leprivier G 2013). In aggressive breast cancers, AMPK activation increases tumor glucose metabolism by increasing glycolysis, as well as the non-oxidative pentose phosphate cycle (Laderoute KR 2014). Hypoxia-induced AMPK activation is reported to regulate autophagy and may contribute to cell survival in prostate cancer (Rishi Raj Chhipa 2011).

1.7 Rationale

Hypoxia plays a critical role in radio- and chemoresistance, angiogenesis, and metastasis. It also activates Akt signaling to promote cell survival. However, how cells sense the oxygen and activate Akt is largely unknown. The sensing mechanism could start from membrane receptors/ channels, nuclear DNA damage, mitochondria or other reported stress kinases such as JNK or AMPK. We could screen many stress-related kinases that might be upstream of Akt activation. Since AMPK is reported to be activated by hypoxia through mitochondria ROS and it also shares many substrates with Akt, such as TSC, Foxo, and mTOR, it will be interesting to explore the possible links between AMPK and Akt under hypoxia. In addition, since Akt polyubiquitination is
important for IGF-1- and EGF-induced Akt phosphorylation, it may be also involved in hypoxia-induced Akt activation. Therefore, the dissertation is mainly focused on the questions that how Akt is activated, whether AMPK or other kinases are involved and whether Akt ubiquitination contributes its activity under hypoxia.
CHAPTER 2

MATERIAL AND METHODS
2.1. Cell culture and reagents

HEK293T, HEK293, MDA231, HUVEC cells were purchased from American Type Culture Collection. Skp2 WT and KO MEFs were prepared as previously described (Hui-Kuan Lin 2010). WT and AMPKα1/α2 knockout immortalized MEFs were from Dr. Kun-Liang Guan from the University of California, San Diego. HEK293 and HEK 293T cells were cultured in F12 medium supplemented with 10% FBS. HUVEC cells were maintained in EGM-plus media from Lonza. All other cells were cultured in DMEM/high glucose medium supplemented with 10% FBS. Kinase inhibitor library was purchased from Selleck Chemicals cherry pick customized library. 2-NBDG used for glucose uptake assay is from Sigma, H2O2, compound C and STO609 is from Sigma as well. Plasmids: His6-ubiquitin (His-Ub), pcDNA3-Flag-Skp2, pBabe-Skp2 constructs were previously described (Hui-Kuan Lin 2010). Skp2 and Cul1 mutants were generated using site-direct mutagenesis. Primers for mutagenesis are listed in Table 2-1.
### Table 2-1 Primers for site direct mutagenesis

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skp2 S238A-F</td>
<td>5’-GCGACTTAACCTTGATGGGTGTTCTG</td>
</tr>
<tr>
<td>Skp2 S238A-R</td>
<td>5’-CAGAACACCCATCAAGGTTAAGTCGC</td>
</tr>
<tr>
<td>Skp2 S256A-F</td>
<td>5’-CTTTGCTAAGCAGCTGTGCCCAGACTGGATGAG</td>
</tr>
<tr>
<td>Skp2 S256A-R</td>
<td>5’-TCATCCAGTCTGAACAGCTGATGAG</td>
</tr>
<tr>
<td>Skp2 S256D-F</td>
<td>5’-GCCTAAGCAGCTGATGAG</td>
</tr>
<tr>
<td>Skp2 S256D-R</td>
<td>5’-TCATCCAGTCTGACAGCTGATGAG</td>
</tr>
<tr>
<td>Skp2 S367A-F</td>
<td>5’-GGATCCGGTTGGACGCTGACATCGGATGCCCTC</td>
</tr>
<tr>
<td>Skp2 S367A-R</td>
<td>5’-GAGGGCATCCGGTTGGACGCTGACATCGGATGCCCTC</td>
</tr>
<tr>
<td>Skp2 S382/383A-F</td>
<td>5’-GAACCTTCCAATATTCCAAGGGCCGCCCATAGGCTTATTTTC</td>
</tr>
<tr>
<td>Skp2 S382/383A-R</td>
<td>5’-GAAACCTTCCAATATTCCAAGGGCCGCCCATAGGCTTATTTTC</td>
</tr>
<tr>
<td>Cul1 K221R-F</td>
<td>5’-GATGATGCAACGGCCCTACGTAAACAG</td>
</tr>
<tr>
<td>Cul1 K221R-F</td>
<td>5’-CTGTTAAGGGCTGCATGGCAATTCATCATC</td>
</tr>
</tbody>
</table>
2.2. Immunoprecipitation and immunoblotting

Cells were lysed in RIPA buffer (50mM Tris-HCl pH 7.4, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, 150mM NaCl, 2mM EDTA, and 50mM NaF). SDS sample buffer was added to the lysate for immunoblotting. For IP, Cells were lysed and quantified for protein concentration. The primary antibody was added and incubated in 4-degree rotation rack overnight. Protein A/G beads were then added for additional 3 hours followed by multiple wash step and immunoblotting. The following antibodies were used: anti-phosphor-Akt(T308), anti- phosphor-Akt(S473), anti-phosphor-ACC, anti-phosphor-AMPKα, anti-phospho-GSK3β, anti-Akt, anti-AMPKα, anti-ACC, anti-GSK3β, anti-Cullin1 are all purchased from Cell Signaling Technology; anti-Ubiquitin, anti-Skp1 are from Santa Cruz; anti-HA, anti-Flag, anti-β actin are from Sigma; anti-CaMKKβ and anti-Glut1 are from Abcam. The customed phosphor-Skp2 S256 antibody is from Jiaxing Xinda Biotechnology Co., Ltd.

2.3. In vitro kinase assay

Recombinant GST-Skp2 WT, S256A, and S256D proteins were expressed in BL21 bacteria and purified with Glutathione beads. HA-AMPKα active and kinase-dead proteins were immunoprecipitated from 293T cells with HA antibody, and then elute with HA peptide. Recombinant GST-Skp2 and HA-AMPKα were incubate for 30 minutes at 30°C in 20 μl of in vitro kinase reaction buffer (2mM DTT, 10mM MgCl2, 25mM Tris-HCl at pH 7.5, 0.1mM Na3VO4 and 5mM β-
glycerophosphate, 0.5mM ATP). The reaction was stopped after indicated time by SDS sample buffer and subjected to immunoblotting analysis.

2.4. Viral infection

For AMPK, CaMKKβ, LKB1 and Skp2 knockdown, lentiviral packing plasmid and pLKO.1-puro-shRNA constructs (Sigma) were transfected into 293T cells using calcium phosphate method. After 36 hours, the supernatant was collected, and MDA231 parental cells were infected and 2µg/ml puromycin selected for a week. Knockdown efficiency was detected by western blot. For Skp2 WT, S256A and S256D restore cell, Skp2 was cloned into pLKO.1 vector (Sigma). Mutants were generated by site-direct mutagenesis. The lentiviral shRNAs (Sigma) for those genes are listed in Table 2-2.
<table>
<thead>
<tr>
<th>shRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>shLuc</td>
<td>5'-CGCTGAGTACTTCGAAATGTC</td>
</tr>
<tr>
<td>shSkp2-1</td>
<td>5'-GATAGTGTCTGCTAAAGAAT</td>
</tr>
<tr>
<td>shSkp2-2</td>
<td>5'-GCCTAAGCTAAATCGAGAA</td>
</tr>
<tr>
<td>shCaMKKβ</td>
<td>5'-CCGGGTGAAGACCATGATACGTTAAACTGAGTTTACGTTTACATGGTGCTTTCACTTTTT</td>
</tr>
<tr>
<td>shLKB1</td>
<td>5'-CATCTACACTCGGACTTCAC</td>
</tr>
<tr>
<td>shAMPKα1-1</td>
<td>5'-CCGGGTAGGTCTCACCATTGACATCTCAGATTTGAGATTGAGTAGGGAGCAACTTTTT</td>
</tr>
<tr>
<td>shAMPKα1-2</td>
<td>5'-CCGGGTAGCTGTGAAGATCTCAATCTGAGATTGAGTAGGACGTCTTCACTTTTTTTT</td>
</tr>
</tbody>
</table>
2.5. In Vivo Ubiquitination assay

HEK293T cells were transfected with HA-Akt, His-Ub and Flag-Skp2 mutant plasmids. 48 hours later, cells were then lysed using high-stringency denaturing buffer (10mM imidazole, 0.1M Na2HPO4/NaH2PO4 at pH 8.0 and 6M guanidine-HCl). 10% whole cell lysate was used as input control and the other 90% lysate was incubated the with nickel-agarose beads for 3 hours. Samples were washed carefully for four times and sample buffer is added to elute the protein binding on the nickel beads. Samples were then subjected to immunoblotting analysis with indicated antibody.

2.6. Cell Survival Assay

$10^4$ of cells were seeded in 12-well plates in triplicates and cultured for 24 hours before any treatment. For cell survival under glucose deprivation, the control group was treated with DMEM high glucose medium (10% FBS) and test group was treated with glucose-free medium (10% FBS) for 16 hours. Culture medium with the dead cell was collected and attached cells were also trypsinized and combined. Then all cells were centrifuged at 3000 rpm for 10 min. Supernatants were removed and 100μl PBS were added to each sample. Trypan blue was added to each sample at 1:10 ratio, then cells were resuspended and aliquot 10μl of it were added into hematocounter for cell counting. The ratio of trypan blue stained and unstained cells were counted under a microscope. For cell survival rate under hypoxia, same cells were seeded with the control group in the incubator and test group in a hypoxia chamber (1% O$_2$) for 72 hours. Viable cells
under both conditions were counted using the trypan blue exclusion assay under a microscope.

2.7. Recombinant Protein Purification
Recombinant GST-Skp2 (WT, S256A, and S256D) protein was expressed in BL21 bacteria by transformation and induced with IPTG for 18 hours at room temperature. The bacteria were lysed by sonication and subjected to protein purification. GST-Skp2 (WT, S256A, and S256D) was purified by using glutathione-S-agarose beads (Invitrogen), and then eluted with reduced glutathione (Fisher Scientific). 10-kDa-cutoff Centricon (Millipore) were used for concentrate the proteins. HA-AMPK (CA and KD) were purified by transfecting plasmid into 293T cells, lysate collected after 36 hours and purified with HA antibody followed by elution with HA peptide on the protein A/G beads. Purified protein concentration was determined by Bradford assay and aliquoted to Eppendorf tube.

2.8. In vitro cell migration Assay
Transwell inserts were placed into 24 well plates with 500μl complete DMEM high glucose medium on the bottom of the well. 10^5 cells per insert were seeded and cells were incubated for 6-8 hours with or without EGF 50ng/μl for migrating along the serum gradient. The medium was then moved and inserts were washed with 1XPBS, fixed with 4% paraformaldehyde on the other side of the bottom for 30minutes at room temperature. Q-tips were used to remove any non-
migrated cells inside the bottom of the insert and the other side was stained with crystal violet reagent. The stained cells were then counted and quantified.

2.9. Glucose Uptake and Lactate Production Assay

For glucose uptake assay, cells were seeded in 60 mm plates. After 24 h, cells were refreshed with full serum (10% FBS) and glucose-free DMEM, while the complete DMEM high glucose medium served as control. After 8 hours, cells were treated with 50μM 2-NBDG for 30 minutes and glucose uptake rate was quantified by FACS analysis. For lactate production assay, cells were plated in 24-well plate and cultured overnight in low serum medium (0.1% FBS). After serum starvation, cells were treated with or without EGF (50 ng/ml) for 4 hrs. Culture medium was then transferred from each well to Eppendorf tube. Lactate concentration was determined by using lactate test strips and reading under Accutrend Lactate analyzer (Roche). Next, cells were harvested, stained with trypan blue and viable cell numbers were counted directly under the microscope using hemocytometer. The lactate production was determined by lactate concentration/cells and normalized with the rate detected in the control group without EGF.

2.10. Tube formation Assay

HUVEC endothelial cell was used for in vitro tube formation assay. Matrigel with reduced growth factor (Sigma) was thawed overnight on ice in 4°C freezer. 250μl
matrigel was then added to 24 well plates and incubated 30min in 37°C incubator before seeding cell. 2x10^4 HUVEC cells were seeded each well and cultured with supernatant from indicated cultured cancer cells. After 24 hours, tube formation on each well was examined under bright field microscopy.

2.11. In Vivo Tumorigenesis Assay
1x 10^8 MDA231 cells with AMPK knockdown and Skp2 overexpression (WT, S256A, and S256D) were subcutaneously injected into nude mice. The tumor size was measured by the caliper, and the tumor volume was calculated by the equation: [mm^3] = (length [mm]) x (width [mm])^2 x 1/2. Xenograft tissues isolated were fixed in 10% formalin overnight and then embedded in paraffin. All manipulations on the animal are under Institutional Animal Care and Use Committee (IACUC) approval protocol.

2.12. Patients, human materials and immunohistochemistry
The human material study is under the approval of the Institutional Review Board of the Chi-Mei Medical Center in Taiwan. Immunohistochemistry was conducted under standard process with primary phosphor-Skp2 S256, phosphor-Akt, pAMPK and p27 antibodies. The secondary antibody was incubated for 30 minutes and then developed for 5 minutes with 3-diaminobenzidine. IHC samples were scored manually by Dr. Chien-Feng Li under the multi-headed microscope as previously described (Chia-Hsin Chan 2012).
2.13. Statistical analysis

All data were listed as means +/- SEM. Each experiment was conducted in three independent repeats. The significance is determined by two-tailed and unpaired student t-test. P-values < 0.05 is considered statistically significant.
CHAPTER 3

RESULTS
3.1 AMPK is required for Akt activation under various stress

The PI3K/Akt pathway is an important signaling for cell proliferation and survival. Except for growth factors and cytokines, it can also be activated by many stresses, like hypoxia. However, the underlying mechanism is not clear. To identify possible kinases upstream of PI3K/Akt, we first applied the kinase inhibitors library which includes many key kinases of multiple cellular processes into HEK293 cells under hypoxia (Figure 3-1-1). All the inhibitors and related targets are listed in Table 3-1. We used LY 290034, a PI3K inhibitor as a positive control to inhibit Akt phosphorylation and used pAkt S473 as a readout for Akt activity since it has a stronger signal to minimize experimental variation. The result showed that HEK293 cells treated with LY290034 have less Phosphor-Akt S473 under hypoxia, and some inhibitors from the library also follow the trend, such as PDK1, PIM and receptor tyrosine kinases. Among all the candidate inhibitors, we noticed compound C, which is the inhibitor of AMPK, also suppresses Akt phosphorylation. AMPK is an energy sensor which also has been reported to be activated under various stresses including Hypoxia. We then tested and confirmed that compound C could inhibit both AMPK and Akt phosphorylation also in MDA231 cells (Figure 3-1-2). To further address the requirement of AMPK in hypoxia-induced Akt activation, we generated AMPK stable knockdown cells using shRNA and we found Akt phosphorylation and its downstream GSK3β phosphorylation were all decreased (Figure 3-1-3A), while restoration of AMPK into AMPK knockdown cells could rescue Akt phosphorylation (Figure 3-1-3B). These data suggest that AMPK is required for
hypoxia-induced Akt activation. To test whether AMPK is comprehensively required for Akt under other stress condition, we also tested glucose deprivation (Figure 3-1-4A) and oxidative stress H2O2 treatment (Figure 3-1-4B) and we found AMPK is also required for Akt activation under both conditions. The result suggests that there is a common mechanism for stress-induced Akt activation.
Figure 3-1-1 Screening of kinases in the hypoxia-induced Akt phosphorylation using kinase inhibitor library

293 cells were pretreated with indicated inhibitors at 10μM for 2 hours and then put into hypoxia chamber (1% O₂) for another 4 hours. Cells were lysed and subjected to immunoblotting with indicated antibody. The targets of each inhibitor are listed in Table 3-1.
### Table 3-1 List of kinase inhibitors and their targets

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Targets</th>
<th>Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY294002</td>
<td>PI3K</td>
<td>PI3K/Akt/mTOR</td>
</tr>
<tr>
<td>pp2</td>
<td>Src</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>Imatinib</td>
<td>BCR-ABL</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>BCR-ABL</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>CX-4945</td>
<td>CK2</td>
<td>DNA Repair</td>
</tr>
<tr>
<td>KU-55933</td>
<td>ATM</td>
<td>DNA Repair</td>
</tr>
<tr>
<td>KU-57788</td>
<td>DNAPK</td>
<td>DNA Repair</td>
</tr>
<tr>
<td>Compound C</td>
<td>AMPK</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Quizartinib (AC220)</td>
<td>FLT3</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>GNE-0877</td>
<td>LRRK2</td>
<td>Autophagy</td>
</tr>
<tr>
<td>BMS-536924</td>
<td>IGF1R</td>
<td>Protein tyrosine kinase</td>
</tr>
<tr>
<td>PF-562271</td>
<td>FAK</td>
<td>Angiogenesis</td>
</tr>
<tr>
<td>BI2536</td>
<td>FLK</td>
<td>Cell Cycle</td>
</tr>
<tr>
<td>Palbociclib</td>
<td>CDK</td>
<td>Cell Cycle</td>
</tr>
<tr>
<td>SGI-1776</td>
<td>PIM</td>
<td>JAK/STAT</td>
</tr>
<tr>
<td>ZCL278</td>
<td>RAC</td>
<td>Cell Cycle</td>
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<tr>
<td>SKI II</td>
<td>S1P Receptor</td>
<td>GPCR&amp;G Protein</td>
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<td>Crizotinib</td>
<td>c-Met, ALK</td>
<td>Protein tyrosine kinase</td>
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<tr>
<td>CUDC-101</td>
<td>HDAC,HER2, EGFR</td>
<td>Epigenetics</td>
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<td>OSU-03012</td>
<td>PDK-1</td>
<td>PI3K/Akt/mTOR</td>
</tr>
<tr>
<td>K-Ras(G12C) inhibitor 9</td>
<td>Rho</td>
<td>Cell Cycle</td>
</tr>
<tr>
<td>Nintedanib</td>
<td>VEGFR,PDGF R,FGFR</td>
<td>Protein tyrosine kinase</td>
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<tr>
<td>Cabozantinib</td>
<td>FLT3, Tie-2,c-Kit, c-Met,VEGFR-2</td>
<td>Protein tyrosine kinase</td>
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<tr>
<td>Selumetinib</td>
<td>MEK</td>
<td>MAPK</td>
</tr>
<tr>
<td>SB 203580</td>
<td>p38, MAPK</td>
<td>MAPK</td>
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<tr>
<td>H 89 2HCL</td>
<td>PKA</td>
<td>PI3K/Akt/mTOR</td>
</tr>
<tr>
<td>TWS119</td>
<td>GAK-3</td>
<td>PI3K/Akt/mTOR</td>
</tr>
<tr>
<td>Enzastaurin</td>
<td>PKC</td>
<td>TGF-beta/Smad</td>
</tr>
</tbody>
</table>
Figure 3-1-2 Compound C inhibits both AMPK and Akt phosphorylation

MDA-MB-231 cells were treated with or without compound C (10μM) and put into hypoxia chamber (1% O₂) for 2 and 4 hours. Cells were lysed and subjected to immunoblotting with indicated antibodies.
Figure 3-1-3 AMPK is required for hypoxia-induced Akt phosphorylation and activation

(A) MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were transferred into hypoxia chamber for 4 and 8 hours. Cell lysates were subjected to immunoblotting with indicated antibody.

(B) MDA-MB-231 cells were transfected with mock (PCDNA3) and HA-AMPK (CA, KD) by Lipofectamine and put into hypoxia chamber (1% O₂). Cell lysates were subjected to immunoblotting with indicated antibody.
Figure 3-1-4 AMPK is also required for glucose deprivation and H2O2-induced Akt activation

(A) MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were cultured in DMEM high glucose and no glucose medium for 1 and 2 hours. Cell lysates were subjected to immunoblotting with indicated antibody.

(B) MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were treated with 100μM H2O2 for 1 and 2 hours. Cell lysates were subjected to immunoblotting with indicated antibody.
3.2. AMPK is involved in EGF-induced Akt activation and activated by CaMKKβ.

Since Akt signaling is a major pathway for growth factors induced cell proliferation and AMPK is required for various stress induced Akt activation, we then checked whether AMPK is also involved in the growth factor-induced Akt activation. To our surprise, we found EGF-induced Akt activation is dependent on AMPK (Figure 3-2-1A), while under other growth factors like IGF (Figure 3-2-1B) and PDGF (Figure 3-2-1C), AMPK did not show any effect. Since LKB1 and CaMKKβ are the two major upstream kinases for AMPK activation, we next tested whether EGF-induced AMPK activation acted through these two known kinases. Our results showed that AMPK and Akt activation induced by EGF were impaired in CaMKKβ knockdown MDA231 cells (Figure 3-2-2A) as well as CaMKKβ inhibitor STO609 treated cells (Figure 3-2-2B). However, in LKB1 knockdown cells, Akt signaling was not affected (Figure 3-2-3). CaMKKβ is activated when intercellular Ca$^{2+}$ level changes. To explore how CaMKKβ is involved in the EGF-induced Akt activation, we used Fura-2 AM, a permeable calcium indicator, to trace intercellular Ca$^{2+}$ level upon EGF treatment and we found Ca$^{2+}$ level was rapidly induced when cells treated with EGF for 5 minutes (Figure 3-2-4A). When we used a calcium chelator (BAPTA) to inhibit the evaluated intercellular calcium level, we also saw decreased AMPK and Akt activation (Figure 3-2-4B). These data suggest that EGF-induced Ca$^{2+}$ level change activates CaMKKβ/AMPK is also important for EGF-induced Akt activation.
**Figure 3-2-1 AMPK is involved in EGF but not PDGF or IGF-induced Akt activation**

(A) MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were serum starved for 16 hours and treated with 50ng/ml EGF for 5 and 15 minutes. Cell lysates were subjected to immunoblotting with indicated antibody.

(B) MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were serum starved for 16 hours and treated with 100ng/ml PDGF for 5 and 15 minutes. Cell lysates were subjected to immunoblotting with indicated antibody.

(C) MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were serum starved for 16 hours and treated with 100 ng/ml IGF for 5 and 15 minutes. Cell lysates were subjected to immunoblotting with indicated antibody.
Figure 3-2-2 CaMKKβ is required for EGF-induced AMPK/Akt activation

(A) MDA-MB-231 cells with control (shLuc) and CaMKKβ knockdown were serum starved for 16 hours and treated with 50ng/ml EGF for 5 and 15 minutes. Cell lysates were subjected to immunoblotting with indicated antibody.

(B) MDA-MB-231 cells were serum starved for 16 hours and treated with 50ng/ml EGF and 10μg/ml STO609 for 15 minutes. Cell lysates were subjected to immunoblotting with indicated antibody.
Figure 3-2-3 LKB1 is not required for EGF-induced AMPK/Akt activation

MDA-MB-231 cells with control (shLuc) and LKB1 knockdown were serum starved for 16 hours and treated with 50ng/ml EGF for 5 and 15 minutes. Cell lysates were subjected to immunoblotting with indicated antibody.
Figure 3-2-4 EGF-induced changes in intercellular Ca2+

(A) MDA-MB-231 cells were seeded in a black 96-well plate and serum starved for 16 hours, then 2μM Fura-2AM was preloaded in calcium recording buffer for 1 hour. Cells were washed with calcium recording buffer and treated with 50ng/ml EGF for 5 min. Data presented represent the relative ratio of fluorescence measured at 340 divides by 360. *P<0.05.

(B) MDA-MB-231 cells were serum starved for 16 hours and treated with 50ng/ml EGF and 10μg/ml BAPTA-2AM for 15 minutes. Cell lysates were subjected to immunoblotting with indicated antibody.
3.3. Akt ubiquitination is induced under hypoxia and AMPK/Skp2 are involved in this process.

The unexpected phenomenon that among three growth factors tested (EGF, PDGF, IGF), only EGF-induced Akt activation is dependent on AMPK also drives us to think what might be the factor downstream of AMPK that is unique for EGF signaling. We previously found that Skp2, an E3 ligase for K63-linked Akt polyubiquitination, activated Akt specifically upon EGF treatment but not under other growth factors, like IGF (Chan, 2013). Therefore, we hypothesized that Skp2 might serve as an AMPK downstream effector. We then use primary Skp2 null MEFs as well as Skp2 stable knockdown cells in MDA231 cell line to check whether Skp2 is also needed under stress conditions, such as hypoxia. Indeed, we found Skp2 deficient cells also have less Akt activation upon hypoxia (Figure 3-3-1A/B). Since Skp2 is an E3 ligase for Akt ubiquitination under EGF induction, we next examine whether Akt ubiquitination is also induced by hypoxia. As shown in Figure 3-3-2A, Akt ubiquitination is dramatically increased during hypoxia and this ubiquitination is dependent on Skp2 (Figure 3-3-2B). As we previously found that AMPK is important for EGF-induced Akt activation (Figure 3-2-1A) and AMPK deficiency inhibits Akt activation. To check whether this impaired Akt activation is due to reduced Akt ubiquitination, we then examined whether AMPK inhibition affects Akt ubiquitination. This result showed that the induction of ubiquitination in hypoxia was inhibited when cells were treated with the AMPK inhibitor Compound C (Figure 3-3-3A). To further ensure this process is not due to the off-target effect of compound C, we also used AMPK knockdown
cells to confirm this phenomenon and the result also supports our previous observations that AMPK is required for hypoxia-induced Akt ubiquitination (Figure 3-3-3B).
Figure 3-3-1 Skp2 is also involved in hypoxia and glucose deprivation induced Akt ubiquitination

(A) WT and Skp2 null MEFs were put into hypoxia chamber (1% O₂) for 4 and 8 hours. Cell lysates were subjected to immunoblotting with indicated antibody.

(B) MDA-MB-231 cells with control (shLuc) and Skp2 knockdown were cultured in DMEM high glucose and no glucose medium for 1 and 2 hours. Cell lysates were subjected to immunoblotting with the indicated antibody.
Figure 3-3-2 Skp2 E3 ligase is important for Hypoxia-induced Akt ubiquitination

(A) 293 cells were treated with hypoxia (1% O₂) 4 hours and harvested for immunoprecipitation with Ig G control and anti-Ub antibody, followed by immunoblotting with Akt antibody. Input was 10% of whole cell lysate.

(B) MDA-MB-231 cells with control (shLuc) and Skp2 knockdown were transferred into hypoxia chamber for 4 hours. Cell lysates were subjected to immunoprecipitation with Akt antibody, followed by immunoblotting with the anti-Ub and Akt antibody. Input was 10% of whole cell lysate.
Akt ubiquitination is induced under hypoxia and AMPK is required for Akt ubiquitination

(A) 293 cells were transfected with HA-Akt plasmid and put into hypoxia chamber with or without compound C (10μM). Cell lysates were subject to immunoprecipitation with HA antibody and immunoblotting with indicated antibody.

(B) MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were transferred into hypoxia chamber for 4 hours. Cell lysates were subjected to immunoprecipitation with Ubiquitin antibody, followed by immunoblotting with Akt antibody. Input was 10% of whole cell lysate.
3.4. AMPK directly phosphorylates Skp2 at S256

Our previous results indicate that there is a strong link between AMPK and Skp2 in stress-induced Akt activation. To test whether Skp2 is a direct substrate of AMPK, we purified GST-Skp2 from bacteria, as well as HA-AMPK constitutively activated form from 293T cells and did in vitro kinase assay. We found active AMPK phosphorylated Skp2 in vitro (Figure 3-4-1). To map the phosphorylation region, we transfected cells with active AMPK and truncated forms of Skp2, and we found that Skp2 was phosphorylated at the C-terminal truncation (205-436) but not the N-terminal truncation (1-200) (Figure 3-4-2). Furthermore, we used online software Sitescan to predict the phosphorylation site of Skp2 based on the putative substrate conserve region of AMPK (Figure 3-4-3A). We then mutated those predicted sites and found Skp2 S256A mutant was no longer phosphorylated (Figure 3-4-3B/C), suggesting that AMPK phosphorylates Skp2 at S256. To better demonstrate the importance of this site, which is conserved among various species (Figure 3-4-4A), we generated Skp2 S256 phosphor-specific antibody. This antibody was validated in cells transfected with the active form of AMPK and Skp2. Skp2 S256 phosphorylation could be detected in WT Skp2 transfected cells but not in cells transfected with Skp2 S256A (Figure 3-4-4B). To examine the direct phosphorylation of Skp2 at S256 by AMPK, GST-Skp2 WT or S256A mutant and HA-AMPK proteins were purified and in vitro kinase assay was conducted. The active form of AMPK, but not the AMPK kinase-dead mutant, phosphorylated Skp2 WT in vitro (Figure 3-4-5A). In
contrast, Skp2 S256A could not be phosphorylated by active AMPK (Figure 3-4-5B).
**Figure 3-4-1 AMPK phosphorylates Skp2 in vitro**

Immunoprecipitates of exogenous HA-AMPKα1 from HEK293T cells and GST-Skp2 purified from bacteria were subjected to *in vitro* kinase assay followed by Immunoblotting.
Figure 3-4-2 AMPK phosphorylates Skp2 at residues after 200

HEK293 cells transfected with Skp2 ΔN, 1-200 and the indicated HA-AMPKα (CA) constructs were subjected to immunoprecipitation followed by immunoblotting.
Figure 3-4-3 Screening of Skp2 Serine residue phosphorylated by AMPK

(A) Skp2 putative phosphorylation site by AMPK id predicted by Scansite (http://scansite.mit.edu/)

(B) HEK293 cells transfected with Skp2 WT, S256, 367, 382/383A and the indicated HA-AMPKα (CA) constructs were subjected to immunoprecipitation followed by immunoblotting.
(C) HEK293 cells transfected with Skp2 WT, S256A/D, and the indicated HA-AMPKα (CA) constructs were subjected to immunoprecipitation followed by immunoblotting.
Figure 3-4-4 AMPK phosphorylates Skp2 at S256 in vivo using S256 phosphor-specific antibody

(A) The sequence of Skp2 in different species are listed. The conserved Serine (S) residues at 256 in different species are highlighted.

(B) HEK293 cells transfected with Skp2 WT, S256, and the indicated HA-AMPKα (CA) constructs were subjected to immunoprecipitation followed by immunoblotting.
Figure 3-4-5 AMPK also phosphorylates Skp2 at S256 in vitro using S256 phosphor-specific antibody

(A) Immunoprecipitates of exogenous HA-AMPKα1 (CA/KD) from HEK293T cells and GST-Skp2 WT purified from bacteria were subjected to in vitro kinase assay followed by Immunoblotting.

(B) Immunoprecipitates of exogenous HA-AMPKα1 (CA) from HEK293T cells and GST-Skp2 WT, S256A purified from bacteria were subjected to in vitro kinase assay followed by Immunoblotting.
3.5. S256 phosphorylation stabilizes Skp2 SCF E3 ligase complex integrity and is important for its E3 ligase activity

Since Skp2 is an E3 ligase and triggers substrate ubiquitination, we next examined the role of S256 phosphorylation in Skp2 E3 activity. We previously found that Skp2 is an E3 ligase for Akt ubiquitination upon EGF treatment and this ubiquitination facilitates Akt membrane localization, phosphorylation, and activation. To examine whether AMPK mediated Skp2 S256 phosphorylation is important for Akt ubiquitination and activation, we performed the in vitro ubiquitination assay from cells transfected with Skp2 WT and S256A/D mutant together with HA-Akt and His-Ub. We found Akt ubiquitination was induced in WT and phosphor-mimic S256D Skp2 cells but not in S256A mutant cells (Figure 3-5-1). Given that Skp2 functions as an F-box protein interacting with Skp1 and Cullin1 to form an SCF complex and the complex integrity are important for its E3 ligase activity, we then tested whether Skp2 S256 phosphorylation affects Skp1 and Cullin1 binding. We found that Cullin1 binding to Skp2 was enhanced by AMPK CA mutant and this binding was less in AMPK KD mutant transfected cells, while Skp1 binding to Skp2 was slightly affected (Figure 3-5-2A), indicating that the kinase activity of AMPK is required for Skp2- Cul1 binding. Although the crystal structure of Skp2 SCF complex shows previously that Skp1 served as a linker between Skp2 and Cul1, It cannot exclude the possibility of Skp2- Cul1 interaction under certain stress condition where AMPK is activated to phosphorylate Skp2. Therefore, we tested the function of Skp2 S256 phosphorylation on Skp2- Cul1 binding and found that Skp2 S256A mutant no
longer bound to Cul1 whereas the phosphor-mimic S256D mutant still bound (Figure 3-5-2B). In order to know how phosphorylation of Skp2 S256 site affects Cullin1 binding, we modeled the 3D structure of Skp2 and Cul1 and found there is a putative interaction between Skp2 S256 and Cul1 K221 (Figure 3-5-3A). To elucidate that the protein interaction is due to negative charge provided by a phosphate group, we eliminated the positive charge on this site by using Cul1 K221R mutant and found the interaction between Skp2 and Cul1 was much less compared to WT Cul1 (Figure 3-5-3B). All this finding suggest that Skp2 S256 phosphorylated by AMPK is important for Skp2 E3 integrity and affected Skp2 mediated Akt ubiquitination.
Figure 3-5-1 Skp2 S256 phosphorylation is important for its E3 ligase function.

*In vivo* ubiquitination assay in HEK293T cells transfected with the indicated plasmids was followed by immunoblotting.
Figure 3-5-2 Skp2 S256 phosphorylation stabilizes Skp2 SCF complex integrity.

(A) HEK293T cells transfected with Flag-Skp2 WT, S256A/D, and the indicated HA-AMPKα (CA) constructs were subjected to immunoprecipitation with Flag antibody and followed by immunoblotting.

(B) HEK293T cells transfected with Flag-Skp2 and the indicated HA-AMPKα (CA/KD) constructs were subjected to immunoprecipitation with Flag antibody and followed by immunoblotting.
Figure 3-5-3 Skp2 S256 interacts with Cullin1 K221 to promote SCF complex stability

(A) The potential binding pockets on the interface of Skp2-Cullin1. Skp2 is shown in green and Cullin1 is displayed in yellow.

(B) HEK293T cells transfected with Flag-Cullin1 WT and K221R were subjected to immunoprecipitation with Flag antibody and followed by immunoblotting
3.6. AMPK is important for EGF-induced glucose uptake, survival under stress as well as glucose deprivation induced VEGF secretion through phosphorylating Skp2 and activating Akt.

To investigate the biological function of S256 phosphorylation in cells, we first examined whether pSkp2 S256 can be detected under physiological condition. We found that Skp2 S256 phosphorylation was induced in many conditions, including EGF treatment, hypoxia, glucose deprivation as well as H₂O₂ oxidative stress (Figure 3-6-1). AMPK is known to regulate glucose metabolism during energy stress. However, whether AMPK is also important for EGF-induced glucose uptake is not known. To test this, we examined the glucose uptake rate induced by EGF. We found that glucose uptake induced by EGF was greatly impaired in AMPK knockdown cells and when we restored Myr-Akt (constitutively activated form) to AMPK knockdown cells, glucose uptake was also restored (Figure 3-6-2). Consistent with the glucose uptake rate, EGF-induced glucose transporter Glut1 expression was also decreased in AMPK knockdown cells (Figure 3-6-3A). By transfecting Myr-Akt, the basal level Glut1 expression was dramatically increased (Figure 3-6-3B). All the data suggest that AMPK regulates EGF-induced glucose uptake through Akt. To test whether it is possibly through Skp2 phosphorylation, we also restored Skp2 WT and S256 A/D mutant into AMPK knockdown cells and found without S256 phosphorylation, glucose uptake could not be restored (Figure 3-6-4).

To test whether AMPK is required for cell survival under stress, we treated cells with hypoxia and glucose deprivation and found cells with deficient AMPK...
survived less under both conditions while Akt could rescue the survival under hypoxia (Figure 3-6-5A) and glucose deprivation (Figure 3-6-5B). We then restored AMPK knockdown cells with Skp2 and found Skp2 S256A phosphorylation-deficient mutant could not rescue the survival under hypoxia (Figure 3-6-6A) and glucose deprivation, but WT could (Figure 3-6-6B).

We next test whether AMPK-Skp2-Akt regulates glucose deprivation-induced VEGF expression. We first checked the VEGF transcription and found MDA 231 cells with AMPKα1 knockdown transcribed less VEGF and Myr-Akt restored cell rescued VEGF mRNA level (Figure 3-6-7A). Moreover, MDA 231 AMPKα1 knockdown cells with Skp2 S256A restored could not rescue VEGF transcription (Figure 3-6-7B). These results indicate that AMPK regulates VEGF transcription through Skp2 S256 phosphorylation and Akt activation. Since VEGF is also secreted out of the cell, we also checked the VEGF amount in the culture medium by Elisa. Similar to its expression, VEGF concentration in the culture medium was also less in AMPK knockdown cells and rescued by Akt overexpression (Figure 3-6-8A) and Skp2 S256 phosphorylation (Figure 3-6-8B). VEGF is a growth factor that stimulates vascularization. Therefore, we examined the in vitro tube formation of endothelial HUVEC cells when applying the above mention culture medium. We found that HUVEC cells cultured with supernatant from AMPK knockdown cells had less tube and branch number but the restoration of Skp2 WT AND S256D phosphor-mimic mutant could partially rescue the branch number of HUVEC cells(Figure 3-6-9).
Figure 3-6-1 Skp2 S256 phosphorylation is induced by EGF, hypoxia, glucose deprivation and H2O2 treatment

MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were treated with EGF, hypoxia, glucose deprivation and H2O2. The cell lysates were subjected to immunoprecipitation with Skp2 antibody, followed by immunoblotting with Akt antibody.
Figure 3-6-2 EGF-induced glucose uptake is impaired in AMPK knockdown cells and can be restored by activated Akt

MDA 231 cells with control (shLuc), AMPKα1 knockdown and Myr-Akt restored were starved in DMEM glucose-free medium for 4 hours and add 2NBDG for 30 min. Cells were then subjected to FACS analysis. Each value represented the mean ± SEM (n = 8 per group) in three independent experiments. **P<0.01.
Figure 3-6-3 AMPK regulates EGF-induced Glut1 expression through Akt.

(A) MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were serum starved and treated with EGF for 15 and 30 minutes. Cell lysates were subjected to immunoblotting with indicated antibody.

(B) Immunoblotting for MDA 231 cells with control (shLuc), AMPKα1 knockdown and Myr-Akt restored.
Figure 3-6-4 EGF-induced glucose uptake impairs in AMPK knockdown cells and can be restored by Skp2 S256 phosphorylation.

MDA 231 AMPKα1 knockdown cells restored with control (Vector), Skp2 WT, S256A/D were starved in DMEM glucose-free medium for 4 hours and add 2NBDG for 30 min. Cells were then subjected to FACS analysis. Each value represented the mean ± SEM in three independent experiments. **P<0.01.
Figure 3-6-5 AMPK promotes cell survival under hypoxia and glucose deprivation through Akt

MDA 231 cells with control (shLuc), AMPKα1 knockdown and Myr-Akt restored were treated with (A) hypoxia and (B) glucose deprivation. Relative cell survival rate was measured by trypan blue exclusive assay. Each value represented the mean ± SEM in three independent experiments. **P<0.01.
AMPK promotes cell survival under hypoxia and glucose deprivation through Skp2 S256 phosphorylation

MDA 231 AMPKα1 knockdown cells with vector control, Skp2 WT and S256A/D restored were treated with (A) hypoxia and (B) glucose deprivation. Relative cell survival rate was measured by trypan blue exclusive assay. Each value represented the mean ± SEM in three independent experiments. **P<0.01.
Figure 3-6-7 AMPK-Skp2-Akt upregulates glucose deprivation induced VEGF transcription

(A) MDA 231 cells with control (shLuc), AMPKα1 knockdown and Myr-Akt restored were treated with the glucose-free medium. RNA isolated from cells were subjected to RT-PCR analysis. Each value represented the mean ± SEM in three independent experiments. **P<0.01.
(B) MDA 231 AMPKα1 knockdown cells with vector control, Skp2 WT and S256A/D restored were treated with the glucose-free medium. RNA isolated from cells were subjected to RT-PCR analysis. Each value represented the mean ± SEM in three independent experiments. **P<0.01.
Figure 3-6-8 AMPK-Skp2-Akt upregulates glucose deprivation induced VEGF secretion

(A) MDA 231 cells with control (shLuc), AMPKα1 knockdown and Myr-Akt restored (B) MDA 231 AMPKα1 knockdown cells with vector control, Skp2 WT and S256A/D restored were treated with a glucose-free medium for 8 hours and supernatant were collected for Elisa analysis. Each value represented the mean ± SEM in three independent experiments. **P<0.01.
Figure 3-6-9 AMPK / Skp2 regulates glucose deprivation induced VEGF secretion in MDA231 cells, which affect the endothelial cell tube formation ability

MDA 231 cells with control (shLuc), AMPKα1 knockdown and vector control, Skp2 WT and S256A/D restored were treated with a glucose-free medium for 8 hours and supernatant were collected for HUVEC tube formation assay. Each value represented the mean ± SEM in three independent experiments. **P<0.01
3.7. Xenograft and clinical relevance of Skp2 S256 phosphorylation.

To further determine whether AMPK is oncogenic in breast cancer, we generated stable knockdown of AMPK in the human breast cancer cell line MDA231 and then investigated the tumorigenicity of the AMPK knockdown cells in vivo by xenograft assay. The result showed AMPK knockdown suppressed tumor growth of breast cancer in vivo (Figure 3-7-1). To test whether AMPK-mediated Skp2 S256 phosphorylation accounts for the tumorigenesis role of AMPK, we stably overexpressed Skp2 WT, S256A, and the S256D mutants and found overexpression of WT Skp2, as well as the phosphor-mimic S256D mutant, promoted tumor growth in vivo, whereas overexpression of phosphorylation-deficient Skp2-S256A failed to do so (Figure 3-7-2). Hence, our data suggest that AMPK displays oncogenic activity in breast cancer in a manner dependent on phosphorylating Skp2 and activating Akt pathway. To investigate whether pAMPK and pSkp2 (S256) correlates with Akt activation and serves as a marker for poor prognosis in breast cancer patients, therefore, we assessed the expression of pAMPK, pSkp2 (S256), pAkt and another Skp2 substrate, p27, as well as their correlation in human breast cancer patients. In our breast cancer patient cohorts, we detected that both pAMPK, pSkp2 (S256) and pAkt were overexpressed in late-stage breast cancer while p27 expression was decreased because Skp2 ubiquitinates and promotes p27 degradation (Figure 3-7-3, Figure 3-7-4). We used the American Joint Committee on Cancer (AJCC) stage that judges the clinical aggressiveness of a tumor at diagnosis. The increased expression of both pAMPK and pSkp2 (S256) was significantly associated with
pAkt expression (Figure 3-7-5, Table 3-2). Also, univariate survival analysis results revealed that high expression of pAMPK or pSkp2 (S256) was significantly predictive of breast cancer disease-specific and metastasis-free survival (Figure 3-7-6 and Table 3-3). Thus, our data suggest that both pAMPK and pSkp2 (S256) are overexpressed during breast cancer progression, thereby their overexpression status may serve as prognostic markers for poor survival outcome of breast cancer patients.
Figure 3-7-1 Knockdown of AMPKα1 suppresses tumor growth of breast cancer in vivo.

MDA-MB-231 cells with control (shLuc) and AMPKα1 knockdown were subcutaneously injected into nude mice. Tumor size was measured by the caliper, and the result was showed as means ± SEM. (n=5). **P<0.01.
Figure 3-7-2 Overexpression of Skp2 WT and S256D promotes tumor growth of breast cancer in vivo

MDA-MB-231 cells with stable transduction of the indicated pBabe-Skp2 WT, S256A and S256D were subcutaneously injected into nude mice. Tumor size was measured by the caliper, and the result is shown as means ± SEM (n=5). **P<0.01.
Figure 3-7-3 pSkp2 (S256) and pAMPK expression are upregulated in breast cancers

Representative images of histological analysis of pSkp2 (S256), pAkt, pAMPK and p27 staining in early (Left panel) and late-stage breast cancer (Right panel). Scale bar, 200μm. The results were carried out by Dr. Chien-Feng Li.
Figure 3-7-4 Overexpression of pSkp2 (S256), pAMPK and in an advanced stage of breast cancer patient

Box plot represents pSkp2 (S256), pAMPK and pAkt expression in different stages of breast cancer patient. The results were carried out by Dr. Chien-Feng Li. **$P<0.01$.**
Figure 3-7-5 pAMPK and pAkt expression both correlate with pSkp2 (S256) in breast cancer patients

Scatter plots of pSkp2 (S256) expression versus pAMPK and pAkt expression in breast cancer patient were presented. The results were carried out by Dr. Chien-Feng Li.
Figure 3-7-6 Overexpression of pSkp2 or pAMPK predicts poor survival outcome of Breast cancer patients

(A) (B) Kaplan-Meier plots showed that high expression of pAMPK (A) or pSkp2 (S256) (B) was significantly predictive of disease-specific survival.

(C) (D) Kaplan-Meier plots showed that high expression of pAMPK (A) or pSkp2 (S256) (B) was significantly predictive of local recurrence-free survival. The results (A–D) were carried out by pathologist Dr. Chien-Feng Li.
Table 3-2 Associations between pSkp2 expressions with important clinic pathologic variables and pAkt, p27, pAMPK expression

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¹, Kruskal-Wallis H test; ², Mann-Whitney U test; ³, Pearson Correlation test; *, Statistically significant
### Table 3-3 Univariate survival analysis

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DSS, disease-specific survival; MeFS, metastasis-free survival; *, Statistically significant
CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS
In our kinase inhibitors screening data as showed in Figure 3-1-1, we noticed that PDK1 inhibitor OSU-03012, PIM inhibitor SGI-1776, a Src inhibitor as well as inhibitors that target cell membrane receptors: IGF-1R, VEGFR, PDGFR, FGFR, and c-MET can also inhibit Akt phosphorylation under hypoxia. PDK1 is a directed kinase to trigger T308 phosphorylation on the plasma membrane, followed by S473 phosphorylation in the cytoplasm. It is not surprising if PDK1 inhibitor could abolish Akt S473 phosphorylation under hypoxia. Activation of growth factor receptors usually leads to activation of Src and PI3K. All of the inhibitors target those kinases could inhibit Akt activation under hypoxia, suggesting that hypoxia-induced Akt activation also acts through cell membrane growth factor receptors. AMPK-mediated Skp2 phosphorylation triggers Akt ubiquitination and activation. This route still requires upstream receptors and PI3K. However, how hypoxia induces the membrane growth factor receptors without ligand binding needs further investigation. The future direction could focus on whether hypoxia affects receptor dimerization, aggregation or internalization.

AMPK is activated by both LKB1 and CAMKKβ. LKB1 mediated AMPK activation suppresses cell growth in certain cancers, such as NSCLC and cervical cancer (Shaw 2009). However, inhibition of the CAMKKβ decreases cell proliferation, migration and invasion in prostate cancer cells (Racioppi 2013). In addition, CaMKKβ is amplified in prostate cancer, invasive breast cancer, and ovarian cancer as shown in the TCGA data set (Ethan Cerami 2012, Gao J 2013). These observations support the oncogenic effect of CaMKKβ. Therefore, its
downstream substrate, AMPK, is likely involved in the oncogenic functions of CaMKKβ. Interestingly, we found that CaMKKβ deficient cell also had decreased Akt activation under EGF treatment, which is similar to what happened in AMPK knockdown cells, suggesting the CaMKKβ/AMPK might promote tumor progression through Akt activation. In contrast, LKB1 has not involved EGF-induced AMPK and Akt activation. Therefore, our findings indicate that oncogenic role of AMPK is through Akt activation and may largely drive by CaMKKβ under EGF treatment while LKB1 might only keep the basal AMPK activation for energy balance in breast cancer cell line MDA-MB-231. Our findings also suggest that AMPK targeting might be therapeutically beneficial for advanced breast cancer.

Future directions can also focus on the role of CaMKKβ in breast cancer progression, either by xenograft mouse model or tissue-specific knockout of CaMKKβ.

EGF binds to its membrane receptor EGFR and activates it through receptor dimerization. Downstream of EGFR include MAPK, PI3K/Akt, PLCγ and STAT-mediated pathway, which ultimately promote many cellular events including cell proliferation, migration, differentiation, and anti-apoptosis. We surprisingly found that EGF also activated AMPK within a short time (~2 min). Also, AMPK deficient cell displayed decreased EGF-induced Akt activation. This observation brings AMPK to the traditional EGF-PI3K/Akt pathway. Moreover, we found AMPK activated by EGF through CaMKKβ but not LKB1. EGF-induced AMPK activation is rapid and without any changes in ATP level but intercellular Ca2+ level. Therefore, our data suggest that CaMKKβ triggers rapid AMPK
activation in a Ca2+ dependent manner while LKB1 activates AMPK when cell face decreased AMP/ADP: ATP ratio. Activated EGFR activates PLCγ to catalyze PIP2 to generate secondary message DAG and IP3. IP3 then binds to its receptor IP3R, a calcium channel on the membrane of ER, which induces calcium release from (endoplasmic reticulum) ER calcium stores and increases intercellular calcium level. Calcium binds to the protein calmodulin and activates CaMKK. Our findings not only indicate the rapid changes of intercellular calcium level is important for EGF-induced Akt signaling, but also brings AMPK as a new component of EGF-PI3K/Akt pathway. The future direction would be to confirm and further explore the importance of AMPK in EGF-PI3K/Akt pathway. Whether both AMPK and PI3K are needed for Akt activation? Could AMPK rescue Akt activity if PI3K is inactivated? Moreover, many studies have revealed that EGFR is overexpressed in MDA231 cells, but EGFR targeting has limited effect in clinical trials due to the resistant mechanism. It will be also worthy of examining whether AMPK activation may account for one of the resistance mechanism for EGFR targeting therapy. It is possible that targeting AMPK together with EGFR is beneficial for triple negative breast cancer treatment.

PI3K/Akt is a classic pathway to promote cell proliferation, survival and inhibit apoptosis. In contrast, AMPK’s role in cancer is context-dependent. However, both of them are activated when the cell faces various stress like hypoxia, oxidative stress, and glucose deprivation. AMPK and Akt share many common substrates but different outcomes. The role of AMPK in Akt regulation is not clear at the current stage. Reports indicate that AMPK can either activate or
inhibit Akt. However, those studies were performed under AICAR and metformin treatment which might have many other off-target effects. Importantly, our study uncovered a novel mechanism that AMPK activates PI3K/Akt pathway by phosphorylating Skp2 and promoting Akt ubiquitination. It improves our current understanding of relationships of AMPK and Akt. Importantly, it indicates that as the tumor progresses and encounters limited resources, activation of AMPK is important to phosphorylate Skp2 and turn on Akt survival pathway to resist immediate death. However, we cannot exclude other Skp2 E3 ligase substrates might contribute to increasing survival. In the future, it may be worth to test other Skp2 substrates, given that Skp2 also ubiquitinates and promotes degradation of p27 (Keiko Nakayama 2004, Uta Kossatz 2004). Our result in the human breast cancer patient sample also showed downregulation of p27 and its correlation with poor survival outcome. It is possible that Skp2 S256 phosphorylation targeting may also affect p27 or other downstream substrates due to its defect E3 ligase activity. We could test whether, under certain stimuli, AMPK activation leads to more Skp2-mediated p21 or p27 ubiquitination in cancer cells to regulates the cell cycle.

Ubiquitination either leads to protein degradation or promote signaling transduction depending on how the ubiquitin is linked. While K48 linked ubiquitination targets protein for proteasome degradation, K63 linked ubiquitination have important functions in signaling transduction. Previous work in our lab revealed that K63 linked Akt ubiquitination is important for its activation by growth factors stimulation. Our finding that Akt ubiquitination was greatly induced.
by hypoxia provides a better picture of how Akt is activated under stress. K63 linked polyubiquitination may recruit other components to facilitate Akt membrane localization. One of the putative molecules that may be relevant is Ubl4A, an ubiquitin-like protein that directly binds to actin filaments. A Recent study has shown that upon insulin treatment, Ubl4a binds to actin related protein 2/3 and accelerates actin branching and Akt membrane localization. It provides a missing link of Akt activation under insulin. It also gives us a hint that there might be other adaptor proteins exist to facilitate Akt membrane localization and activation under stress, like hypoxia. Akt ubiquitination may be critical to recruiting various adaptors for Akt membrane localization and activation under the diverse situation.

Skp2-SCF complex consists of an F-box protein Skp2, an adaptor Skp1, and Cul1. Crystal structure of Skp2-SCF (Figure 4-1) revealed that Skp1 act as a linker to bridge both Skp2 and Cul1. However, the crystal structure used a truncated F-box domain of Skp2 instead of full-length Skp2 (Ning Zheng 2002). Skp2 contains an F-box domain which binds Skp1 and an LRR domain which is important for substrate recognition. The S256 residue is located inside the LRR domain. We found that Skp2 S256 phosphorylation is important for Cul1 binding and Akt ubiquitination. It suggests Skp2 may also interact with Cul1 to keep the SCF complex integrated and more functional. However, whether Skp1 is required for Cul1 and Skp2 interaction is still not clear. To this end, we could examine the interaction of Skp2 and Cul1 without Skp1. The crystal structure with Skp1, Cul1, and full-length Skp2 will be beneficial to examine the interaction between Skp2 and Cul1. It is also possible that Skp2 S256 phosphorylation may
affect the substrate recognition. Previous studies showed that Cks1 (CDK subunit 1) is required for Skp2 to recognize phosphorylated p27 (Ganoth D 2001). Therefore, the interaction between Skp2 and Cks1 or Akt can also be tested for a better understanding of the function of Skp2 S256 phosphorylation.
Figure 5 Crystal structure of Skp2-SCF complex
Our study also showed that expression level of Skp2 S256 phosphorylation was correlated with AMPK and Akt phosphorylation and poor survival outcomes in breast cancer patients. These results indicate the in vivo relevance of AMPK in breast cancer progression. Our findings also showed the pronounced effect of AMPK and Skp2 S256 phosphorylation in mouse xenograft models. It suggests that AMPK and Skp2 S256 phosphorylation targeting might be a promising strategy for inhibiting breast cancer progression. In order to further dissect the significance of Skp2 S256 phosphorylation in cancer progression, we can use MMTV/Skp2 WT and S256A/D transgenic mice as a tool to study whether the phosphorylation is important to increase tumor incidence. We can also use Skp2 WT and S256A/D knock-in mice and cross with PTEN conditional knockout mice to further address the oncogenic role of Skp2 S256 phosphorylation. Meanwhile, we can also generate small molecular inhibitors to target Skp2 S256 phosphorylation and evaluate its efficacy in blocking tumor progression.

Another interesting finding of our work is that AMPK mediated Skp2 phosphorylation and Akt activation is involved in the glucose deprivation induced VEGF secretion from cancer cells to the culture medium. When we apply the supernatant to the endothelial cell grown in matrix-gel, it will cause the formation of tube-like structure. These results suggest that as tumor quickly expands and encounters short supplies of glucose, AMPK might be also important for cancer cells to modify the tumor microenvironment by promoting angiogenesis and obtain more glucose in the blood vessel. Future work is needed to examine angiogenesis in vivo by detecting markers for vascularization within xenograft
tissue. By doing this, we can potentially prove the role of AMPK and Skp2 phosphorylation in angiogenesis and promote tumor progression. Since angiogenesis will facilitate tumor migration from the original location to distal organs, we will also check the role of AMPK and Skp2 S256 phosphorylation in metastasis.

In summary, our study shows that AMPK is required for various stress induced Akt activation and promotes cell survival, including hypoxia and glucose deprivation. AMPK is also important for EGF-induced Glut1 expression and glucose uptake, as well as glucose deprivation induced VEGF expression and secretion. AMPK directly phosphorylates Skp2 at S256 residue and this phosphorylation is important for Skp2 SCF complex integrity and E3 ligase activity to ubiquitinate and activates Akt upon various stresses, as well as EGF growth factor stimulation. The Skp2 phosphorylation status is important for tumor progression and correlated with poor survival of breast cancer patient. The working model of my study is shown in Figure 4-2.
Figure 4-6 Hypothetic model of the role of pAMPK in Stress and EGF-induced Akt activation and tumorigenesis.


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