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UNDERSTANDING THE ROLE OF SUMOYLATION IN REGULATING LKB1 FUNCTION

Joan W. Ritho

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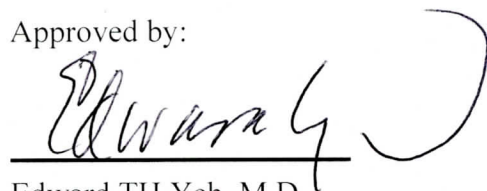
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**UNDERSTANDING THE ROLE OF
SUMOYLATION IN REGULATING
LKB1 FUNCTION**

by

Joan Ritho B.A.

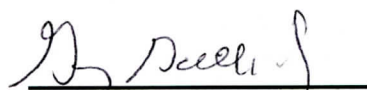
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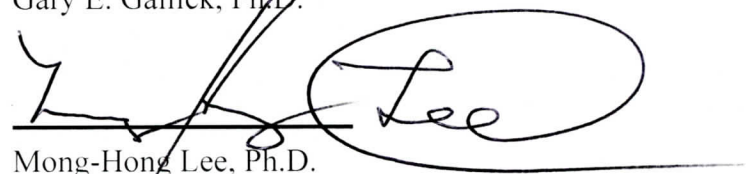
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**UNDERSTANDING THE ROLE OF
SUMOYLATION IN REGULATING
LKB1 FUNCTION**

**A
DISSERTATION**

Presented to the Faculty of
The University of Texas
Health Science Centre at Houston
Graduate School of Biomedical Sciences
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences

In Partial Fulfillment
Of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

By
Joan Ritho, B.A.
Houston, Texas
May, 2015

DEDICATION

To God for His love, grace, and giving me the strength to push through this arduous journey.
To my mother and father, Margaret G. Gatere and Colonel Samuel R. Mwangi, and my brothers,
Captain Ronnie Ritho and Andrew Ritho. Thank you for your unconditional love and support.

I love you!

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Abstract

UNDERSTANDING THE ROLE OF SUMOYLATION IN REGULATING LKB1 FUNCTION

Joan Ritho, B.A.

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Energy homeostasis in a cell is critical for its survival during metabolic stress. Liver kinase B1 (LKB1), one of the key regulators of cellular energy balance, was initially discovered as a tumor suppressor mutated in patients with Peutz-Jeghers syndrome. Germline mutations in LKB1 predispose patients to develop several benign and malignant tumors including gastrointestinal and lung cancers. In 2003, several groups demonstrated that LKB1 is a major upstream kinase of the energy sensor AMP-activated protein kinase (AMPK), directly associating it with the regulation of energy balance in cells. During energy stress, LKB1 phosphorylates AMPK at threonine 172 (T172) resulting in AMPK activation. This leads to the inhibition of anabolic pathways such as fatty acid synthesis and activation of energy-producing pathways such as glycolysis. Some of the proteins targeted include: ACC1 (fatty acid synthesis), ACC2 (fatty acid oxidation), mTORC1 (protein synthesis), ULK1 (mitophagy) and GLUT1 (glucose uptake). Therefore, LKB1 is strategically positioned as an essential kinase in maintaining cellular energy balance.

A number of studies have described the influence of covalent post-translational modifications in governing LKB1 activity. However, the role of SUMOylation in regulating LKB1 function is unknown. SUMOylation is the reversible covalent modification of a SUMO

(small ubiquitin-related modifier) protein to a specific lysine on the target protein. This process has been implicated in important processes such as transcription, protein stability, and protein subcellular localization. At the molecular level, SUMOylation can (1) inhibit the interaction between the target and its interacting partner, (2) enhance this interaction through creation of a binding surface where the target would recognize the partner via a SUMO-interacting motif (SIM), or (3) change the conformation of the target, thereby altering its function. Given the diverse roles SUMOylation plays in the eukaryotic cell, we hypothesized that, during energy stress, SUMOylation regulates the LKB1-AMPK interaction and that this accordingly affects the kinase activity of AMPK.

Our findings here demonstrate that energy deficit triggers an increase in the modification of LKB1 by SUMO1 despite a global reduction in both SUMO1 and SUMO2/3 conjugates. During metabolic stress, LKB1 is specifically modified by SUMO1 at lysine 178 (K178) but not by SUMO2/3, acetylation, or ubiquitination. This modification is essential in promoting LKB1-AMPK interaction. On the basis of the crystal structure depicting the non-covalent recognition of SUMO1 by RanBP2, we identified a SIM in the N-terminal region of AMPK. Mutation of the hydrophobic residues necessary for SUMO1 interaction prevented LKB1 from recognizing and activating AMPK. Finally, we observed that cells with the LKB1 K178R SUMO mutant had defective AMPK signaling and mitochondrial function, inducing apoptosis in energy-deprived cells. Thus, we propose a model in which energy stress upregulates the modification of LKB1 by SUMO1, thereby facilitating its interaction with AMPK. This enhances the rate at which AMPK can respond to the metabolic needs of the cell.

TABLE OF CONTENTS

Approval Sheet.....	i
Title Page	ii
Dedication	iii
Acknowledgements	iv
Abstract	vii
Table of Contents	ix
List of Figures	xi
Abbreviations	xiv
 CHAPTER I: Background and Significance.....	1
Liver Kinase B1 (LKB1)	2
LKB1 as a tumor suppressor.....	5
The role of LKB1 as a master regulator of the cell's energy balance.....	8
LKB1 post-translational modifications	11
SUMO (small ubiquitin-like modifier)	13
The SUMO conjugation pathway	14
DeSUMOylation	14
SUMO acceptor sites	16
Consequences of SUMOylation.....	16
SUMO Interacting Motifs (SIMs).....	18
Hypothesis and aims	19

CHAPTER II: Materials and methods	20
CHAPTER III: LKB1 is SUMOylated	27
CHAPTER IV: SUMO1 modification of LKB1 is upregulated during energy stress	36
CHAPTER V: LKB1 K178 is modified by SUMO1 and is critical in activating AMPK.....	51
CHAPTER VI: SUMOylation promotes LKB1-AMPK interaction.....	67
CHAPTER VII: LKB1 K178 SUMOylation is essential in the AMPK signaling pathway ..	82
CHAPTER VIII: List of conclusions	93
CHAPTER IX: Discussion	96
CHAPTER X: Future direction.....	105
Acknowledgements and funding.....	108
Bibliography	109
Vita	125

LIST OF FIGURES

Figure 1. <i>LKB1</i> (<i>STK11</i>) gene structure	3
Figure 2. LKB1-STRAD α -MO25 α heterotrimeric complex	4
Figure 3. LKB1-AMPK signaling pathway	7
Figure 4. Model of LKB1-dependent regulation of the energy balance of a cell	9
Figure 5. Examples of pathways regulated by AMPK.....	10
Figure 6. Post-translational modifications of LKB1	12
Figure 7. SUMO conjugation pathway	15
Figure 8. The three general non-exclusive consequences of SUMOylation.....	17
Figure 9. Predicted SUMO Interacting Motifs (SIMs)	18
Figure 10. Endogenous SUMOylation of LKB1 in HEK293 cells.....	29
Figure 11. SUMO protease, SENP1, decreases endogenous LKB1 SUMOylation	30
Figure 12. Exogenous SUMO1 and SUMO2/3 modification of LKB1.....	32
Figure 13. SENP1 decreases exogenous LKB1 SUMO1 modification.	33
Figure 14. <i>In vitro</i> SUMO1 modification of LKB1	34
Figure 15. LKB1 SUMOylation changes that occur when HEK293 cells are subjected to various metabolic stress agents.....	39
Figure 16. LKB1 SUMOylation changes that occur in HEK293 cell during energy stress	40
Figure 17. LKB1 SUMOylation changes that occur in mouse myoblast cell line, C2C12, during energy stress.....	41
Figure 18. Validation of LKB1 SUMOylation via the siRNA knockdown of LKB1	42
Figure 19. Global SUMOylation changes that occur in a cell during metabolic stress	44
Figure 20. The role of SUMOylation in regulating AMPK activation	46

Figure 21. Endogenous and exogenous SENP1 and SENP2 protein levels decrease during metabolic stress	47
Figure 22. SENP1 regulates the catalytic activity of LKB1	49
Figure 23. LKB1 SUMOylation predicted sites.....	54
Figure 24. Characterization of the consensus LKB1 SUMOylation site	55
Figure 25. The role of energy stress in regulating LKB1 lysine 178 SUMOylation	56
Figure 26. Lysine 178 of LKB1 is SUMO1 but not SUMO2/3 modified during energy stress ...	57
Figure 27. The acetylation or ubiquitination status of LKB1 lysine 178 is not altered during energy stress.....	58
Figure 28. The physiological implication of the LKB1 K178R SUMO1 mutant during energy stress.....	60
Figure 29. Ribbon diagram showing structural features of LKB1.....	62
Figure 30. LKB1 K178R SUMO mutant is not a catalytic mutant.....	63
Figure 31. Speculative structural model of K178-SUMOylated LKB1.....	64
Figure 32. LKB1 K178R SUMO1 mutant forms an active heterotrimeric complex with STRAD and MO25	65
Figure 33. LKB1 K178R SUMO1 mutant interaction with AMPK during energy stress	70
Figure 34. Role of SUMO protease, SENP1, in regulating LKB1-AMPK interaction	71
Figure 35. AMPK interacts with endogenous and exogenous SUMO1	72
Figure 36. SUMO1 recognizes AMPK via a SUMO interacting motif.....	73
Figure 37. Identification of AMPK SIM.....	76
Figure 38. Characterization of AMPK SIM.....	79
Figure 39. <i>In vivo</i> characterization of AMPK SIM and its physiological relevance	80

Figure 40. LKB1 K178 SUMOylation is important in regulating the activation of AMPK and its downstream targets during metabolic stress	86
Figure 41. The role of LKB1 K178 SUMOylation in maintaining intracellular ATP levels during energy stress.....	87
Figure 42. LKB1 K178 SUMOylation is important in preventing apoptosis during metabolic stress.....	88
Figure 43. The role of LKB1 K178 SUMOylation in regulating mitophagy	89
Figure 44. LKB1 K178 SUMOylation is important in regulating mitochondrial health.....	91
Figure 45. Proposed model illustrating the SIM mediated activation of AMPK.....	95
Figure 46. LKB1 is a master kinase that activates AMPK and 12 of its related kinases.....	107

List of abbreviations

ACC: acetyl CoA carboxylase

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ATP: adenosine triphosphate

CaMKK β : Ca²⁺/calmodulin-dependent protein kinase kinase β

cDNA: complementary deoxyribonucleic acid

CRD: C-terminal regulatory domain

DMEM: Dulbecco's modified Eagle's medium

FACS: fluorescence-activated cell sorter

GLUT: glucose transporter

HA: hemagglutinin

IgG: immunoglobulin G

kb: kilobase

kDa: kilodalton

LKB1: liver kinase B 1

LOH: loss of heterozygosity

MEFs: mouse embryonic fibroblasts

MO25: scaffolding mouse 25

mRNA: messenger ribonucleic acid

mTOR: (mammalian target of rapamycin)

NRD: N-terminal regulatory domain

NSCLC: non-small cell lung carcinomas

OCR: oxygen consumption rate

p27: p27 cell cycle inhibitor (Cyclin-dependent kinase inhibitor 1B)

p53: p53 tumor suppressor

PCR: polymerase chain reaction

PI3K: phosphatidyl inositol-3-kinase

PJS: Peutz-Jeghers syndrome

PTEN: phosphatase and tensin homologue

qRT-PCR: quantitative real-time PCR

RanBP: Ran-binding protein

RanGAP: Ran GTPase-activating protein

ROS: reactive oxidative species

SEM: standard error of mean

SENP: sentrin/SUMO-specific protease

Ser: serine

shRNA: short-hairpin ribonucleic acids

SIM: SUMO-interacting motif

siRNA: silencing ribonucleic acids

STK11: serine/threonine kinase 11

STRAD α : STE20-related adaptor alpha

STUbL: SUMO-targeted ubiquitin ligase

SUMO: small ubiquitin-like modifier

Thr: threonine

ULK1: UNC-51-like kinase 1.

WT: wild-type

CHAPTER I: Background and Significance

Liver Kinase B1 (LKB1)

In 1996, Jun-ichi Nezu of Chugai Pharmaceuticals made the seminal discovery of the *LKB1* gene (also known as *STK11*- Serine Threonine kinase 11) while screening for new kinases (Hemminki et al., 1998). Unfortunately, he deposited the sequence in a database without characterizing the gene or writing a research paper describing his findings. A year later, Lauri Aaltonen's laboratory in Helsinki identified it as a gene mutated in patients suffering from Peutz-Jeghers syndrome (PJS) (Hemminki et al., 1998). PJS, described and characterized earlier by Dr. Johannes Peutz and Dr. Harold Jeghers, is an autosomal dominant disease (Jeghers, 1944, Westerman et al., 1999). It increasingly predisposes patients to develop multiple benign and malignant tumors in various tissues including the gastrointestinal (GI) tract and lung (Hemminki, 1999, Tomlinson and Houlston, 1997, Westerman et al., 1999).

In rare cases where sporadic LKB1 mutations are exhibited, patients tend to develop ovarian and pancreatic cancers among others (Sanchez-Cespedes, 2007). These observations therefore led to the conclusion that LKB1 might be an important tumor suppressor. In fact, loss of LKB1 function has been described to be a first or second "hit" according to the Knudson hypothesis in which additional loss of function of tumor suppressors: p53 or PTEN, or gain of function of the oncogene Kras leads to the synergistic instigation of cancer progression (Huang et al., 2008, Morton et al., 2010, Takeda et al., 2006, Wei et al., 2005).

The *LKB1* gene is located on chromosome 19p13.3. It is 23 kb long and contains 10 exons (one of which is noncoding) (Hemminki et al., 1997). In humans, the LKB1 protein is 433 amino acids long and includes an N-terminal regulatory domain (NRD), kinase domain, and a C-terminal regulatory domain (CRD) (Figure1) (Alessi et al., 2006). It exists as a constitutively active heterotrimeric complex with mouse protein 25 (MO25) and pseudokinase STE20-related

adaptor (STRAD) (Figure2) (Zeqiraj et al., 2009). Indeed, these two partners also determine its cellular localization (Boudeau et al., 2003). Studies have illustrated that when LKB1 is solely overexpressed in mammalian cells, it is predominantly located in the nucleus. However, in the presence of STRAD and MO25, the heterotrimeric complex quickly translocates into the cytoplasm (Tiainen et al., 2002, Nezu et al., 1999, Boudeau et al., 2003).

Over the years, LKB1 has emerged as a bona fide tumor suppressor with complex and multi-faceted roles that include cell cycle arrest, cell metabolism, p53-mediated apoptosis, and maintenance of hematopoietic stem cells among others (Alessi et al., 2006). Therefore, many researchers have made it a priority to understand how it is regulated so as to possibly devise therapeutic strategies that will help eliminate cancer.

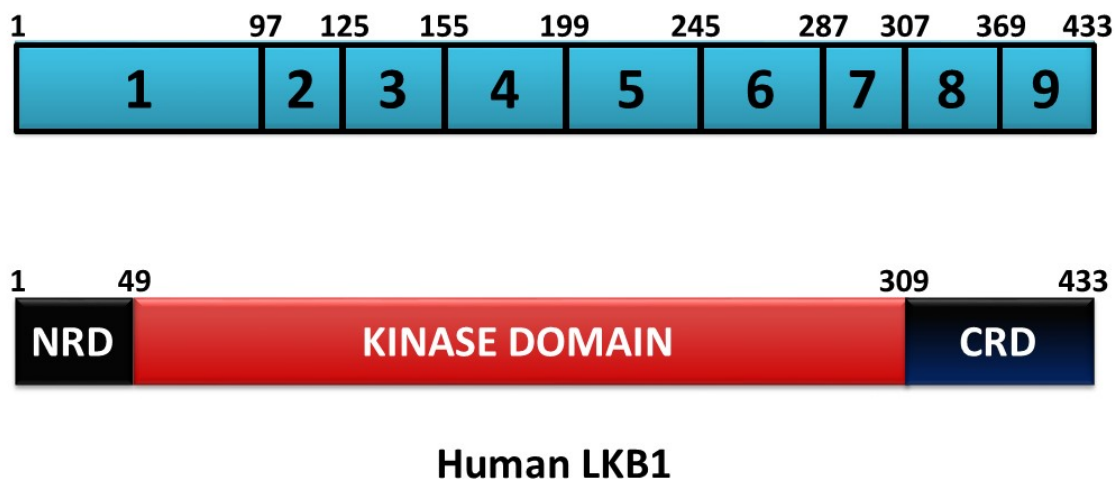


Figure 1. *LKB1* (*STK11*) gene structure.

LKB1 consists of 10 exons, one of which is noncoding (top). It has an N-terminal regulatory domain (NRD), catalytic domain (amino acids 49-309), and a C-terminal regulatory domain (CRD) (bottom).

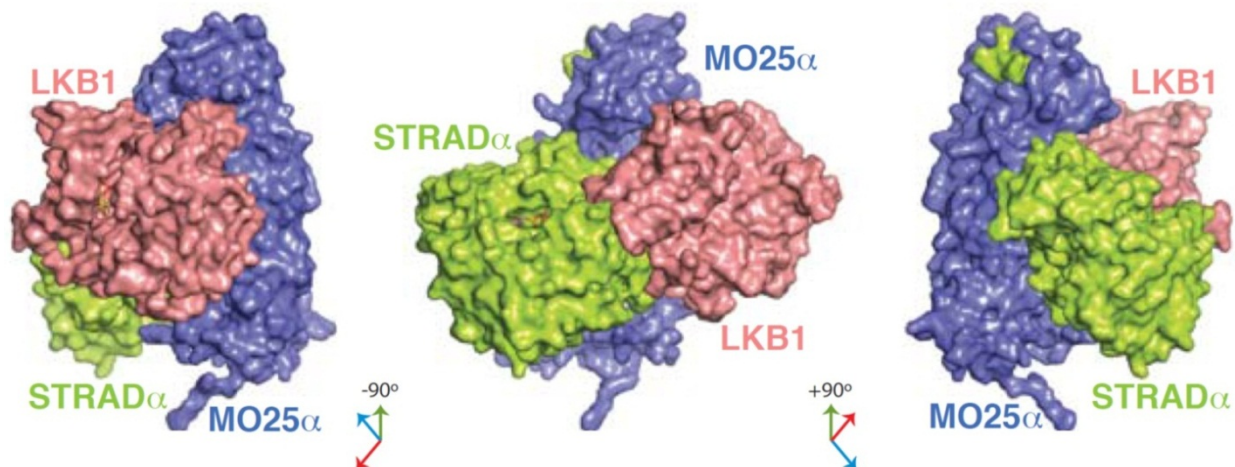


Figure 2. LKB1-STRAD α -MO25 α heterotrimeric complex.

Depicted is an illustration of the crystal structure of the active LKB1-STRAD α -MO25 α heterotrimeric complex at rotation angles of 90° and -90°. STRAD α , a pseudokinase, binds the catalytic domain of LKB1 and MO25 α wraps around LKB1 and STRAD α like a scaffold thereby assembling and stabilizing the complex. LKB1 is active only when in complex with STRAD and MO25 proteins. (**Structure of the LKB1-STRAD-MO25 Complex Reveals an Allosteric Mechanism of Kinase Activation** Elton Zeqiraj, Beatrice Maria Filippi, Maria Deak, Dario R. Alessi, and Daan M. F. van Aalten *Science* 18 December 2009; **326** (5960), 1707-1711. Reprinted with permission from AAAS. License number: 3596531043504).

LKB1 as a tumor suppressor

As alluded to earlier, studies have demonstrated that PJS patients have a higher propensity to develop malignancies of the gastrointestinal tract (38%-93%), gynecologic (13%-18%), and breast (32%-54%) (van Lier et al., 2010). It has been demonstrated that this higher predisposition to the aforementioned cancers is attributed to the PJS missense mutations located in the conserved serine/threonine kinase domain that consequently leads to a decrease in LKB1 kinase activity and function (Jenne et al., 1998). These observations highlighted that LKB1 may be an important tumor suppressor. Therefore, this prompted several mechanistic studies that defined and illustrated the vital role LKB1 plays in inhibiting cancer progression.

Several studies have demonstrated that LKB1 is essential in regulating the cell cycle. Reintroduction of wild type LKB1 in cells that lacked it induced a G1 cell cycle arrest, and the converse was true when LKB1 was knocked down in human embryonic kidney 293T cells (HEK-293T cells) and human umbilical vein endothelial cells (HUVECs) (Liang et al., 2010). This LKB1-dependent G1 cell arrest is mediated through p53-dependent or independent mechanisms (Zeng and Berger, 2006, Tiainen et al., 2002, Xie et al., 2007, Scott et al., 2007). This consequently leads to the induction of cell cycle progression inhibitors, p21 and p27 (Figure3).

Additional studies have illustrated the role of LKB1 in p53-mediated apoptosis. Karuman et al. demonstrated that LKB1 interacts with and phosphorylates p53 leading to apoptosis (Karuman et al., 2001) (Figure3). However, in *Drosophila*, dLkb1 induces p53-independent programmed cell death via the JNK pathway (Lee et al., 2006). In mice, loss of both *LKB1* and *p53* alleles leads to augmented instigation of gastric hamartomas and hepatic

adenomas/carcinomas (Alessi et al., 2006). This provides cogent evidence that supports the cooperation between LKB1 and p53 during tumor progression.

LKB1 also has been shown to have a direct upstream role in regulating PTEN (Phosphatase and Tensin homolog deleted on chromosome TEN), a tumor suppressor, whose function is critical in inhibiting the PI3K/AKT oncogenic survival pathway (Mehenni et al., 2005, Song et al., 2007). LKB1 also facilitates the nuclear export of PTEN, which in turn leads to the inhibition of the initial stages of the PI3K/AKT pathway in the plasma membrane (Korsse, Peppelenbosch, & van Veelen, 2013). Liu et al. demonstrate that this process is independent of AMPK/mTOR signaling (Liu et al., 2011). Furthermore, loss of both *PTEN* and *LKB1* has been shown to accelerate lung squamous cell carcinoma (SCC) progression (Xu et al., 2014). All in all, these studies illustrate the vital role LKB1 plays in inhibiting tumorigenesis via the cooperation and regulation of PTEN.

Altogether, the role of LKB1 in regulating cell growth and apoptosis, in part, highlights its important function as a tumor suppressor. Investigating or identifying loss of function LKB1 mutations or deletions could prove to be important prognostic markers that will provide insight in cancer risk assessment.

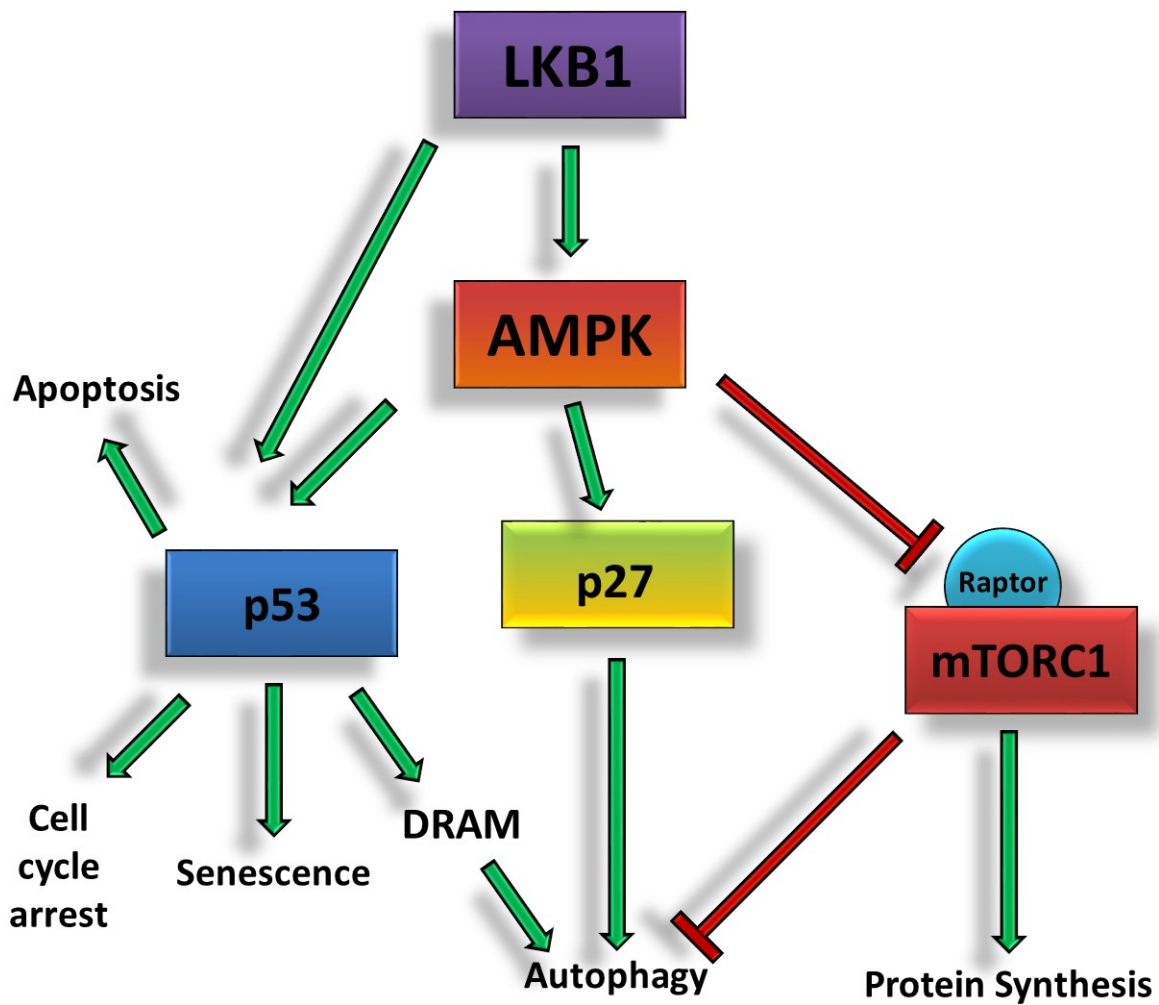


Figure 3. LKB1-AMPK signaling pathway

The diagram shown is a snippet of pathways that are activated and inhibited by the tumor suppressor LKB1 via activated AMPK. This consequently leads to the regulation of cell growth and apoptosis.

The role of LKB1 as a master regulator of the cell's energy balance

When a cell encounters energy stress, different survival mechanisms are employed to ensure the cell's survival. AMP-activated protein kinase (AMPK) is the master energy sensor in control of ensuring that the cell maintains its energy equilibrium (Yeh et al., 1980, Grahame Hardie et al., 1989). During conditions of energy deprivation, the AMP/ATP ratio increases (Hardie et al., 2012). AMP binds the γ subunit of AMPK thereby altering its conformation. The conformation change allows the efficient phosphorylation of threonine 172 located on the activating loop of AMPK α , consequently activating it (Hardie et al., 2012, Xiao et al., 2011, Oakhill et al., 2011). This leads to the inhibition of processes that consume energy while activating catabolic pathways (Figure 4 and 5). For example, AMPK facilitates the translocation of GLUT4 to the plasma membrane, which increases glucose uptake (Yamaguchi et al., 2005) while inhibiting mTORC1, an important protein involved in the anabolic process of protein synthesis (Shackelford and Shaw, 2009).

The activating upstream kinase of AMPK remained elusive until 2003 when three groups identified that the LKB1-STRAD-MO25 heterotrimeric complex was responsible for phosphorylating AMPK on threonine 172 during energy stress subsequently activating it (Woods et al., 2003, Hawley et al., 2003, Shaw et al., 2004). This important observation strategically positioned LKB1 as an important player that assessed and coupled energy surplus or deficit to mediate cell growth and division. Downregulation of LKB1 function results in bioenergetic catastrophe, which leads to cell death. Indeed, the seminal study by the Lewis Cantley group illustrated that LKB1-deficient cells were prone to increased cell death when exposed to energy stressful conditions (Shaw et al., 2004).

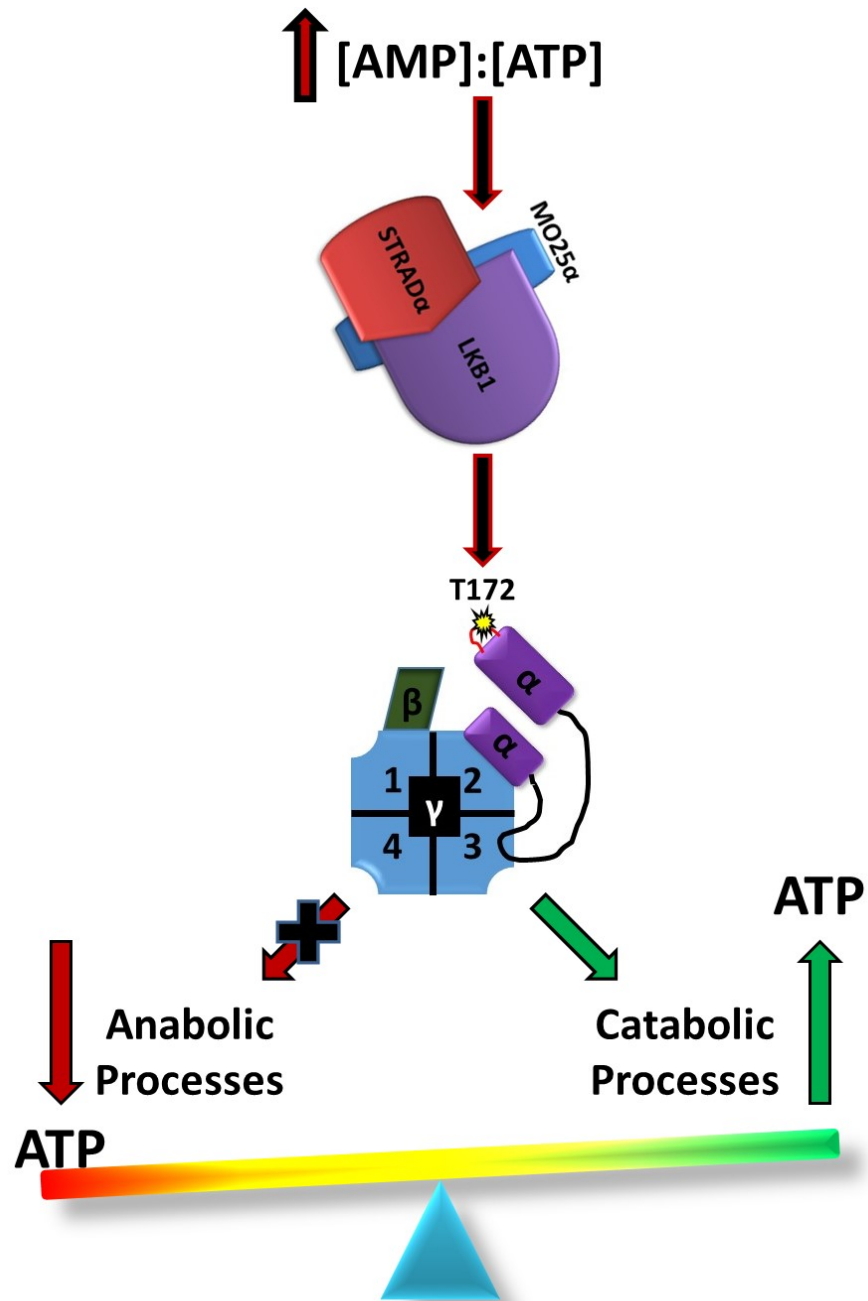


Figure 4. Model of LKB1-dependent regulation of the energy balance of a cell.

In the advent of metabolic stress, LKB1 binds to and activates AMPK by phosphorylating threonine 172. This leads to the activation and inhibition of energy producing and anabolic pathways, respectively.

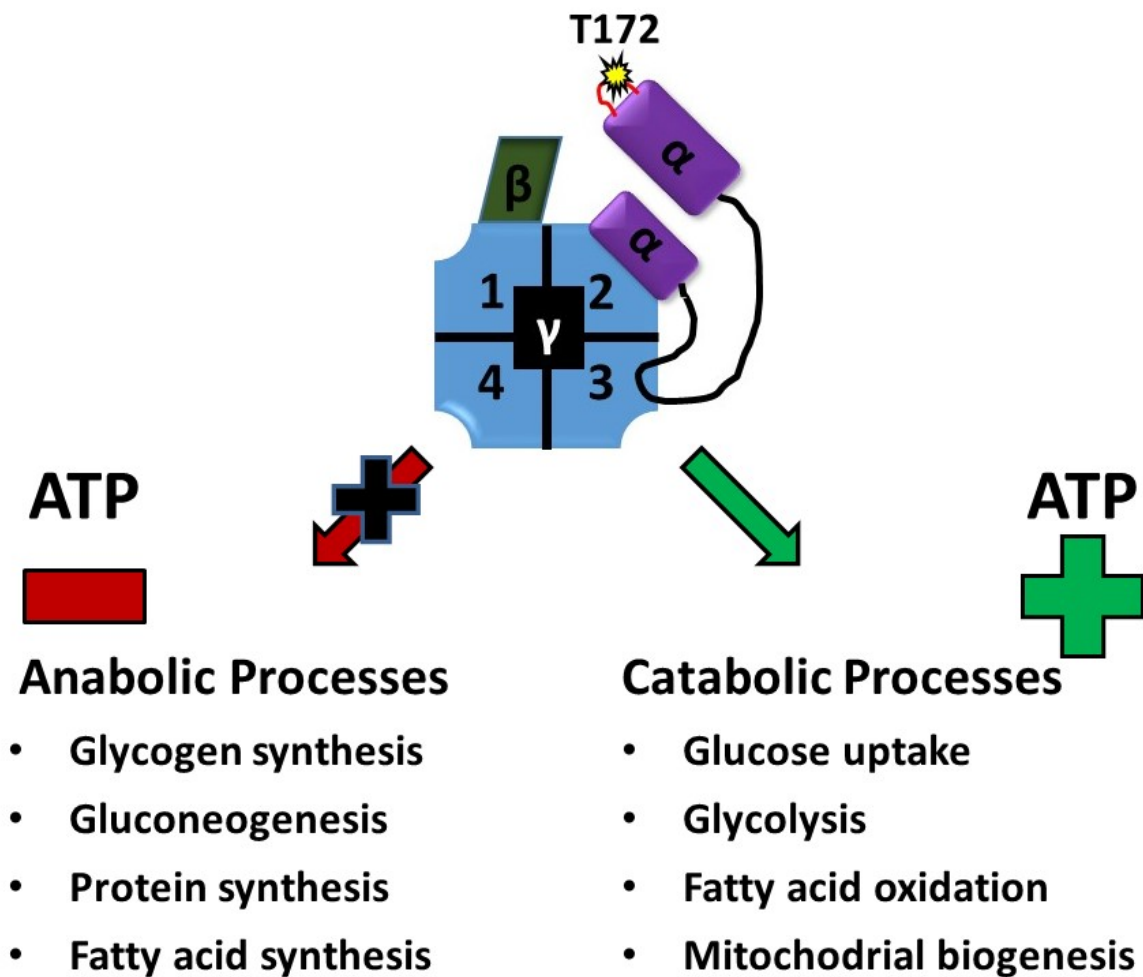


Figure 5. Examples of pathways regulated by AMPK.

To ensure cell survival during energy stress, AMPK activates catabolic processes that generate energy for the cell while curbing pathways that consume energy.

LKB1 post-translational modifications

Several LKB1 post-translational modifications have been described. LKB1 autophosphorylates at threonines 185, 189, 336, and 402 (Figure 6). Moreover, it has been shown that mutations that abrogate or mimic constitutive phosphorylation of these sites by mutating them (alanine or glutamate respectively) has no phenotypic outcome in regards to LKB1 catalytic activity or subcellular localization (Alessi et al., 2006).

Over the years, several studies have identified key kinases that regulate LKB1. For instance, RSK (p90 ribosomal S6 protein kinase) and PKA (cAMP-dependent kinase) phosphorylate LKB1 at serine 428, and mutation of this site to alanine prevented LKB1 from inhibiting cell growth of G361 malignant melanoma cells (Sapkota et al., 2001). In addition, LKB1 is phosphorylated by ATM (ataxia-telangiectasia-mutated) kinase on threonine 363, during DNA damage (Sapkota et al., 2002). Both serine 31 and 325 lie in a phosphorylation consensus sequence and are predicted to be phosphorylated by AMPK/ AMPK related kinases and PDK (proline-directed kinase), respectively. No study as of yet has characterized these two sites.

A study by Collins et al. also demonstrated that LKB1 can be prenylated at cysteine 433 (Figure 6). However, mutation of this site to alanine did not affect the subcellular localization of LKB1, its catalytic activity, or inhibition of cell growth in G361 cells (Collins et al., 2000).

LKB1 is also acetylated on lysine 48 (Figure 6). Deacetylation modulated by SIRT1 increases LKB1 catalytic activity by enhancing its interaction with STRAD subsequently stimulating AMPK activity (Lan et al., 2008). In addition, LKB1 cytoplasmic localization is enhanced when SIRT1 is overexpressed or when lysine 48 is mutated to an arginine, which blocks acetylation (Lan et al., 2008).

SUMOylation has emerged as important post-translational modification that regulates protein function, stability or localization. However, LKB1 SUMOylation has not been described or characterized. This thesis aims to understand the role SUMOylation plays in regulating LKB1 function and ultimately the cellular repercussions of altering LKB1 SUMOylation.

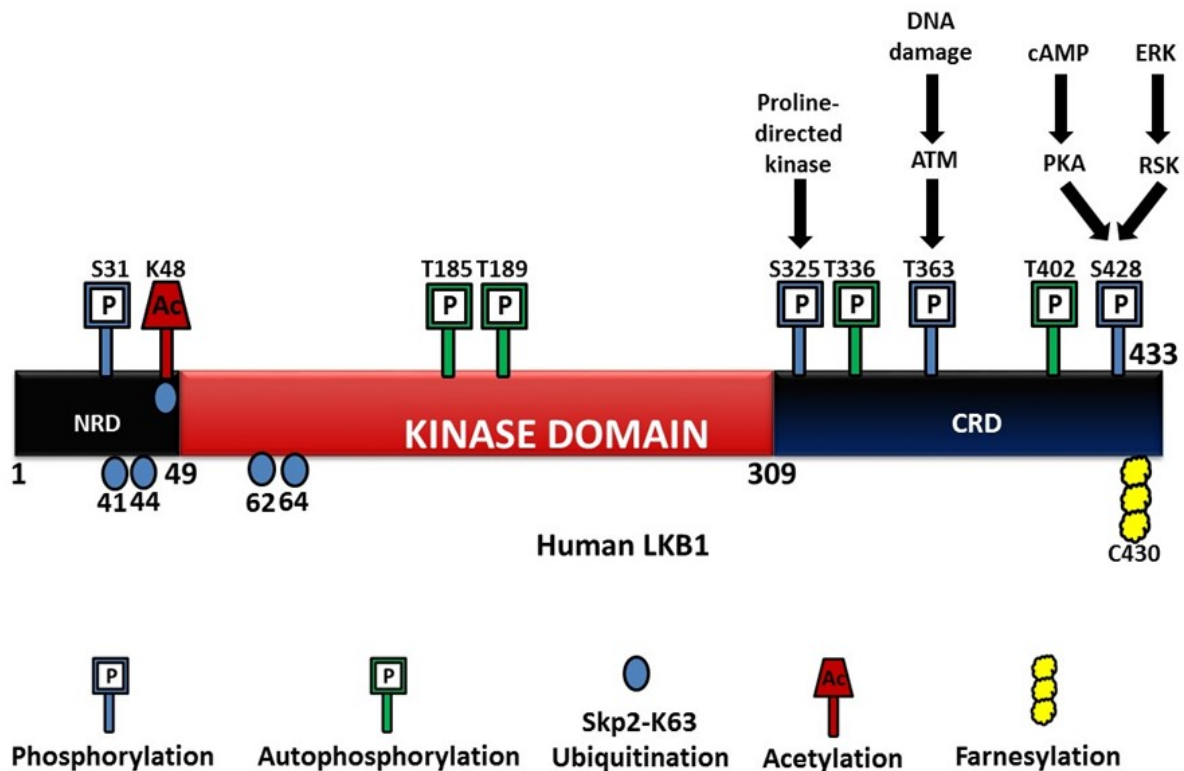


Figure 6. Post-translational modifications of LKB1

A diagram illustrating the post-translational modifications of LKB1 protein. Autophosphorylation sites are shown in green while sites phosphorylated by indicated kinases are depicted in blue. LKB1 is acetylated and farnesylated on lysine 48 and cysteine 430, respectively.

SUMO (small ubiquitin-like modifier)

Around 1996, our lab and several others discovered SUMO protein (Okura et al., 1996, Shen et al., 1996, Boddy et al., 1996, Matunis et al., 1996). It was later shown to bear a striking resemblance to ubiquitin 3-D structure and also the mode of conjugation (Bayer et al., 1998, Haas and Siepmann, 1997). Several functions were attributed to this important protein at the time. Our lab discovered SUMO (or sentrin) bound FAS/Apo thereby protecting the cell from programmed cell death (Okura et al., 1996). The other groups found it to bind RAD51/52 (nucleoprotein filament proteins) (Shen et al., 1996) and also PML which is important in the formation of PML nuclear bodies (Boddy et al., 1996). It was a study by Matunis et al. that originally emphasized and characterized SUMO's role as a reversible covalent protein modifier (Matunis et al., 1996).

Four SUMO proteins exist in mammals: SUMO-1 (also identified as sentrin, PIC1, UBL1, GMP1, or Smt3c), SUMO-2 (also identified as sentrin 2, GMP related protein, or Smt3b), SUMO-3 (also identified as sentrin 3 or Smt3a), and SUMO-4. SUMO-2 and SUMO-3 proteins are ~97% homologous to each other and are thereby referred to as SUMO2/3 (Wilkinson and Henley, 2010). SUMO-1 on the other hand, shares a ~50% semblance in amino acid sequence to SUMO2/3 (Wilkinson and Henley, 2010). SUMO4, which has been proposed to be a pseudogene since it lacks introns, is ~87% homologous to SUMO2/3 (Bohren et al., 2004).

SUMO proteins are expressed as immature precursors. A di-glycine motif is embedded in the C-terminal region of all SUMO proteins that must be exposed for them to conjugate to the specific lysines of their targeted substrates. A family of SUMO proteases referred to as SENP (sentrin/SUMO-specific protease) is responsible for this cleavage (Yeh, 2009).

The SUMO conjugation pathway

SUMOylation is analogous to the ubiquitination process, in that both processes require an E1 activating enzyme, E2 conjugating enzyme and an E3 ligase. The mature SUMO protein is first activated by a heterodimer enzyme containing SAE1 (SUMO-activating enzyme E1) and SAE2, which forms a high energy thioester bond between SUMO's glycine residue in the C-terminal and SAE2's cysteine residue (Gong et al., 1999). SUMO protein is then transferred to the active cysteine of the E2 conjugating enzyme UBC9 (ubiquitin-conjugating 9) (Johnson and Blobel, 1997, Gong et al., 1997, Desterro et al., 1997, Schwarz et al., 1998, Saitoh et al., 1998, Lee et al., 1998). The SUMO E3 ligase has the ability to recognize both the intended SUMO substrate and the E2 enzyme loaded with SUMO (Geiss-Friedlander and Melchior, 2007). This thereby allows the E3 ligase to align them in a suitable conformation, as a scaffold protein would, and facilitate an efficient conjugation of the SUMO protein to the specific lysine on the target protein (Figure 7). Studies have further illustrated that SUMO2/3 can form polymeric chains due to the presence of internal lysines (Tatham et al., 2001). SUMO1 does not have this ability although it has been reported that it can cap and terminate SUMO2/3 chains (Matic et al., 2008).

DeSUMOylation

SUMOylation is a dynamic process that is reversible with the help of the same SUMO proteases, SENPs (Figure 7). Hence, this catapults SENPs as important regulators of SUMOylation since they possess a hydrolase activity that facilitates SUMO's maturation via the cleavage and exposure of its C-terminal glycine and secondly, through their isopeptidase activity that aids the cleavage of SUMO proteins off their substrate. Six SENPs exist in the mammalian

system: SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7. Their preference or specificity of SUMO paralogues varies during the deconjugation process (Yeh, 2009). SENP1 and SENP2 have the ability to cleave both SUMO1 and SUMO2/3 from their substrates (Gong et al., 2000, Hang and Dasso, 2002, Zhang et al., 2002). On the other hand, SENP3, SENP5, SENP6, and SENP7 prefer to deconjugate SUMO2/3 chains (Nishida et al., 2000, Di Bacco et al., 2006, Gong and Yeh, 2006, Mukhopadhyay et al., 2006, Shen et al., 2009).

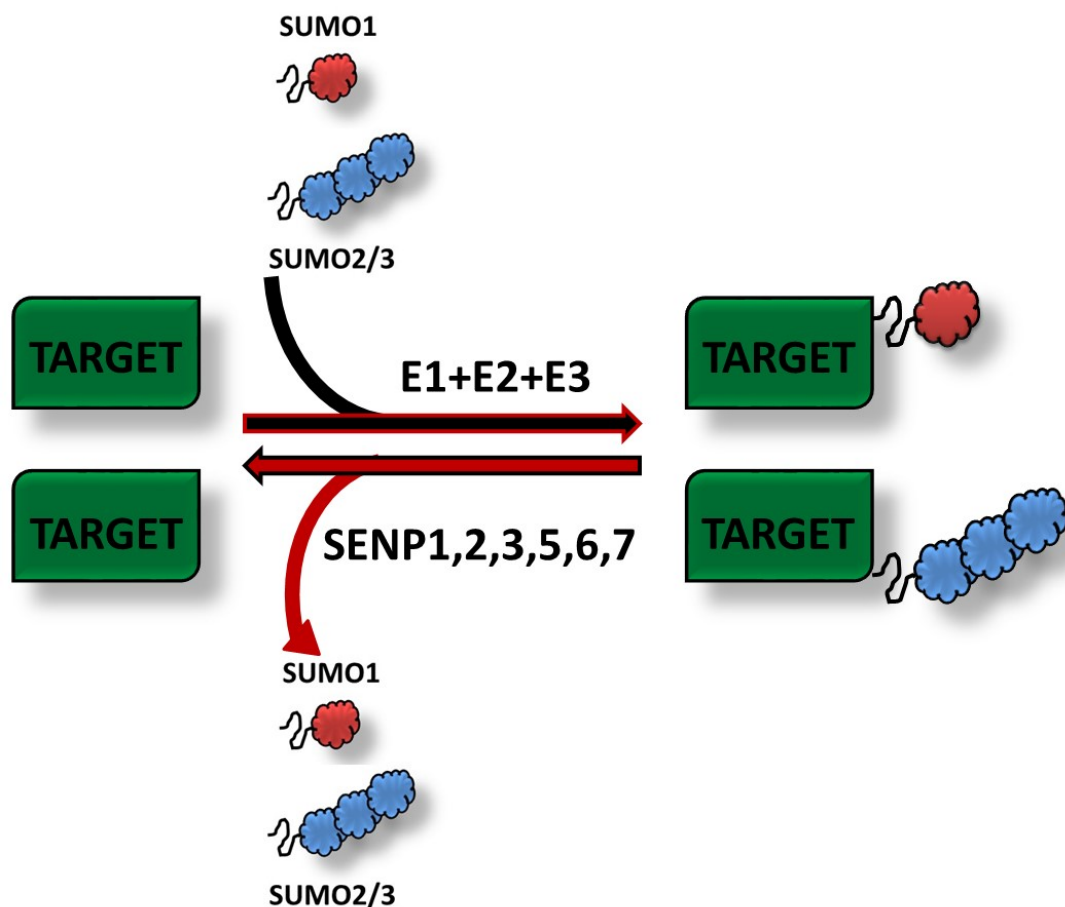


Figure 7. SUMO conjugation pathway.

The covalent conjugation of SUMO1, or SUMO2/3 proteins on a target protein. SUMO proteases, SENPs, facilitate the deSUMOylation /cleavage of the SUMO proteins off the target protein.

SUMO acceptor sites

Different parameters define the lysine site that will be modified by the SUMO protein. The general consensus is that the acceptor lysine lies in the sequence, Ψ KXE, whereby Ψ is a hydrophobic amino acid (isoleucine, leucine, or valine) and X is any amino acid (Xu et al., 2008). Two other modifications to this rule have been proposed. The first one is SUMOylation of a lysine found in the consensus motif that is followed by a phosphorylated serine and a proline residue (Ψ KXEXXpSP) (Hietakangas et al., 2006). This is termed as phosphorylation-dependent SUMOylation. The second SUMO motif proposed involves the presence of negatively charged amino acids after the consensus motif (Yang et al., 2006). Indeed, the common feature in these additional modifications is the presence of a negative charge adjacent to the SUMO acceptor, which studies have illustrated is important in regulating SUMOylation. While the consensus motifs mentioned may determine the SUMOylation status for a majority of substrates, many others do not follow this rule and are modified in a non- consensus manner (Geiss-Friedlander and Melchior, 2007).

Consequences of SUMOylation

SUMOylation has been implicated in regulating protein stability, function and subcellular location. At the molecular level, there are generally three nonexclusive consequences to SUMOylation (Figure 8). First, SUMOylation can mask and inhibit the interaction between partner proteins as observed when SUMOylated CtBP cannot bind its interacting partner, nNos (Lin et al., 2003). Second, SUMOylation can change the conformation of a protein thereby altering its function. This is the case with TDG (thymine DNA glycosylase), which when SUMOylated, changes its conformation consequently inhibiting its interaction with DNA (Baba

et al., 2005). Third, SUMOylation can enhance protein-protein interaction by enabling the non-covalent recognition of a SUMO interacting motif of the target partner. The most classic example of this scenario is the interaction between SUMOylated RanGAP1 and nucleoporin RanBP2 subsequently leading to its translocation from the cytoplasm to the nuclear pore (Matunis et al., 1996, Mahajan et al., 1997).

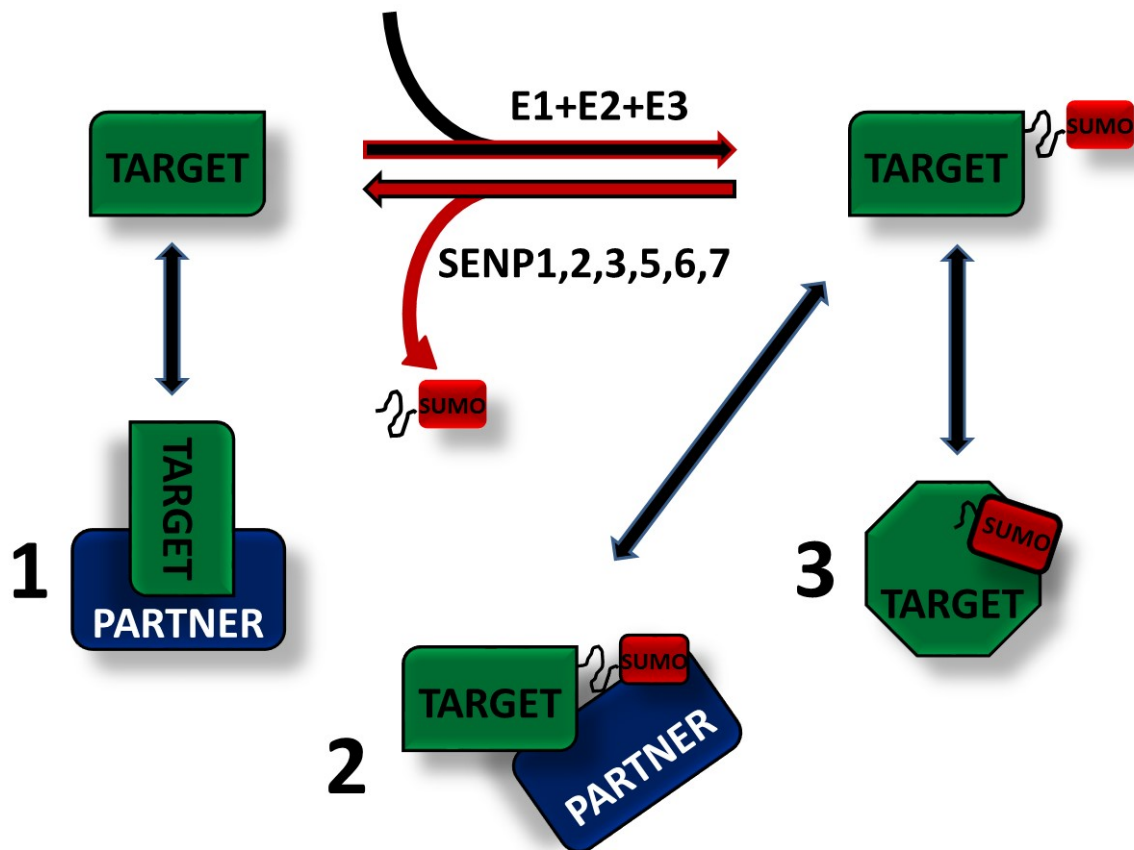


Figure 8. The three general non-exclusive consequences of SUMOylation

SUMOylation can lead to the (1) obstruction of protein-protein interaction, (2) enhancement of protein-protein interaction through the recognition of the partner protein's SUMO interacting motif (SIM), or (3) change in conformation of a target protein.

SUMO Interacting Motifs (SIMs)

As previously discussed, SUMO can non-covalently interact with a specific substrate via a SIM. Several motifs have been described. In 2000, Minty et al. were the first to demonstrate this interaction using a two-hybrid approach that SUMOylated p73 recognized a common sequence in its binding partners, S-x-S (Serine-any amino acid-Serine), which was abutted to acidic amino acids on one side and a hydrophobic core on the other (Minty et al., 2000). Four years later, Song et al reported a largely hydrophobic sequence, V/I-X-V/I-V/I (Valine/Isoleucine - any amino acid - Valine/Isoleucine - Valine/Isoleucine) was important in enhancing the recognition by SUMO (Song et al., 2004). Figure 9 summarizes the current predicted SIM motifs. In addition, other studies have demonstrated that the SIM peptide aligns in the groove of SUMO's α - helix and β -strand and that the mutation of a hydrophobic residue in the motif greatly inhibits its interaction with SUMO (Baba et al., 2005, Hecker et al., 2006). All things considered, it is widely agreed that SIMs are generally characterized by the presence of an indispensable hydrophobic core flanked by a stretch of acidic amino acids (Hannich et al., 2005, Hecker et al., 2006).

Predicted SUMO interacting motifs (SIMs)		
❖	Motif #1: h-h-X-[S]-[X]-[S/T]-a-a-a	(Minty et al)
❖	Motif #2: [V/I]-X-[V/I]-[V/I]	(Song et al)
❖	Motif #3: K-X₃₋₅-[I/V]-[I/L]-[I/L]-X₃-[D/E/Q/N]-[D/E]-[D/E]	(Hannich et al)

Figure 9. Predicted SUMO Interacting Motifs (SIMs)

A figure illustrating the predicted SIMs characterized by the indicated authors

Hypothesis and aims

Hypothesis: Given the diverse roles SUMOylation plays in the eukaryotic cell, we hypothesized that, during energy stress, SUMOylation regulates the LKB1-AMPK interaction and that this accordingly affects AMPK catalytic activity.

Specific Aims:

Specific Aim 1: Characterize the SUMOylation of Liver kinase B1 (LKB1) during energy stress

- 1.1 Examine whether LKB1 is SUMOylated
- 1.2 Investigate whether there are changes in LKB1 SUMOylation during energy stress
- 1.3 Identify the LKB1 lysine residues that are SUMO1 modified during energy stress

Specific Aim 2: Establish the role of SUMOylation in regulating LKB1-AMPK interaction

- 2.1 Examine whether the LKB1 K178R mutant has a decreased affinity to AMPK
- 2.2 Determine that SUMO1 is responsible for the increase in LKB1-AMPK interaction during energy stress
- 2.3 Identify the AMPK SUMO-interacting motif (SIM) that regulates LKB1-AMPK interaction
- 2.4 Evaluate the physiological relevance of the AMPK SIM mutant

Specific Aim 3: Investigate the phenotypic readout when LKB1 SUMOylation is altered during metabolic stress

- 3.1 Examine whether the LKB1 K178R SUMO mutant has a defective AMPK signaling during energy stress
- 3.2 Verify that LKB1 K178 SUMOylation is essential in ensuring effective mitochondrial clearance during metabolic stress
- 3.3 Investigate the oxygen consumption rates as a measure of mitochondrial respiration in LKB1 K178R SUMO mutant-expressing cells

CHAPTER II: Materials and Methods

Cell culture

Non-small-cell lung carcinoma A549 cells, cervical cancer HeLa cells, HEK293 cells, and Phoenix cells were obtained from the American Type Culture Collection. The AMPK α wild-type and knockout MEF cell lines were gifts from Dr. Hui-Kuan Lin (M.D. Anderson Cancer Center, Houston, TX). All cell lines were maintained in DMEM (Thermo Fisher Scientific) containing 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Sigma-Aldrich), and incubated at 37°C at 5% CO₂ levels.

Reagents

Phenformin hydrochloride (P7045-10G), CAMKK inhibitor STO-609-acetic acid (S1318-25MG), puromycin dihydrochloride (P9620-10ML), polybrene (H9268-5G) were purchased from Sigma-Aldrich. Antibodies used for immunoblotting, including LKB1 (D60C5; #3047), phospho-AMPK Thr172 (#2531), total AMPK α 1/2 (#2532), phospho-Raptor Ser792 (#2083), total Raptor (#2280), phospho-ULK1 (S555; #5869), total ULK1 (#8054), MO25 α /CAB39 (C49D8; #2716S) and SUMO1 (#4930), were obtained from Cell Signaling Technology. STRADA (HPA031637-100UL) was purchased from Sigma-Aldrich. Actin (sc-47778) and AMPK α 1 (ab32047) antibodies were obtained from Santa Cruz Biotechnology, Inc., and Abcam, respectively. GST-AMPK alpha 1 recombinant protein (H00005562-P01) was obtained from Abnova. His6-SEN1 Catalytic Domain recombinant protein (E-700) was purchased from Boston Biochem. LKB1/MO25/STRAD Active human recombinant protein (SRP0246-20UG) was obtained from Sigma-Aldrich.

Plasmids

pBabe-FLAG-LKB1 (#8592), pBabe-FLAG-LKB1-KD (#8593), and pcDNA3-FLAG-LKB1 (#8590) were obtained from Addgene. The LKB1 mutants were generated by using QuikChange II XL Site-Directed Mutagenesis Kit (#200522) purchased from Agilent. HA-SUMO1, HA-SUMO2, Flag-SENP1, and Flag-SENP1 catalytic mutant plasmids were generated in our laboratory. Myc-AMPK and catalytic mutant Myc-AMPK (D157A) were gifts from Dr David Carling (Imperial College London), and pBabe empty vector was a gift from Dr. Hui-Kuan Lin (M.D. Anderson Cancer Center, Houston, TX).

RNA interference

Nontargeting siRNA (ON-TARGETplus #2, catalog number D-001810-02-20) and SENP1 siRNA (ON-TARGETplus SMARTpool Human SENP1, catalog number L-006357-00-0010) were purchased from Dharmacon RNAi Technologies. LKB1 siRNA (CCAACGUGAAGAAGGAAAUUC) were designed and obtained from SigmaAldrich. Cells were grown to ~40% confluency and 5 μ M siRNA was transfected into cells using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Cells were harvested after 48 hours for further analysis.

Viral infection

For retroviral infection, Phoenix cells were transfected with the plasmids encoding pBabe empty vector, pBabe-FLAG-LKB1, pBabe-FLAG-LKB1 (K178R), or pBabe-FLAG-LKB1-KD. Forty-eight hours after transfection, the viral supernatants were collected, spun for 10 minutes at 1500 rpm, filtered, and infected into A549 cells in the presence of 4 μ g/mL polybrene. After 48 hours, cells were selected for another 48 hours with puromycin (1.5 μ g/mL). To induce metabolic

stress, cells were treated with 2 mM phenformin and 10 µg/mL CAMKK inhibitor STO609 (both, Sigma-Aldrich) for 6 hours unless otherwise indicated.

Western blot analysis

Cells were harvested, washed twice with cold PBS, and subjected to lysis in a buffer containing 20 mM Tris-HCl at pH 7.5, 0.5% NP-40, 150 mM NaCl, and protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined via Bradford Protein Assay (Bio-Rad). 50µg of the protein samples were heated in 1X SDS-PAGE sample buffer at 95 °C for 5 minutes and loaded onto 4-15% Mini-PROTEAN® TGX™ precast gels (Bio-Rad). The protein samples were separated via electrophoresis at a constant voltage of 90v in 1X Tris-glycine running buffer for around an hour and 30 minutes. The separated protein samples were transferred to polyvinylidene fluoride (PVDF) membrane at a constant voltage of 50v for 2 hours. Membranes were blocked with 5% Bovine serum albumin (BSA) when detecting phosphoproteins or 5% non-fat dry milk diluted in 1X TBS-T (1% Tween) for 1 hour at room temperature. Membranes were probed overnight at 4°C with respective primary antibodies at indicated dilutions: LKB1 (1:1000), phospho-AMPK Thr172 (1:1000), total AMPK α 1/2 (1:1000), phospho-Raptor Ser792 (1:1000), total Raptor (1:1000), phospho-ULK1 (1:1000), total ULK1 (1:1000), SUMO1 (1:1000), Actin (1:3000), AMPK α 1 (1:1000), STRADA (1:1000), and MO25 (1:1000). Membranes were briefly washed with TBS-T (1%Tween) and incubated for an hour with appropriate secondary antibodies (Santa Cruz 1:5000), diluted in 2% non-fat milk in TBS-T. Membranes were then washed 5 times for 5 minutes, respectively using TBS-T. Chemiluminescent detection was then performed using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) and HyBlot CL autoradiography film (Denville Scientific Inc.).

Immunoprecipitation analysis

Cells were harvested and subjected to lysis in a buffer containing 20 mM Tris-HCl at pH 7.5, 0.5% NP-40, 150 mM NaCl, and protease inhibitors. The extracts were then incubated with the appropriate antibody overnight at 4°C and subsequently with the respective agarose beads for an additional 2 hours. After the beads were washed three times with lysis buffer, the immunoprecipitates were heated in 2× Laemmli sample buffer, cooled, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were analyzed by the standard western blot described above.

***In vitro* kinase assay**

Purified LKB1 (wild-type LKB1 or LKB1 SUMO mutant [K178R]) were suspended in 50 µL of 1× Kinase Buffer (Cell Signaling Technology #9802) supplemented with 200 µM ATP (Cell Signaling Technology #9804) and incubated for 30 minutes at 30°C. The reaction was terminated with 50 µL 2× SDS sample buffer. The samples were heated in SDS-PAGE sample buffer for 5 minutes at 95°C. The samples were then loaded on an SDS-PAGE gel. A standard western blot was conducted and the membrane probed with the indicated antibodies.

***In vitro* LKB1 SUMOylation assay**

250ng of recombinant LKB1 protein was incubated with E1 activating enzyme (SAE1/SAE2), E2 conjugating enzyme (ubc9), and in the presence SUMO1 or SUMO1 mutant protein according to the manufacturer's guidelines (Active Motif). The reactions were incubated at 30°C

for 3 hours. The samples were heated in 2× Laemmli sample buffer, cooled, and subjected to a standard western blot as described above.

SIM competitive assay

GST-AMPK (Abnova-H00005562-P01; 150 ng) and a SIM peptide were added sequentially to 400 µL of lysis buffer. A sample without added peptide was included. SUMO-1 agarose beads (40 µL) were added to each tube. Samples were incubated and rotated at 4°C for 2 hours. After the beads were washed three times with lysis buffer, the immunoprecipitates were heated in SDS-PAGE sample buffer at 95°C for 5 minutes and subjected to standard western blot. Membranes were probed for SUMO1 and GST.

Peptide pull-down assay

HEK293 cells were harvested and lysed in a buffer containing 20 mM Tris-HCl at pH 7.5, 0.5% NP-40, 150 mM NaCl and protease inhibitors. The biotinylated wild-type or mutant AMPK SIM peptides were conjugated to avidin bead to make a resin. 40ul of the peptide-bound resin were added to each tube. As a negative control, unconjugated avidin beads were used in the experiment. Samples were incubated and rotated at 4 °C for 2 hours. After washing three times with lysis buffer, the beads were heated in SDS-PAGE sample buffer at 95 °C for 5 minutes. A standard western blot was conducted afterwards and membranes were probed for LKB1

Quantitative real-time polymerase chain reaction

RNA was extracted from cells by using the RNeasy Mini Kit (Qiagen #74104) and reverse-transcribed by using the iScript cDNA synthesis kit (Bio-Rad #170-8891). For SENP1 transcript

change analysis, quantitative real-time polymerase chain reaction was conducted in triplicate by using TaqMan Gene Expression Master Mix (Invitrogen #4369016) and SENP1 TaqMan Gene Expression Assay (Invitrogen #Hs01060367_m1) per the manufacturer's instructions.

Intracellular ATP measurement

Cells were harvested and washed twice with cold PBS. Their intracellular ATP levels were quantified using abcam's ATP Assay Kit (Colorimetric/Fluorometric) (ab83355) per manufacturer's instructions.

XF bioenergetic assay

Cellular oxygen consumption rates were obtained with a Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience). Experiments were performed as previously described (Wu et al., 2007). The data were analyzed by using Graphpad Prism 6 (GraphPad Software) and are expressed as means and S.E.M. for at least three independent experiments.

Flow cytometry

To quantify mitochondrial content, cells were harvested and stained with MitoTracker Red CMXRos dye (Invitrogen V35116). To detect apoptosis in the treated cells, we used annexinV Alexa Fluor 488 (Invitrogen V35116) and 7-AAD (BD Pharmingen #559925), following the manufacturer's instructions. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). The apoptosis and MitoTracker Red data were analyzed by FlowJo VX (FlowJo Software) and FCS Express 4 Flow software (DeNovo Software), respectively.

CHAPTER III: LKB1 is SUMOylated

Introduction

SUMOylation is an important regulatory post-translational modification implicated in a wide number of processes in the eukaryotic cell including metabolism (Agbor and Taylor, 2008, Flotho and Melchior, 2013). However, the SUMOylation status of LKB1, a key protein involved in maintaining the cell's energy balance, has not been established. Therefore, it was vital that we determine that LKB1 is in fact SUMOylated. The following experiments were designed and conducted in this regard:

1. Knockdown or overexpression of a deSUMOylating enzyme

Approach: As reviewed in chapter 1, SENP1 can cleave both SUMO1 and SUMO2/3 off a substrate protein. We therefore decreased or increased SENP1 expression levels in HEK293 cells using SENP1-specific siRNA or plasmid encoding FLAG-SENP1 or catalytic mutant FLAG-SENP1, respectively.

Results: We noted an increase in modification of LKB1 by SUMO1 in HEK293 cells in which endogenous SENP1 was knocked down by SENP1-specific siRNA and conversely decreased in SENP1-overexpressing cells (Figures 10 and 11). We observed a similar decrease in SUMO2/3 levels (Figure 11), but knocking down SENP1 did not alter the conjugation of LKB1 by SUMO2/3 indicating there may be a compensatory effect by other SUMO2/3-specific deSUMOylases (SENP3, 5, 6, or 7).

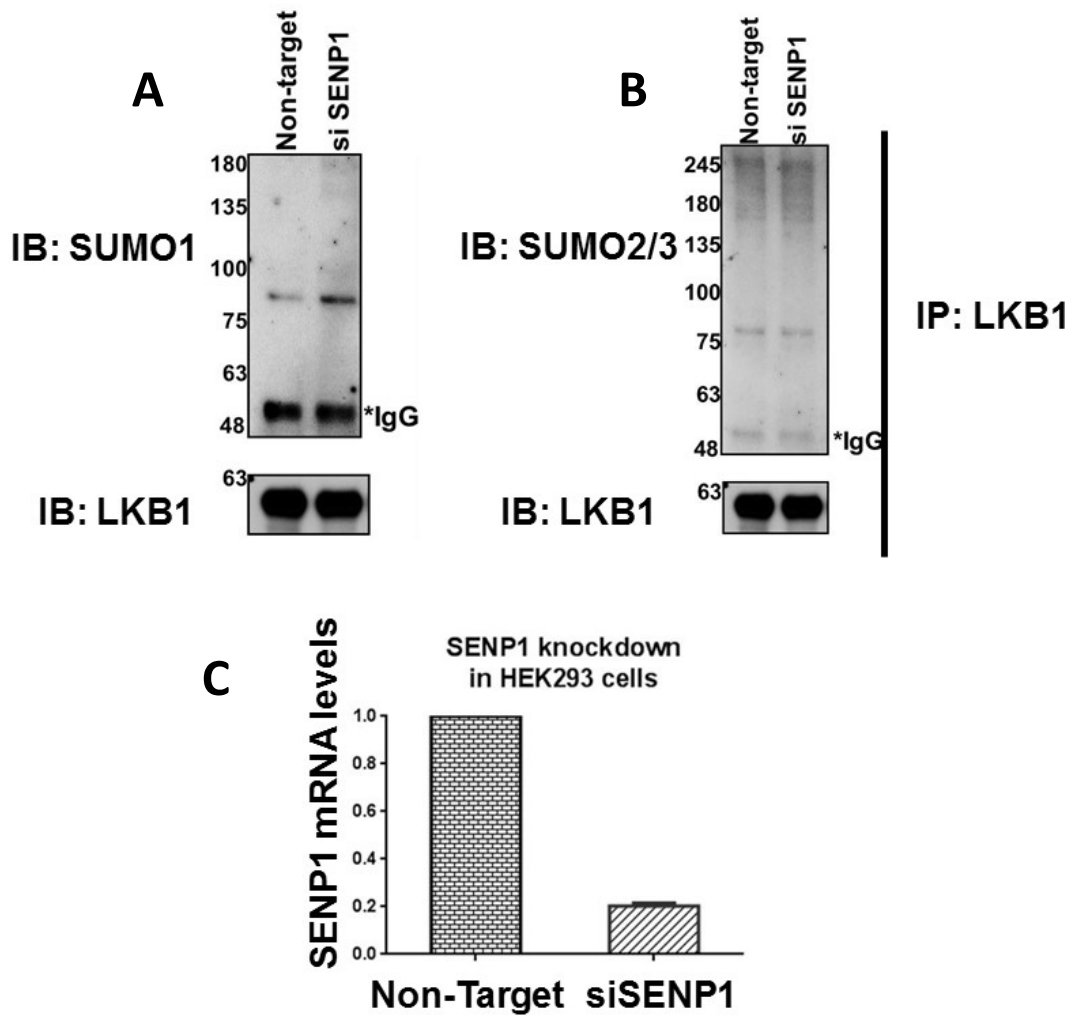


Figure 10. Endogenous SUMOylation of LKB1 in HEK293 cells. (A, B) SENP1 expression level was knocked down by SENP1-specific siRNA in HEK293 cells. LKB1 immunoprecipitates were subjected to western blot using the indicated antibodies. (C) Messenger RNA levels of SENP1 were measured by quantitative real-time polymerase chain reaction in triplicate. Data are presented as the mean \pm S.D. (error bars).

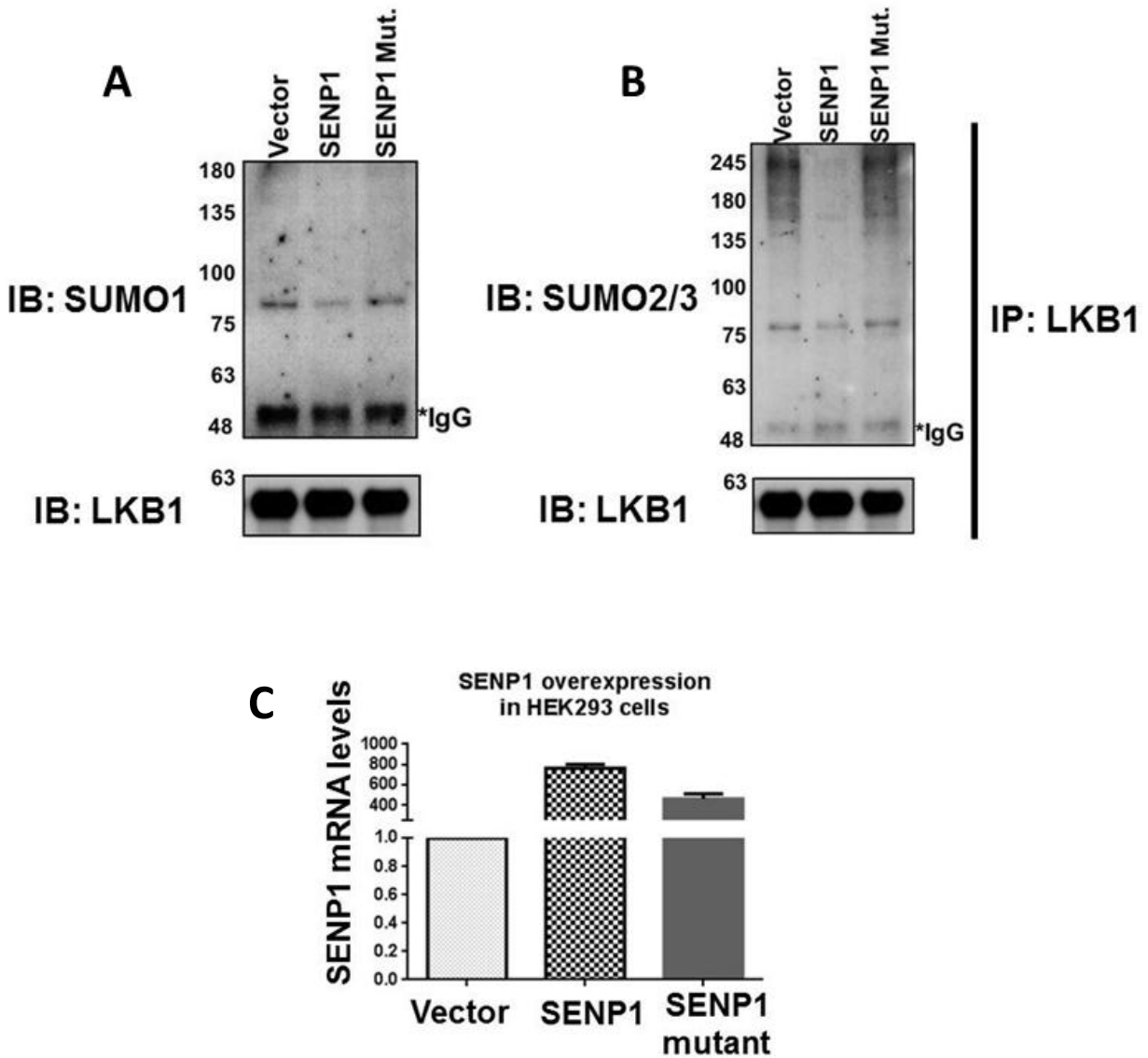


Figure 11. SUMO protease, SENP1, decreases endogenous LKB1 SUMOylation

(A, B) FLAG SENP1 or its catalytic mutant was transiently expressed. Immunoprecipitates were subjected to western blot using the indicated antibodies. (C) Messenger RNA levels of SENP1 were measured by quantitative real-time polymerase chain reaction in triplicate. Data are presented as the mean \pm S.D. (error bars).

2. Overexpression of SUMO1 or SUMO2/3

Approach: We utilized an overexpression system to further validate the SUMOylation of LKB1. HA-tagged SUMO1 or HA-tagged SUMO2 was overexpressed together with LKB1 cDNA in LKB1-null HeLa cells. In another set of experiments, we included SUMO protease SENP1 to conclusively illustrate that LKB1 is SUMOylated.

Results: Our data demonstrates that LKB1 is modified by both SUMO1 monomeric and SUMO2/3 polymeric chains (Figure 12). This observation was additionally validated when we increased exposure time of blots shown in Figure 13 and detected the SUMO1 monomeric modification of LKB1. This SUMO1 modification diminished in the presence of SENP1.

3. *In vitro* SUMOylation of LKB1

We also demonstrated that LKB1 is a potential SUMOylation substrate by utilizing a commercially available *in vitro* SUMOylation kit. Recombinant LKB1 protein was incubated with E1 activating enzyme (SAE1/SAE2), E2 conjugating enzyme (UBC9), and in the presence SUMO1 or SUMO1 mutant protein. As a control, we also included recombinant SENP1 in the assay that had wild type SUMO1 protein.

Results: We observed that LKB1 is SUMOylated when wild type SUMO1 protein was included. In addition, the SUMO protease SENP1 diminished the SUMO1 modification further validating our claim that LKB1 is SUMOylated (Figure 14).

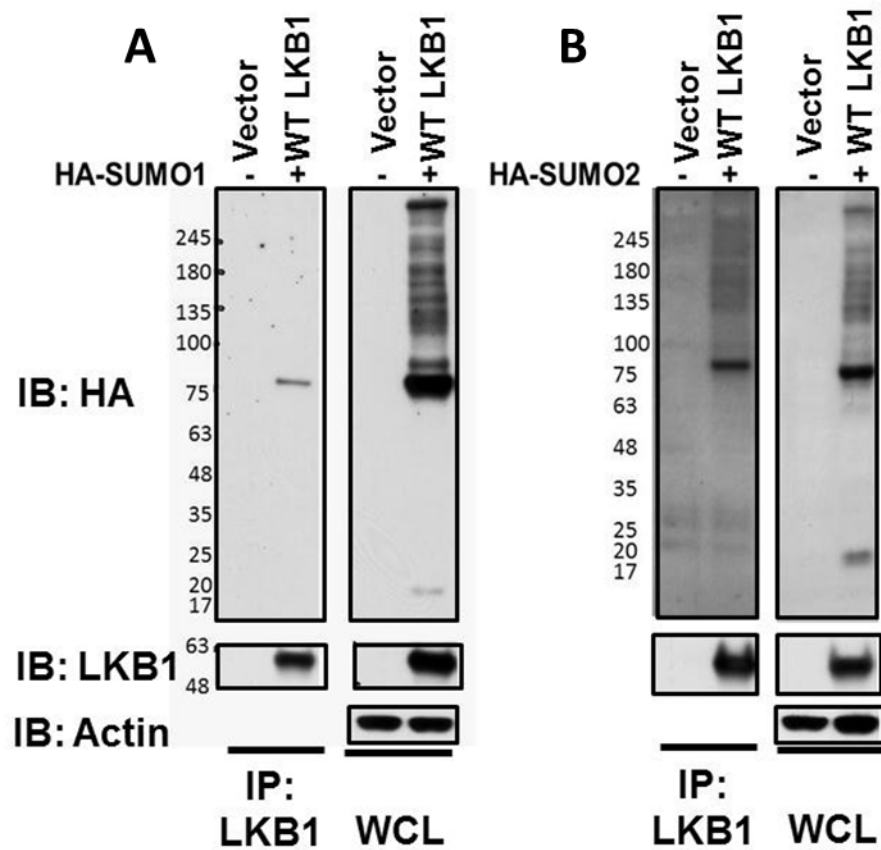


Figure 12. Exogenous SUMO1 and SUMO2/3 modification of LKB1

SUMOylation of LKB1 via co-expression of HA-SUMO1 (A) or HA-SUMO2/3 (B) and LKB1 cDNA in HeLa cells (LKB1 null). LKB1 was immunoprecipitated from these cells and the immunoprecipitates were subjected to western blot using the indicated antibodies.

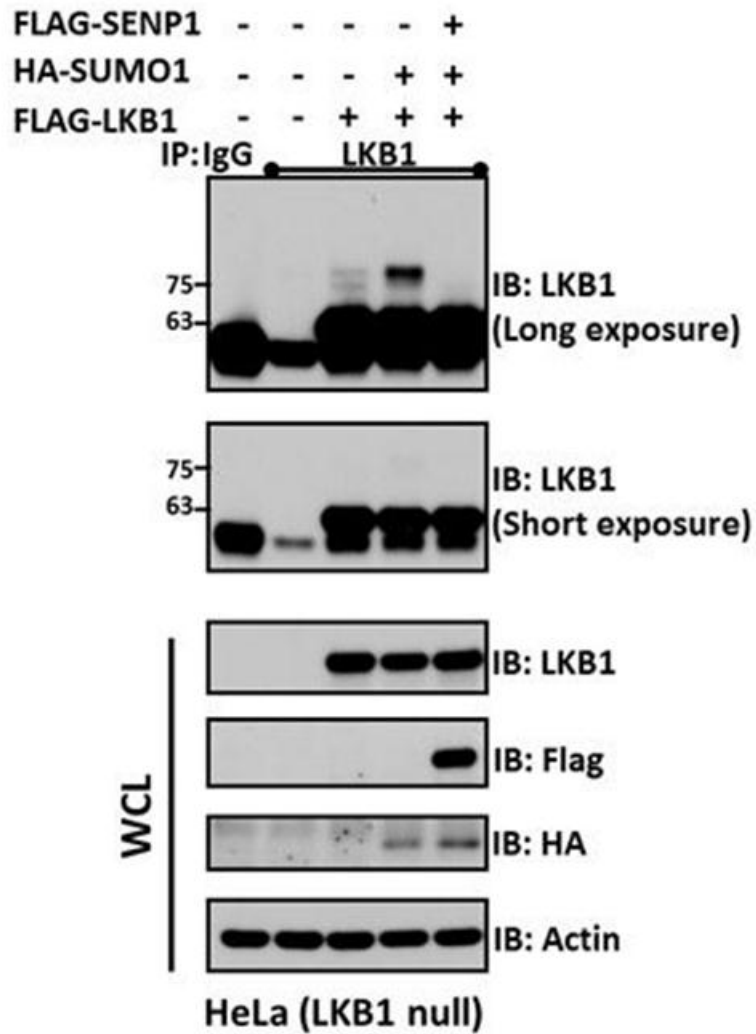


Figure 13. SENP1 decreases exogenous LKB1 SUMO1 modification

SUMOylation of LKB1 via co-expression of HA-SUMO1 and LKB1 cDNA with or without FLAG SENP1 in HeLa cells (LKB1 null). LKB1 was immunoprecipitated from these cells and the immunoprecipitates were subjected to western blot using the indicated antibodies.

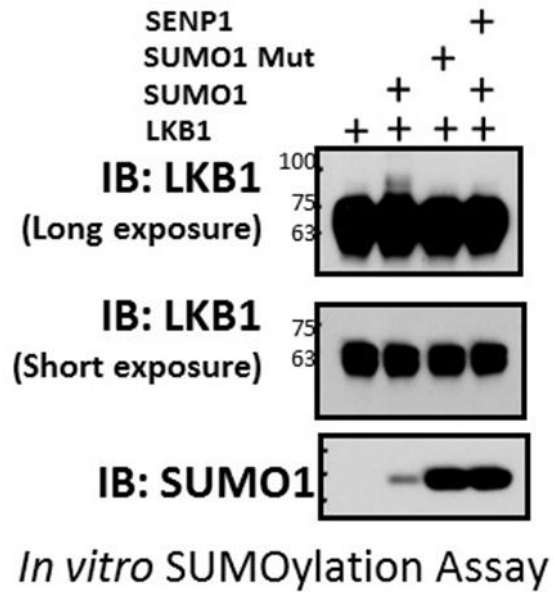


Figure 14. *In vitro* SUMO1 modification of LKB1

In vitro SUMOylation of recombinant LKB1 in the presence of wild type SUMO1 or SUMO1 mutant recombinant protein. Recombinant SENP1 was included as control.

Conclusion

Our study demonstrates for the first time that LKB1 is indeed SUMOylated. Endogenous studies revealed that LKB1 is both SUMO1 and SUMO2/3 modified (Figures 10 and 11). Additionally, we corroborated this observation by using an overexpression system and also via *in vitro* SUMOylation (Figures 12-14). Therefore, we have laid the important foundation upon which we can further characterize and understand the role SUMOylation plays in regulating LKB1 function.

CHAPTER IV: SUMO1 modification of LKB1 is upregulated during energy stress

Introduction

SUMOylation has been implicated in the response of cellular metabolic changes and homeostasis (Agbor and Taylor, 2008, Wilkinson and Henley, 2010, Tempe et al., 2008). However, insights into its fundamental role in regulating metabolism are still in its nascent stages. Therefore, considering the focus of my project, key questions arose:

1. What happens to the SUMOylation status of LKB1 during energy stress?
2. Are there global SUMOylation changes that occur in a cell during metabolic stress?
3. What are the roles of SUMO proteases during energy stress?
4. What are the consequences of deregulating SUMOylation during energy stress?

1. LKB1 SUMOylation status during metabolic stress

SUMOylation has been implicated in cellular stress adaptation (Wilkinson and Henley, 2010). Since LKB1 is a critical player involved in regulating the cell's energy balance when ATP levels decline, we questioned whether SUMOylation may be involved in regulating its function during metabolic stress. We therefore investigated whether the SUMOylation status of LKB1 changed upon metabolic stress.

Approach:

- Energy stress was induced in HEK293 (expressing empty vector or FLAG SENP1) through glucose restriction, treatment of cells using 2-DG (a glycolysis inhibitor) or treatment with phenformin (a biguanide compound that inhibits complex 1 of the mitochondria). CAMKK inhibitor STO 609 was included in the treatment regimen since CAMKK β can compensate for LKB1 by phosphorylating AMPK on the same site

(Woods et al., 2005). We also concurrently examined the intracellular ATP levels of these cells subjected to the various metabolic stress agents. LKB1 was immunoprecipitated from the cells and its SUMOylation status was assessed.

- To broaden the physiological relevance of our study, we also examined the SUMOylation status of LKB1 in a mouse myoblast cell line, C2C12, during stress. C2C12 cells actively depend on AMPK to regulate energy stress and were thus a good platform that would further validate the observations made from HEK293 cells.
- We also knocked down LKB1 in HEK293 cells as a control experiment to confirm the LKB1 SUMOylation changes that occur during stress.

Results: Interestingly, we observed that SUMO1-modified LKB1 levels increased as the intracellular ATP levels declined (Figure 15). As a control, we also noted that immunoprecipitates from cells transfected with FLAG-SENP1 exhibited a diminished expression of the modification. On the other hand, there was a decrease in LKB1 conjugation by SUMO2/3 when the cells were subjected to energetic stress by phenformin (Figure 16). We also noted an increase in LKB1 SUMO1 modification when C2C12 cells were subjected to phenformin treatment (Figure 17). Our observations were further validated when we observed a marked reduction of the energy stress–induced SUMO1 modification of LKB1 in HEK293 cells in which LKB1 was knocked down by a specific siRNA (Figure 18). This control experiment illustrated that the bands shown are indeed LKB1 and they diminish when LKB1 siRNA is introduced in these cells. These results demonstrate that, during stress, the SUMO1 modification of LKB1 might be important in maintaining the energy homeostasis in a cell.

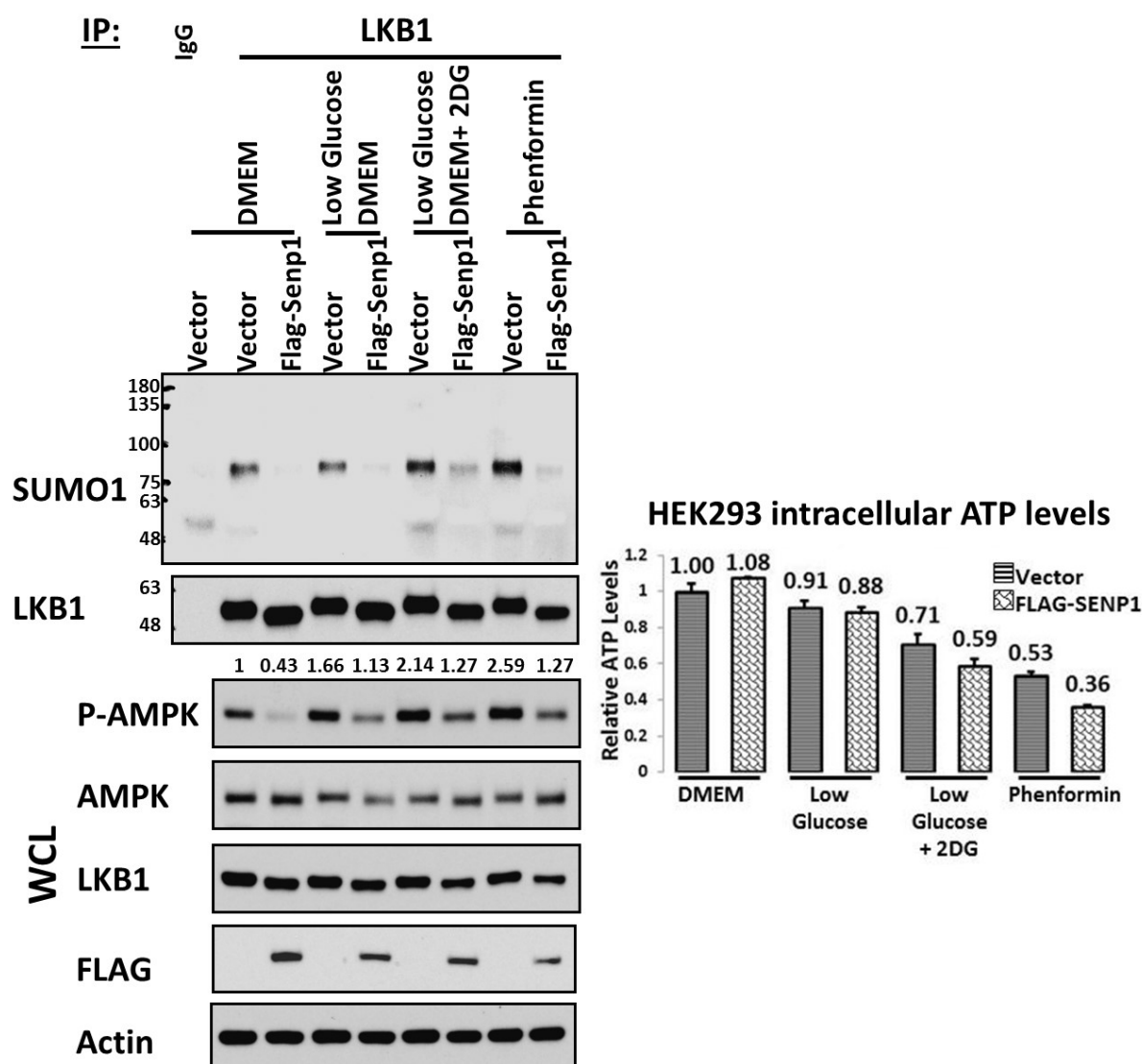


Figure 15. LKB1 SUMOylation changes that occur when HEK293 cells are subjected to various metabolic stress agents. HEK293 cells (expressing empty vector or FLAG SENP1) were subjected to energy stress for 6 hours through low glucose conditions, low glucose together with glycolysis inhibitor 20 mM 2-DG, or treatment with 5 mM phenformin. 10 μ g/mL CAMKK inhibitor STO 609 was included in the treatment regimen. LKB1 was immunoprecipitated and its SUMO1 conjugation levels were assessed. Whole cell lysates were subjected to western blot using the indicated antibodies. In addition, intracellular ATP levels were evaluated these cells.

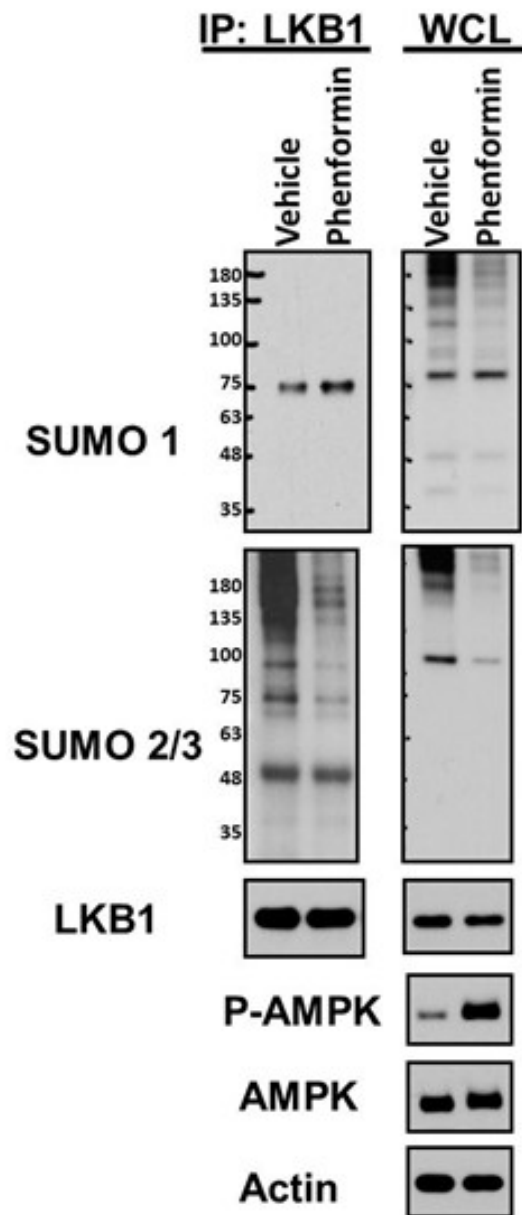


Figure 16. LKB1 SUMOylation changes that occur in HEK293 cell during energy stress.

HEK293 cells were treated with vehicle (10 μ g/mL STO609) or STO609 together with 5 mM phenformin/ 20mM 2-DG for 6 hours. LKB1 was immunoprecipitated and their SUMO1 or SUMO2/3 conjugation levels were assessed.

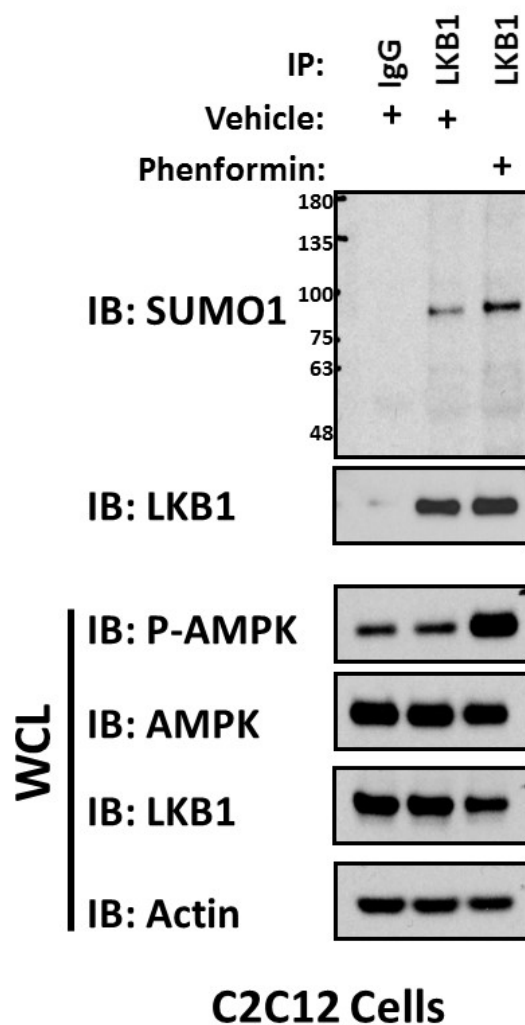


Figure 17. LKB1 SUMOylation changes that occur in mouse myoblast cell line, C2C12, during energy stress. C2C12 were treated with vehicle (10 µg/mL STO609) or STO609 together with 5 mM phenformin for 6 hours. LKB1 was immunoprecipitated and its SUMO1 conjugation levels were assessed.

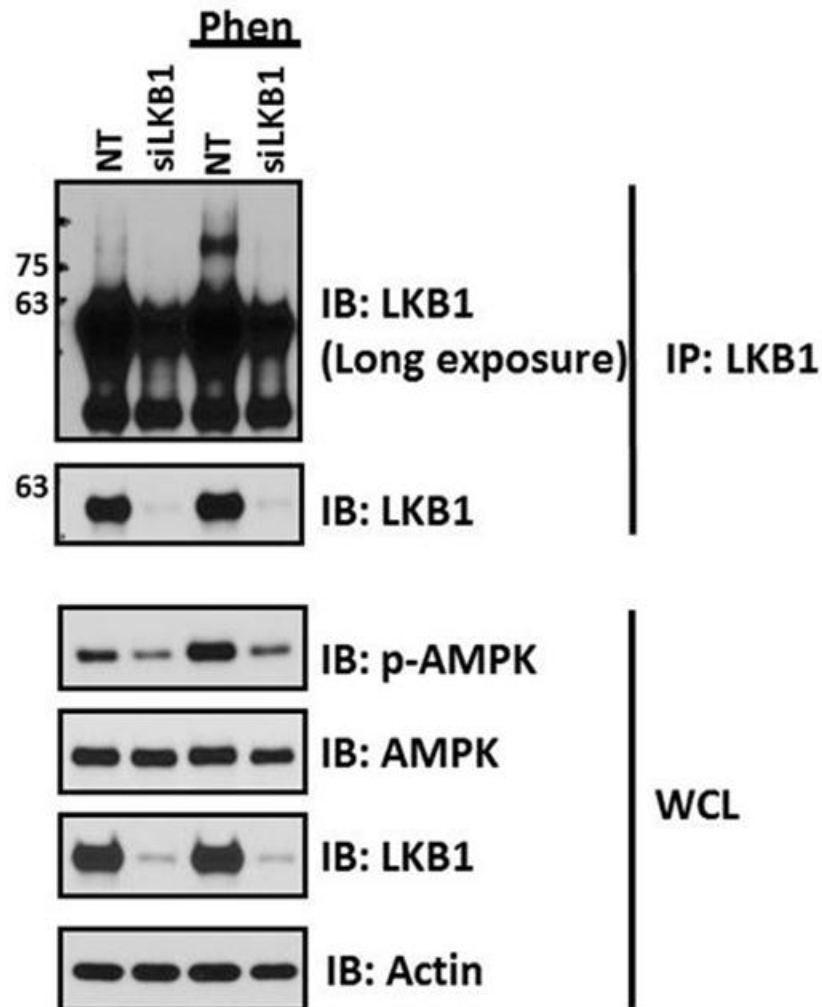


Figure 18. Validation of LKB1 SUMOylation via the siRNA knockdown of LKB1

HEK293 cells expressing either non-targeting or LKB1-specific siRNA were treated with vehicle (10 μ g/mL CAMKK inhibitor STO609) alone or with 5 mM phenformin for 6 hours. LKB1 was immunoprecipitated and its SUMO1 conjugation was assessed.

2. Global SUMOylation changes that occur in a cell during energy stress

We also investigated the global SUMOylation changes that occur in a cell during energy stress. We conducted a time course experiment where we simultaneously treated HEK293 cells with phenformin and CAMKK inhibitor STO 609 for 30 minutes, 1 hour and two hours.

Results: We observed that energy depletion led to a global decrease in both SUMO1- and SUMO2/3-conjugated proteins (Figure 19 A and B). This phenotype was reversed when MG132, a proteasome inhibitor, was added to the treatment regimen (Figure 19B). No changes were observed when the energy stressed cells were treated with chloroquine, an agent that inhibits autophagy. These data imply that the proteasome-dependent degradation of proteins, in general, is a downstream consequence of metabolic stress, and this may be vital in ensuring that the cell meets its energy demands. The fact that we observe an increase in SUMO-1 modified LKB1 while there's a global decrease in both SUMO1- and SUMO2/3-conjugated proteins, speaks to the specificity of this modification during energy stress. This therefore provides a theoretical consideration that SUMO-1 modification of LKB1 is important in AMPK activation during energy stress.

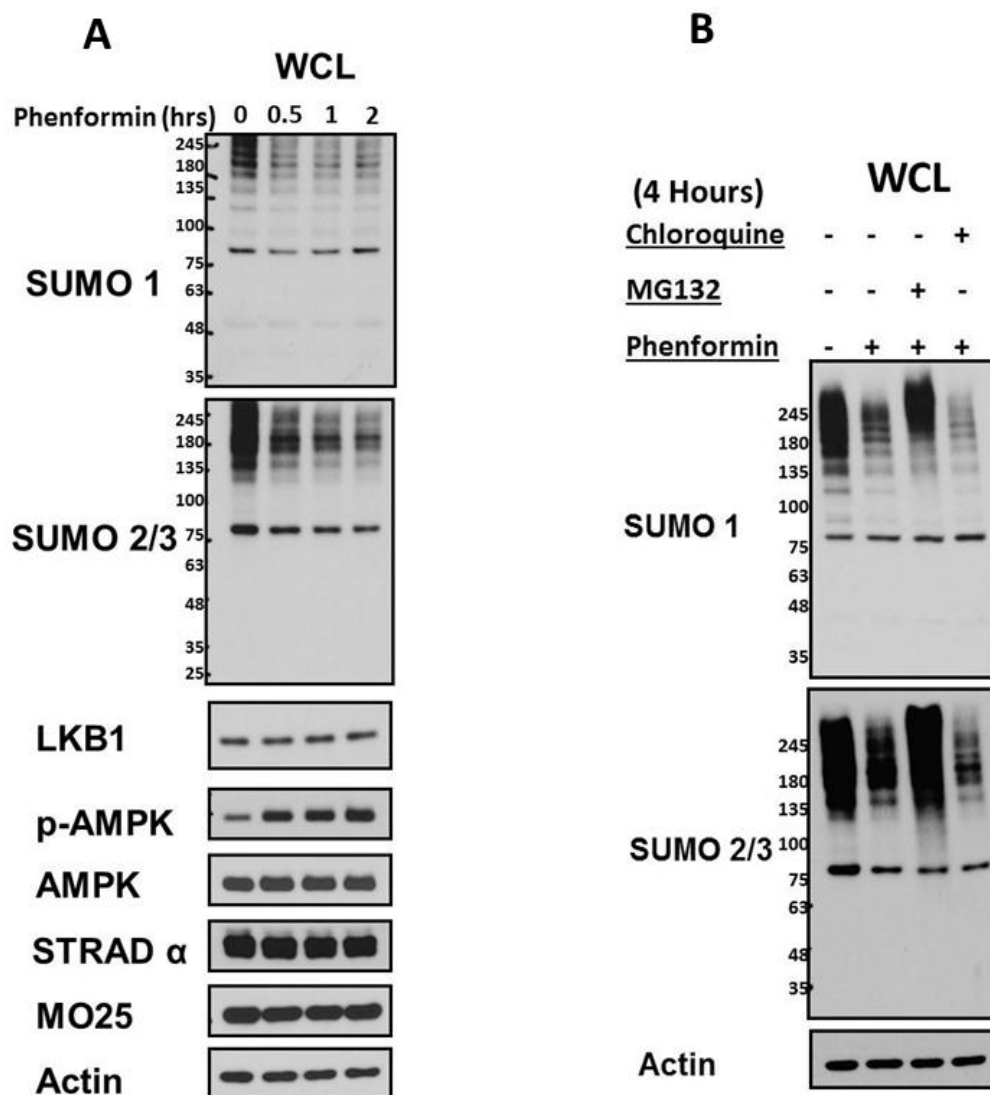


Figure 19. Global SUMOylation changes that occur in a cell during metabolic stress.

(A) HEK293 cells were treated with CAMKK inhibitor (10 μ M ST0609) together with 5 mM phenformin for the indicated periods of time. (B) HEK293 cells were treated with 5 mM phenformin, 10 μ M MG132, and/or 100 μ M chloroquine, as indicated, for 4 hours. Whole-cell lysates were subjected to western blot using the indicated antibodies.

3. The role of SUMOylation during energy stress

Since we observed that SUMO1 might have a role in regulating LKB1 function, we then looked into the consequences of deregulating the SUMOylation process, by introducing SUMO protease SENP1, during metabolic stress.

Approach: The phosphorylation and activation of AMPK is important in maintaining energy homeostasis during metabolic stress. We examined whether overexpressing SUMO protease SENP1 would have any effect in regulating the phosphorylation/activation status of AMPK. We overexpressed empty vector (pcDNA3) or SENP1 in HEK293 cells and treated the cell cultures with vehicle (CAMKK inhibitor) alone or with phenformin for 20 hours. Whole cell lysates were blotted for LKB1, AMPK, p-AMPK, SUMO1, SUMO2/3 and Actin.

Results: We detected a decrease in AMPK activation in the SENP1-overexpressing cells compared to the cells expressing vector control in both normal and stress conditions (Figure 20). This indicated that SUMOylation might have a role in regulating the catalytic activity of LKB1. Of note, SENP1's expression levels in this experiment decreased during energy stress. This observation was further verified using a phenformin time-course experiment and correspondingly examined the endogenous and exogenous levels of the SUMO proteases, SENP1 and SENP2, during stress. Indeed, the protein levels of both the SUMO proteases decreased with phenformin treatment (Figure 21 A-C). Future work will confirm whether our observations are due to a synthesis or stability issue by conducting cycloheximide chase experiments or treating the cells with MG-132, respectively. Nevertheless, these important results illustrate that SUMOylation may be an important player involved in regulating the cell's energy balance.

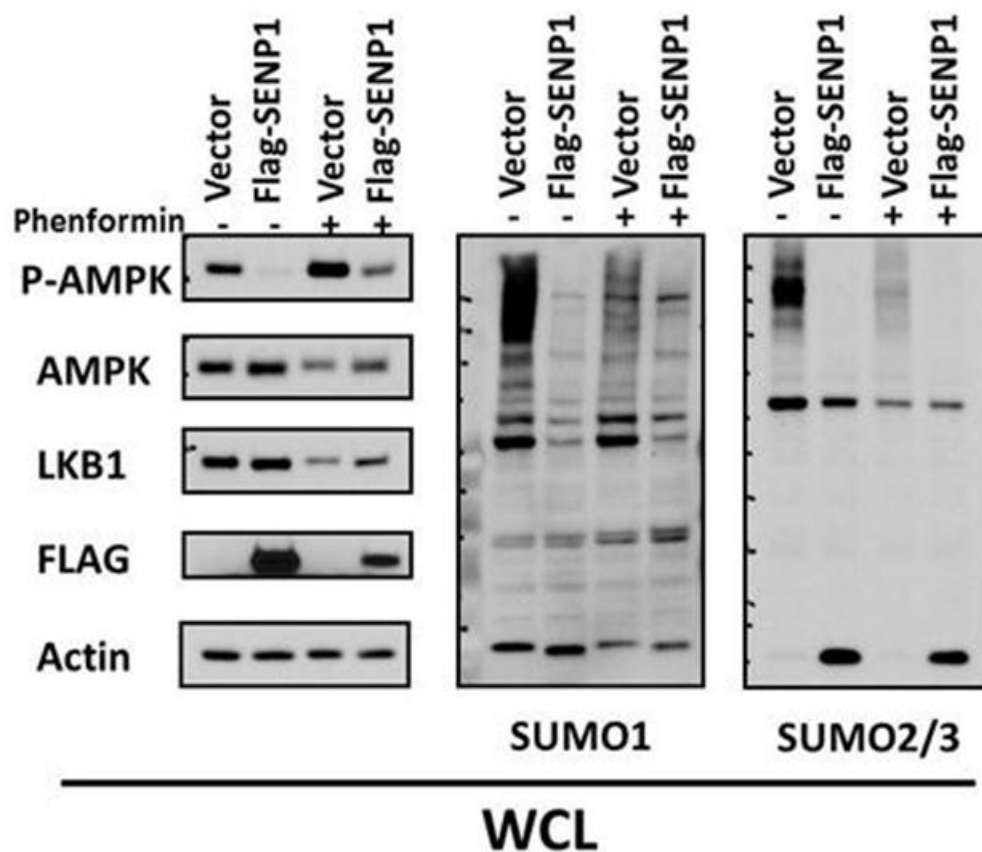


Figure 20. The role of SUMOylation in regulating AMPK activation

HEK293 cells were transiently transfected with either empty vector (pcDNA3) or FLAG SENP1 followed by treatment for 20 hours with vehicle (CAMKK inhibitor) alone or with 5 mM phenformin. Whole cell lysates were subjected to western blot using the indicated antibodies.

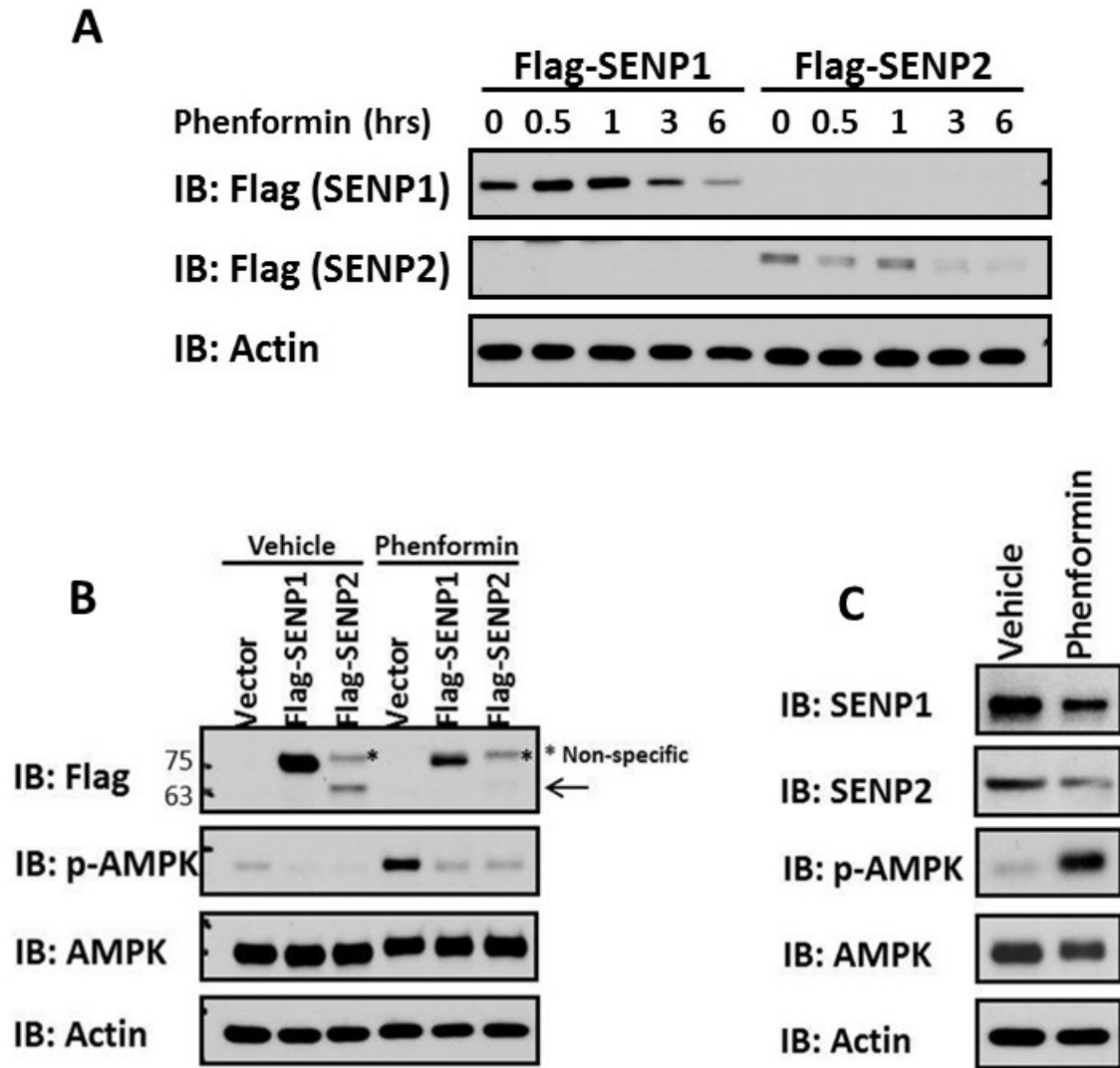


Figure 21. Endogenous and exogenous SENP1 and SENP2 protein levels decrease during metabolic stress. (A) HEK293 cells were transfected with FLAG-SEN1, or FLAG-SEN2 and treated with 5 mM phenformin and CAMKK inhibitor at indicated durations. (B) HEK293 cells transfected with vector, FLAG-SEN1, or FLAG-SEN2. HEK293 cells in Figure 21 (B) and (C) were treated with vehicle (CAMKK inhibitor) alone or with 5 mM phenformin for 15 hours. Whole cell lysates were subjected to western blot using the indicated antibodies.

4. Understanding the role SUMO proteases play in regulating LKB1 function

Owing to the observations made in chapter 4, it was imperative that we understand the direct role SUMOylation or rather the role SUMO proteases play in regulating LKB1 function. Since overexpression of SENP1/SENP2 diminished AMPK activation, we then investigated whether SUMOylation dictated the catalytic activity of its upstream kinase LKB1.

Approach: LKB1 was immunoprecipitated from HEK293 cells expressing either vector control or FLAG SENP1. The precipitates were equally divided and one set was incubated with the catalytic domain of recombinant human His6 SENP1. A kinase assay was performed and the autophosphorylation status of LKB1 was assessed in these samples.

Results: SENP1 diminished the catalytic activity of LKB1 in both *in vivo* and *in vitro* systems (Figure 22). Thus, this helps explain our previous observation where the activation status of AMPK was diminished in unstressed cells. It is well-known that LKB1 is a constitutively active protein and our data indicate that SUMOylation may be important in maintaining this basal activity. Our findings suggest that SENP1 may directly or indirectly inhibit LKB1 autophosphorylation. Therefore, it is plausible to propose that degradation of SENP1 and SENP2 is important in ensuring maximal SUMOylation-mediated LKB1 activation, thereby allowing the appropriate cellular response during energy stress. All things considered, our data suggest that LKB1 SUMOylation is essential in maintaining the cell's energy balance

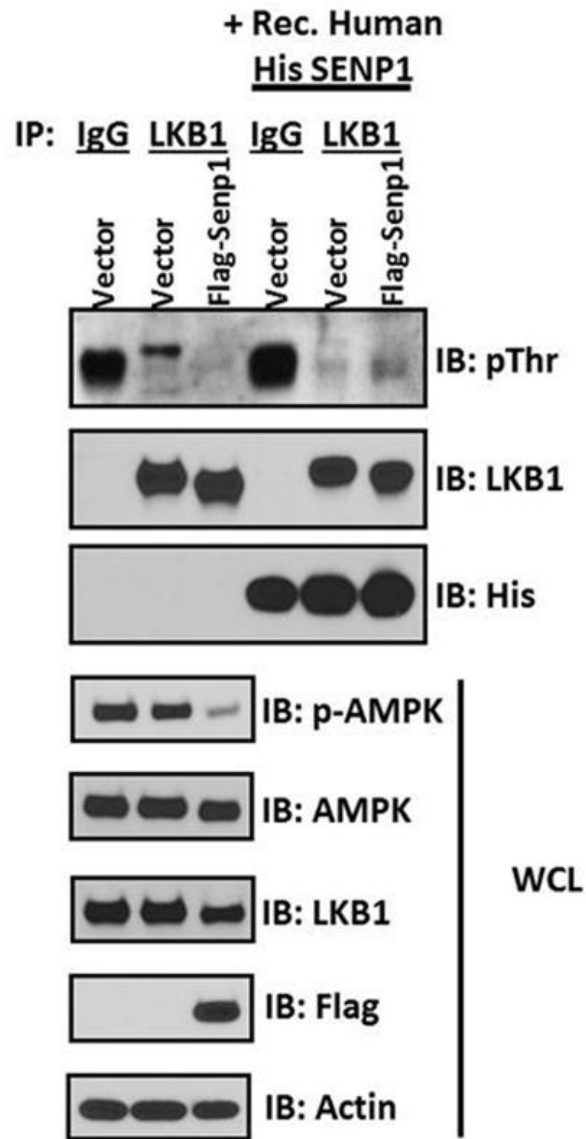


Figure 22. SENP1 regulates the catalytic activity of LKB1

LKB1 was immunoprecipitated from HEK293 cells expressing either vector control or FLAG SENP1. The precipitates were equally divided and one set was incubated with recombinant human His6 SENP1 catalytic domain. LKB1 autophosphorylation status was assessed. Cell lysates were subjected to western blot using the indicated antibodies.

Conclusion

We demonstrate for the first time that SUMOylation and, more specifically, LKB1 SUMO1 modification may play an important role in maintaining cellular energy balance during metabolic stress. We observed that SUMO1 levels of LKB1 increased (Figures 15-18) despite a proteasome-dependent global decrease in both SUMO1- and SUMO2/3-conjugated proteins (Figure 19 B). This therefore highlights the specificity of this modification during energy stress and implies that SUMO1 modification of LKB1 is important in regulating the energy balance in a cell. We also demonstrate that SUMO protease SENP1 may directly or indirectly inhibit LKB1 autophosphorylation thereby hampering its catalytic activity. Future work will look into the specific mechanism responsible for this observation. Nevertheless, our important findings suggest the exciting possibility that SUMOylation is important in the maintenance of energy homeostasis of a cell.

CHAPTER V: LKB1 K178 is modified by SUMO1 and is critical in activating AMPK.

Introduction

We have shown that LKB1 SUMO1 levels increase during energy stress. In this chapter, we identify and characterize the lysine residue that is increasingly SUMO1 modified, thereby helping maintain energy balance in a cell.

1. Identification of the LKB1 lysine site modulated by SUMO1 during energy stress

The covalent conjugation of a SUMO protein to a target's lysine residue is dictated by a consensus motif (Ψ Kxe, in which Ψ is an aliphatic branched amino acid and x is any amino acid) (Yeh et al., 2000, Xu et al., 2008). In some cases, this motif is not observed, and a lysine residue can be modified by SUMO in a non-consensus mode (Wilkinson and Henley, 2010, Geiss-Friedlander and Melchior, 2007).

Approach: To identify the possible LKB1 lysine residues that may be SUMO1 modified during energy stress, we used a SUMOylation site prediction software (Ren et al., 2009) that took into consideration the different types (i.e., through consensus and non-consensus motifs) of SUMO conjugation. The sites identified were mutated to arginine and their SUMO1 and SUMO2/3 modification status was assessed during energy stress. As a control, we also investigated the acetylation and ubiquitination status of the SUMO mutant and checked whether it was altered under conditions of metabolic stress.

Results: The software generated a number of lysine targets and we chose the top three candidates with the best cutoff scores: K96, K122, and K178 (Figure 23). We generated LKB1 K96R, K122R, and K178R mutants and transfected them or their wild-type counterpart into HeLa cells (LKB1 null) together with HA-SUMO1. The cells were subjected to energy stress,

LKB1 was immunoprecipitated, and the lysates were blotted for HA. We observed that, upon metabolic stress, SUMO1 modification of the LKB1 K178R mutant was markedly lower than that of wild-type LKB1 (Figures 25 and 26). However, SUMO1 modification of the LKB1 K96R mutant remained unaltered (Figure 24). The LKB1 K122R mutant was unstable and thus not tested further (data not shown). Notably, when the cells were not subjected to energy stress, levels of SUMO1-modified LKB1 remained the same for both the wild-type and K178R mutant LKB1 (Figure 25). Thus, the SUMOylation status of the K178R mutant only changed upon metabolic stress. These data suggest that, during energy stress, K178 is a critical SUMO1 conjugation site.

Since lysine residues can also be covalently modified by SUMO2/3, acetylation, and ubiquitination, we examined their conjugation levels in the LKB1 K178R mutant during energy stress. We found that the SUMO2/3, acetylation or ubiquitination status of LKB1 lysine 178 was not altered during energy stress (Figures 26 and 27). These results led to the conclusion that, during energy stress, conjugation of K178 with SUMO1 may be important in regulating LKB1 in maintaining the energy equilibrium in a cell.

Predicted sites				
Position	Peptide	Score	Cutoff	Type
96	ANV K KEI	2.825	0	TypeI: Ψ-K-X-E
97	NV K EIQ	1.618	0	TypeI: Non-consensus
108	LRH K NVI	1.015	0	TypeI: Non-consensus
122	NEE K QKM	2.338	0	TypeI: Non-consensus
124	E K QKMYM	1.471	0	TypeI: Non-consensus
146	VPE K RFP	0.956	0	TypeI: Non-consensus
175	IVH K DIK	0.794	0	TypeI: Non-consensus
178	KD I KPGN	2.221	0	TypeI: Non-consensus
191	GT L KISD	1.191	0	TypeI: Non-consensus
235	SGF K VDI	1.5	0	TypeI: Non-consensus
262	NIY K LFE	1.412	0	TypeI: Non-consensus
269	NIG K GSY	1.059	0	TypeI: Non-consensus
287	DLL K GML	1.456	0	TypeI: Non-consensus
296	EPA K RFS	1.162	0	TypeI: Non-consensus
311	WFR K KHP	0.765	0	TypeI: Non-consensus
312	FR K KHPP	0.897	0	TypeI: Non-consensus

Enter sequence(s) in FASTA format

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1 mewdpqqlg mtegelmsv gmdtfihrid steviqpr krakligkyl mgdllegesy
61 gkvkevdse tlcrravkil kkkdriprn geankvkeiq llrrlrknv iqlvdvlyne
121 ekqkmymme yvcgmqeml dspekrfpv cqahgyfcql idgleyltsq givhkdiikpg
181 nlllttgti kisdigvaea lhpfaaddtc rtsqgsfafq ppeiandlt fsgflvdiws
241 agvtlyntt glypfegdni ykfenigkg syaigdcgp plsdlikgml eyepakrfsi
301 rqiqrhswfr kkhppaeapv pippspdkd rwrsmtwpy ledlhgaded edlfdieddi
361 iytqdfvpg qpveeeashn gqrrglpkav cmngteaql stksraegra pnparkacsa
421 sskirrisac kqq

```

Threshold
☐ High
☐ Medium
☐ Low
☒ All

Console

Example

Clear

Submit

Figure 23. LKB1 SUMOylation predicted sites

LKB1 consensus and non-consensus SUMOylation motifs/sites predicted by GPS-SUMO software (<http://sumosp.biocuckoo.org>).

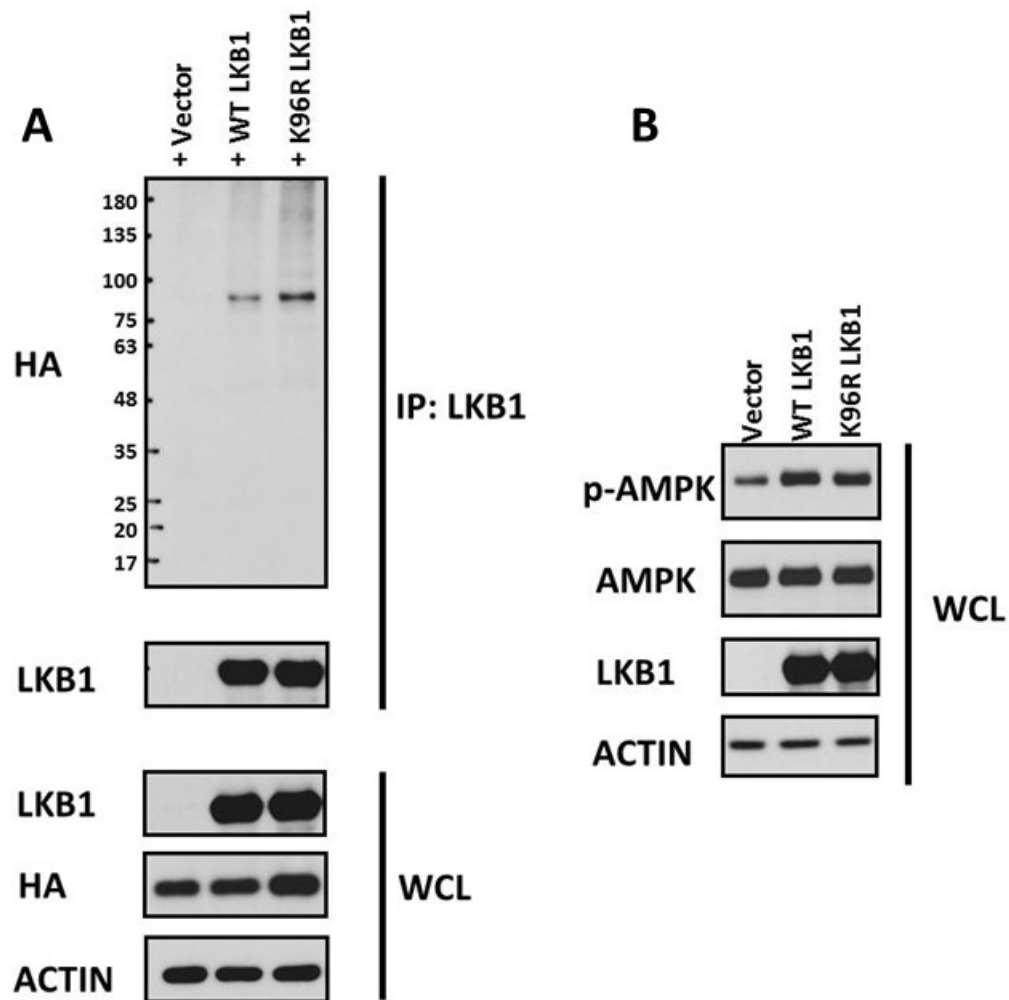


Figure 24. Characterization of the consensus LKB1 SUMOylation site

(**A, B**) HeLa cells transiently expressing empty vector, wild-type LKB1, or LKB1 mutant K96R (predicted SUMO consensus site) were treated with 2 mM phenformin and 10 μ g/mL STO609 for 6 hours. LKB1 was immunoprecipitated and a western blot was conducted using the indicated antibodies.

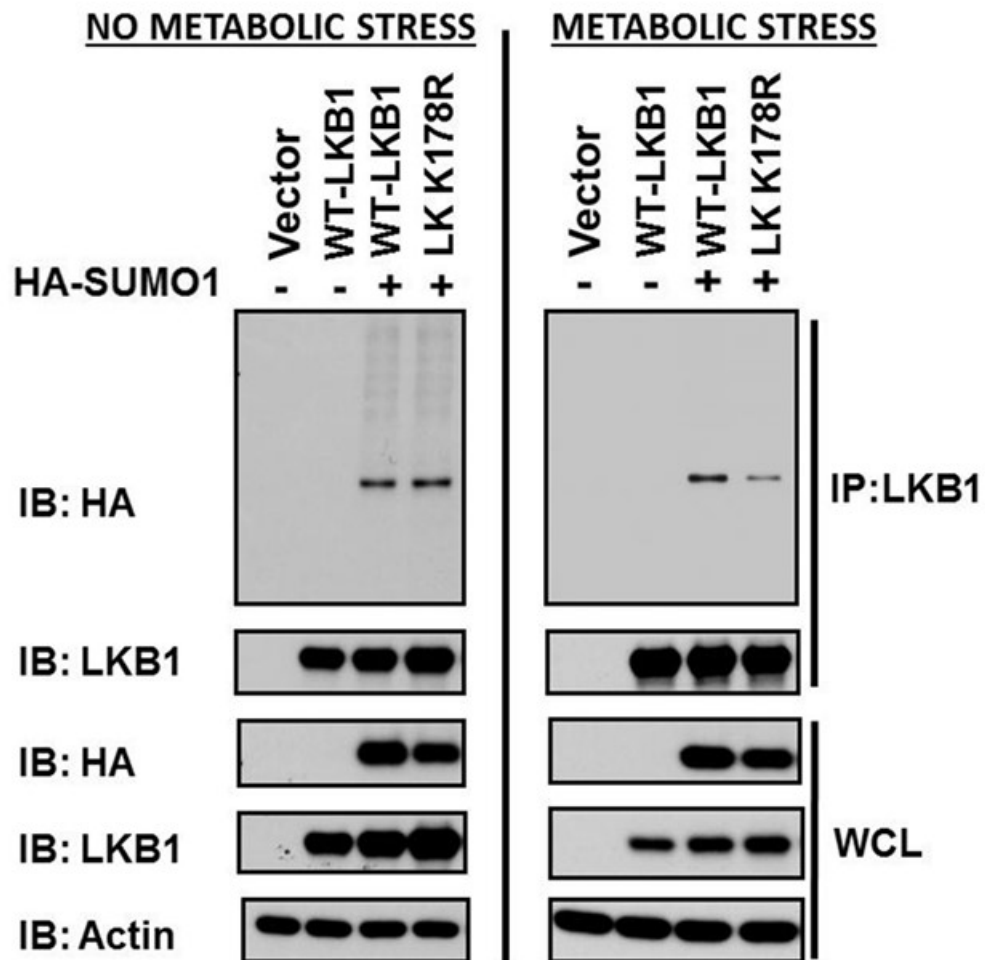


Figure 25. The role of energy stress in regulating LKB1 lysine 178 SUMOylation

HeLa cells transiently expressing empty vector, wild-type LKB1, or LKB1 mutant K178R were either subjected to energy stress or left untreated for 6 hours. LKB1 was immunoprecipitated and a western blot was conducted using the indicated antibodies.

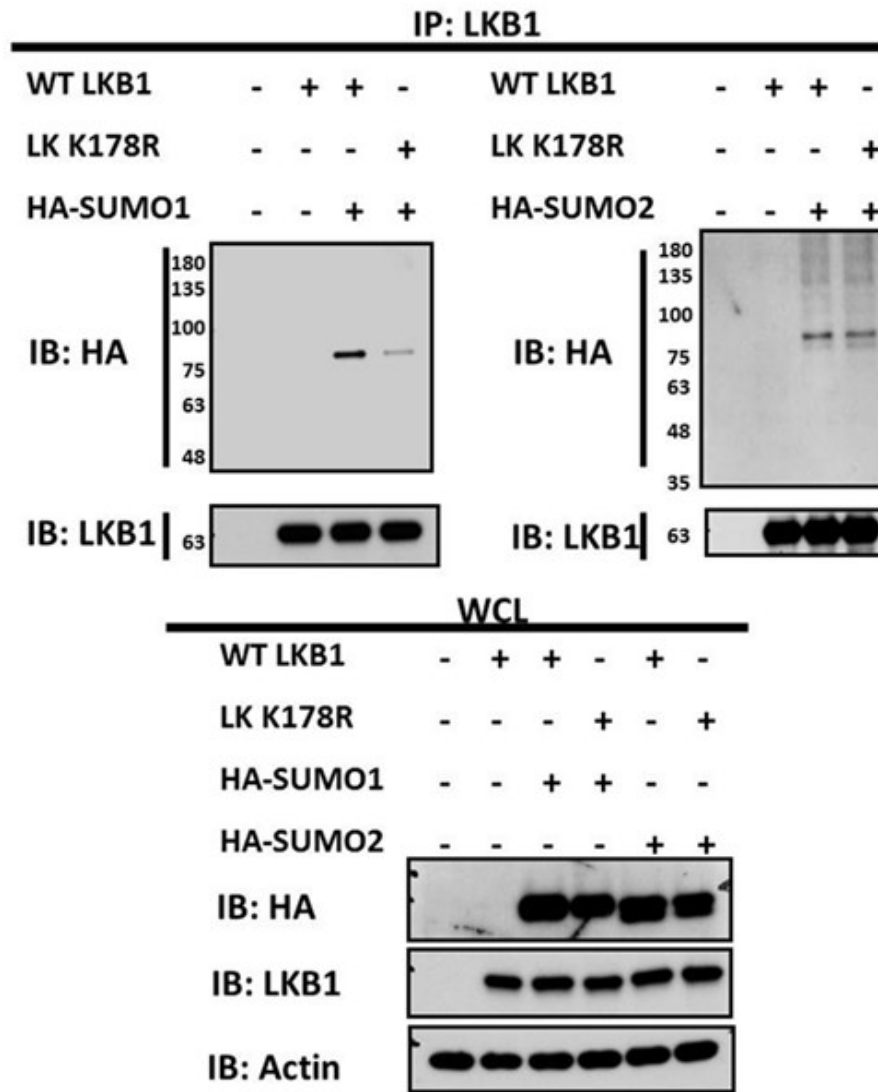


Figure 26. Lysine 178 of LKB1 is SUMO1 but not SUMO2/3 modified during energy stress

Post-translational modification of LKB1 wild-type and K178R mutant by SUMO1 and SUMO2 in HeLa cells during metabolic stress (6 hours treatment with 2 mM phenformin and 10 µg/mL STO609).

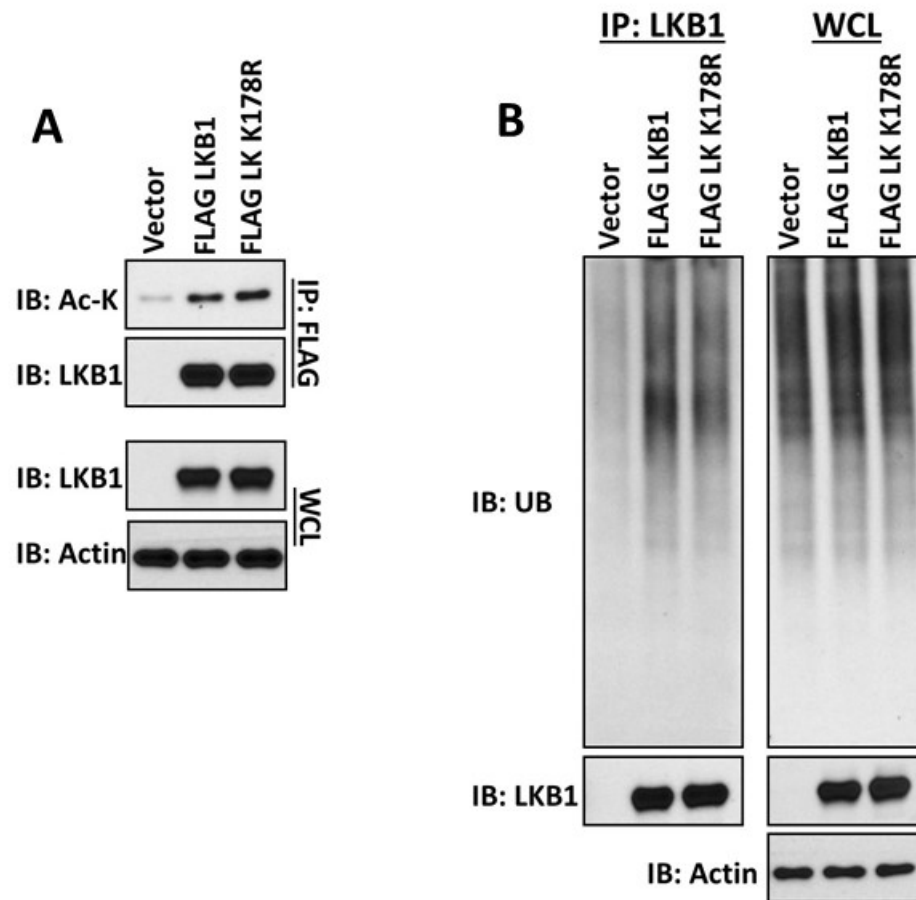


Figure 27. The acetylation or ubiquitination status of LKB1 lysine 178 is not altered during energy stress

Post-translational modification of LKB1 wild-type and K178R mutant by (A) acetylation, and (B) ubiquitin in HeLa cells during metabolic stress.

2. The physiological relevance of the LKB1 K178R SUMO1 mutant

Owing to the fact that we identified a lysine residue that is SUMO1-modified during metabolic stress, it was vital that we confirm whether LKB1 K178R mutant had a physiological role in maintaining energy balance in the cell.

Approach: To evaluate the physiological relevance of the specific LKB1 K178R SUMO1 mutant in maintaining the cell's energy balance, we transfected empty vector (pcDNA3), wild-type LKB1, or LKB1 K178R into LKB1-null HeLa cells and treated the cell cultures with phenformin and STO-609 at the indicated times to induce metabolic stress.

Results: Interestingly, the phosphorylation of endogenous AMPK at T172 was markedly reduced in cells expressing empty vector (pBABE) or the LKB1 K178R mutant (Figures 28 A and B). Notably, in these cells in which the phosphorylation of AMPK is basally high, the CAMKK inhibitor inhibited AMPK activation, suggesting that CAMKK β is the major upstream AMPK-activating kinase in LKB1-null settings. We note that this is a cell type-specific trait, since AMPK phosphorylation increases when HEK293 and C2C12 cells are subjected to metabolic stress (Figures 15-21). This observation is consistent with previous studies demonstrating that activated AMPK can act as an ally in regulating cancer cell survival by maintaining NADPH levels (Jeon et al., 2012). Taken together, these findings demonstrate the importance of the SUMO1 modification of LKB1 K178 in activating AMPK.

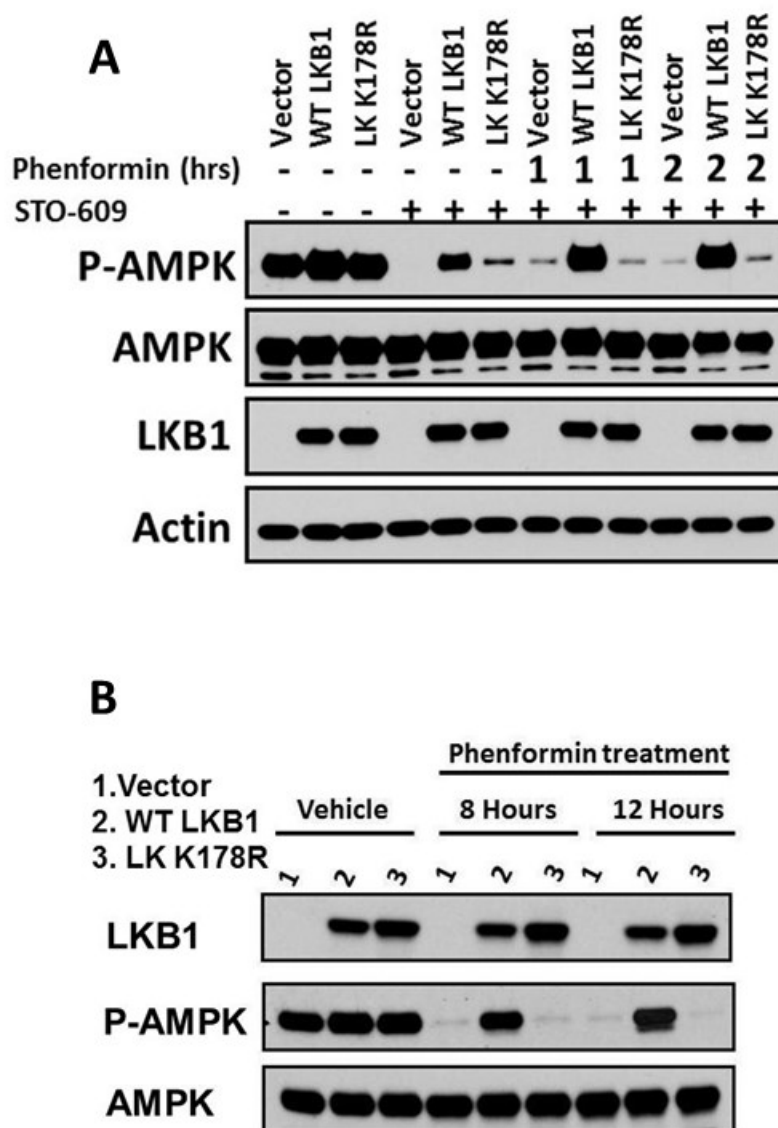


Figure 28. The physiological implication of the LKB1 K178R SUMO1 mutant during energy stress. (A) A549 cells (LKB1 null) stably expressing empty vector (pcDNA3), wild-type LKB1, or LKB1 mutant K178R were treated with 2 mM phenformin and/or 10 μ g/mL STO609 as indicated. (B) HeLa cells transiently expressing empty vector (pcDNA3), wild-type LKB1, or LKB1 mutant K178R were treated with 2 mM phenformin and 10 μ g/mL STO609 for 8 or 12 hours. Cell lysates in (A) and (B) were subjected to western blot using the indicated antibodies.

3. Verification that the LKB1 K178R SUMO mutant is not a catalytic mutant

LKB1 is found in a heterotrimeric complex with STRAD and MO25, and this complex constitutively active in the cell (Zeqiraj et al., 2009). Owing to the proximity of lysine 178 to the active site (Figure 29), we had to ascertain that mutating the lysine to arginine did not alter the catalytic activity of LKB1. The following experiments were done to confirm that the LKB1 K178R SUMO mutant is not a catalytic mutant:

- Autophosphorylation status of the SUMO mutant was investigated
- A kinase assay was performed to establish that the SUMO mutant was capable of phosphorylating and activating AMPK
- Since the formation of the LKB1-STRAD α -MO25 heterotrimeric complex is important in the allosteric activation of LKB1 (Alessi et al., 2006), we examined whether the SUMO mutant was able to form this complex

Results: When LKB1 was immunoprecipitated from HeLa cells transiently expressing empty vector (pcDNA3), wild-type LKB1, or LKB1 K178R mutant, the autophosphorylation status and kinase activity of the LKB1 K178R mutant remained unaltered (Figures 30 A and B). The speculative structural model of K178-SUMOylated LKB1 also corroborates that although K178 is close to the active site, SUMOylation of this residue does not necessarily block access to the active site of LKB1 (Figure 31). In addition, while the Peutz-Jeghers syndrome LKB1 mutations (Q170P, I177N, N181E, and L182P) impeded the formation of the LKB1-STRAD α -MO25 heterotrimeric complex, the LKB1 K178R mutant did not (Figure 32). Thus mutation of K178 to arginine did not change the ability of LKB1 to form this active complex. Taken together, these findings demonstrate that LKB1 K178R is not a kinase-dead mutant.

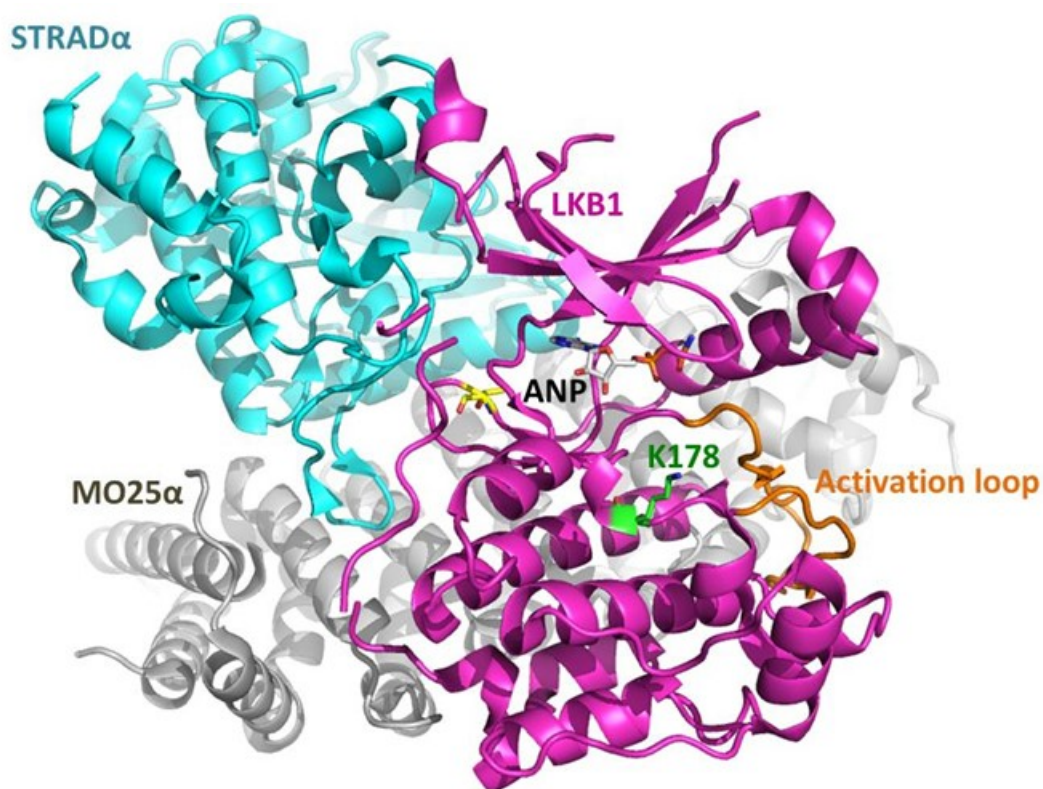


Figure 29. Ribbon diagram showing structural features of LKB1.

LKB1 (magenta) is shown in a complex with STRADα (cyan) and MO25α (gray). Highlighted are K178 (green), the activation loop (orange) and the ADP analogue ANP (white carbons). The structure has been drawn based on PDB entry 2WTK.

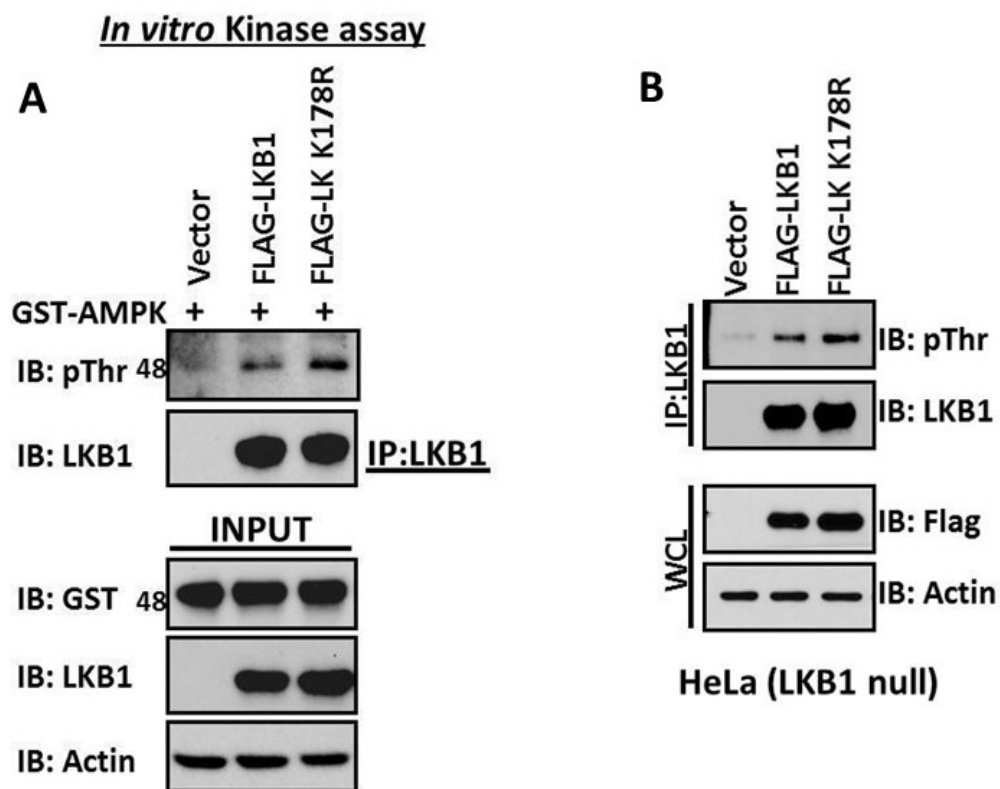


Figure 30. LKB1 K178R SUMO mutant is not a catalytic mutant

LKB1 was immunoprecipitated from HeLa cells transiently expressing empty vector (pcDNA3), wild-type LKB1, or LKB1 K178R mutant. (A) An *in vitro* kinase assay using GST-AMPKA1 recombinant protein was performed and, (B) Autophosphorylation of LKB1 and the LKB1 K178R mutant was analyzed.

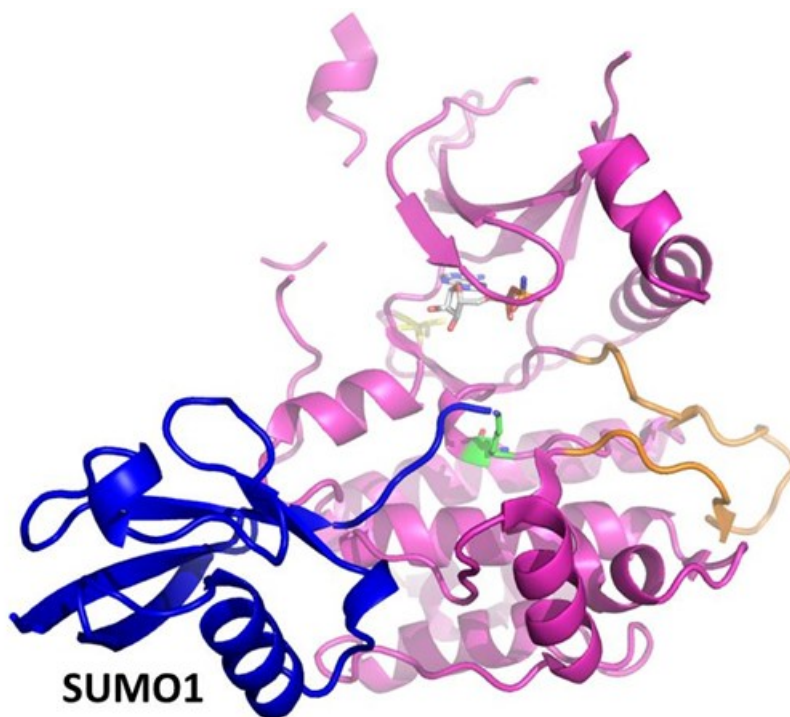


Figure 31. Speculative structural model of K178-SUMOylated LKB1.

LKB1 (cyan) is shown in a similar orientation to Figure 29. For highlighted features, see legend to Figure 29. A SUMO molecule (taken from PDB 3UIP; shown in blue) has been manually positioned.

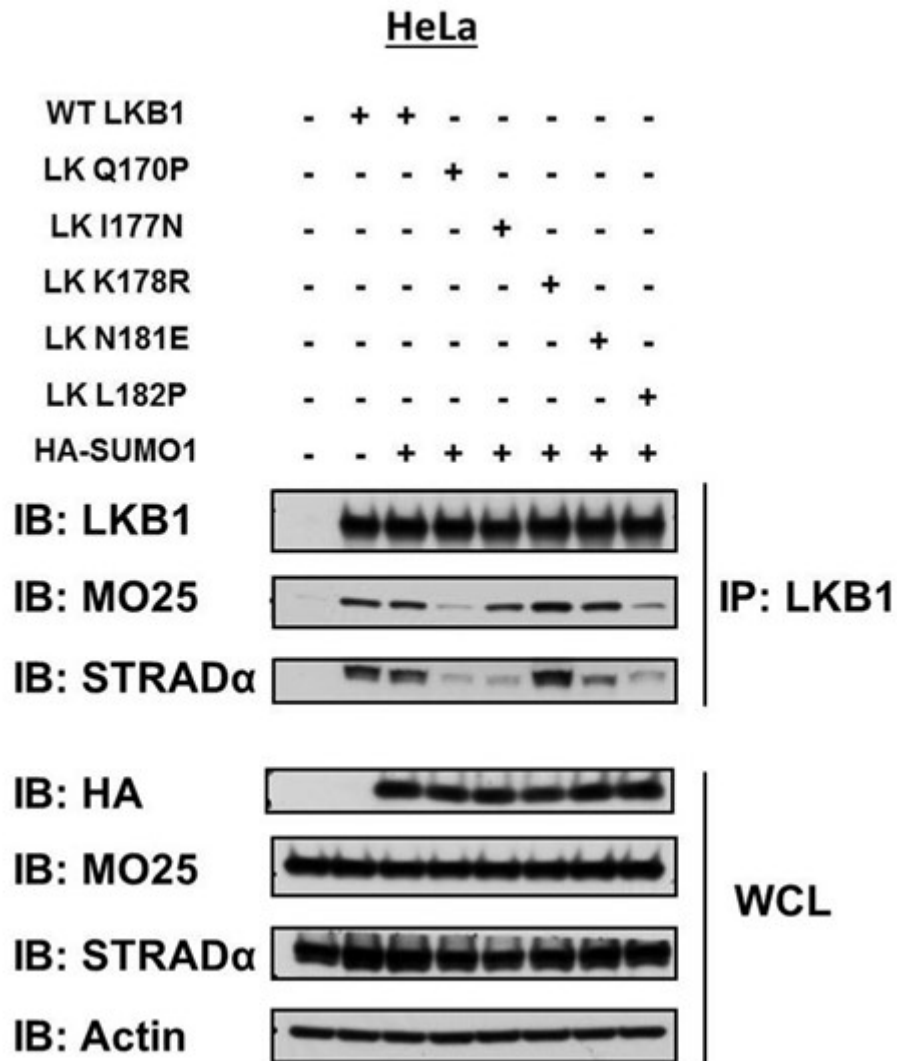


Figure 32. LKB1 K178R SUMO1 mutant forms an active heterotrimeric complex with STRAD and MO25. Wild-type LKB1, LKB1 K178R mutant, and the various Peutz–Jeghers syndrome LKB1 mutants were transfected and immunoprecipitated from HeLa cells, and their interactions with STRADα and MO25 were assessed.

Conclusion

Our studies indicate that the SUMO1 modification of LKB1 is important in maintaining the energy balance of a cell. In this chapter, we identified the SUMO1 specific site that is essential in ensuring that the LKB1-AMPK signaling pathway is activated during energy stress. We demonstrated that lysine 178 of LKB1 is SUMO1 modified during stress and not by SUMO2/3, acetylation, or ubiquitination (Figures 25-27). Mutation of lysine 178 to an arginine inhibited the activation of AMPK during energy stress (Figure 28). Since K178 is close to the catalytic loop of LKB1, we had to confirm that we did not simply generate a catalytic mutant. Indeed, the autophosphorylation and catalytic activity of LKB1 remained intact when lysine 178 was converted to an arginine (Figure 30). Moreover, LKB1 K178R mutant was capable of forming the catalytically active heterotrimeric complex with STRAD and MO25 (Figure 32). Our important results therefore provide more detailed information as to where SUMO1 modifies LKB1 during energy stress ultimately helping the cell maintain energy homeostasis.

CHAPTER VI: SUMOylation promotes LKB1-AMPK interaction

Introduction:

Previous studies have demonstrated that proteins can non-covalently interact with SUMO via their SUMO-interacting motif (SIM) and that this interaction may promote the interaction between the protein and its substrate or partner (Kerscher, 2007). Several divergent SIM motifs have been described and characterized but the common feature in all of them is the presence of a hydrophobic core flanked by a stretch of acidic amino acids (Minty et al., 2000, Song et al., 2004, Hecker et al., 2006, Hannich et al., 2005). In this chapter, we confirm that SUMOylation is important in regulating LKB1-AMPK interaction, thereby enabling the phosphorylation and activation of AMPK. We also identify a SIM in the N-terminal region of AMPK. Mutation of the hydrophobic residues critical for its recognition by SUMO1 impeded the interaction with LKB1 during energy stress thereby deregulating the cell's response to maintain energy balance.

Approach:

- Examine whether the LKB1 K178R mutant has a lower affinity to AMPK than the wild-type LKB1.
- Investigate whether deregulating SUMOylation by overexpressing SENP1 would alter LKB1-AMPK interaction
- Determine whether SUMO1 was responsible for the increase in LKB1-AMPK interaction
- Investigate whether LKB1-AMPK interaction is mediated via a SIM
- Identify and characterize AMPK SIM
- Evaluate physiological function of AMPK SIM in regulating the cell's energy balance

Results:

The importance of SUMOylation in regulating LKB1-AMPK interaction

We observed that, during energy stress, wild-type LKB1 bound AMPK more than the LKB1 K178R mutant (Figure 33). Furthermore, overexpression of the deSUMOylating enzyme SENP1 caused a marked decrease in LKB1-AMPK interaction in stable LKB1-expressing A549 cells (Figure 34A) as well as in HEK293 cells that endogenously express wild-type LKB1 (Figure 34B). To establish that SUMO1 was responsible for the increase in LKB1-AMPK interaction during energy stress, we assessed whether SUMO1 interacted with AMPK. We overexpressed vector control or HA-SUMO1 in A549 cells and conducted a co-immunoprecipitation study. As expected, endogenous AMPK co-precipitated with HA-SUMO1 (Figure 35B) and also with endogenous SUMO1 in HEK293 cells (Figure 35A). We then determined whether this interaction was mediated via a SIM by conducting a competition assay using peptides containing the SUMO E3 ligase PIASy SIM sequence. As we increased the SIM peptide concentration, the affinity of the SUMO1 beads to the GST-AMPK recombinant protein decreased (Figure 36A). As a control, we demonstrate that boiled SUMO1 beads have a lower affinity to AMPK (Figure 36B). This affirmed that AMPK does interact with SUMO1 via a SIM.

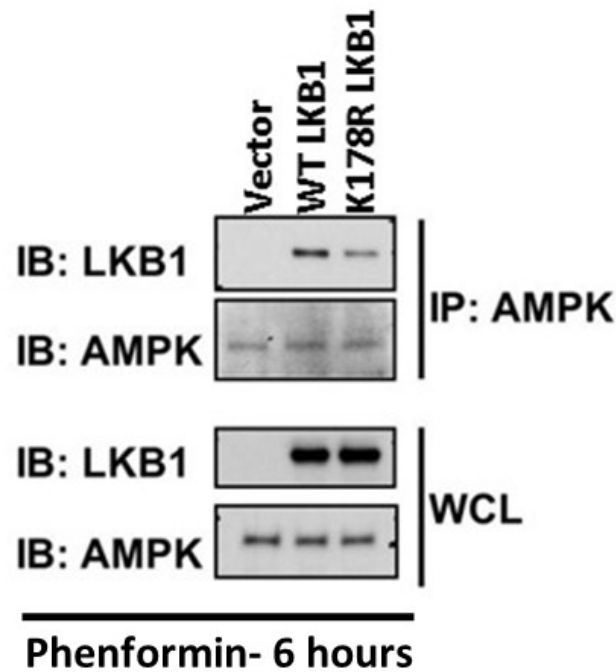


Figure 33. LKB1 K178R SUMO1 mutant interaction with AMPK during energy stress

In vivo assay in HeLa cells of binding between AMPK and LKB1 wild-type or K178R mutant after 6 hours of metabolic stress.

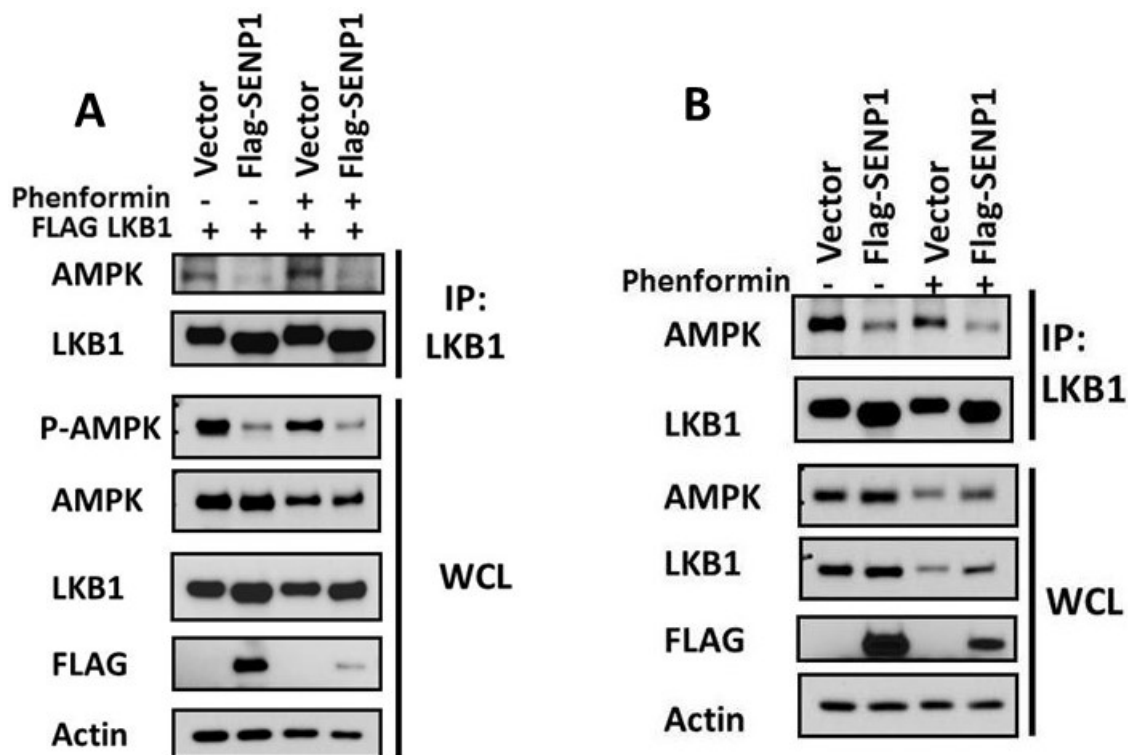


Figure 34. Role of SUMO protease, SENP1, in regulating LKB1-AMPK interaction

(A) Stably LKB1-expressing A549 cells were transiently transfected with either empty vector (pcDNA3) or FLAG SENP1. (B) HEK293 cells were transiently transfected with either empty vector (pcDNA3) or FLAG SENP1. The cells in (A) and (B) were then treated for 20 hours with vehicle (CAMKK inhibitor) alone or with 2 mM phenformin. LKB1 was immunoprecipitated from lysates and its interaction with AMPK was assessed. Cell lysates were also subjected to western blot using the indicated antibodies.

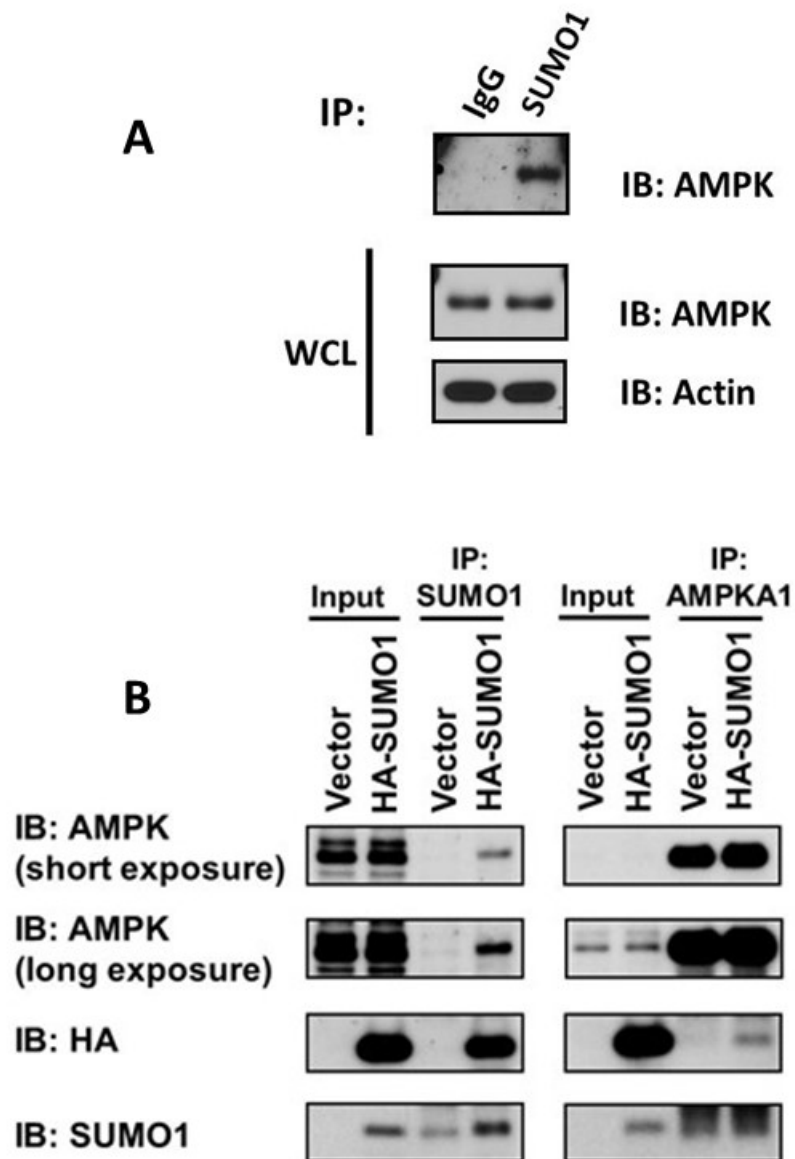
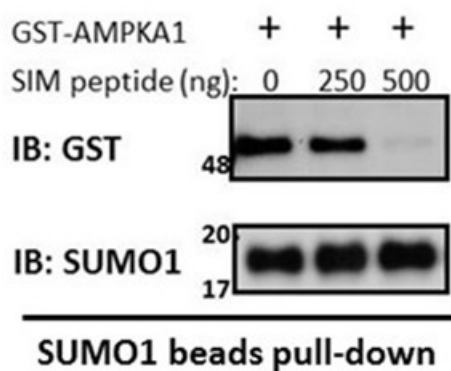


Figure 35. AMPK interacts with endogenous and exogenous SUMO1

(A) Co-immunoprecipitation assay of endogenous AMPK and SUMO1 in HEK293 cells. (B) Co-immunoprecipitation assay of endogenous AMPK and HA-SUMO1 in A549 cells.

A



B

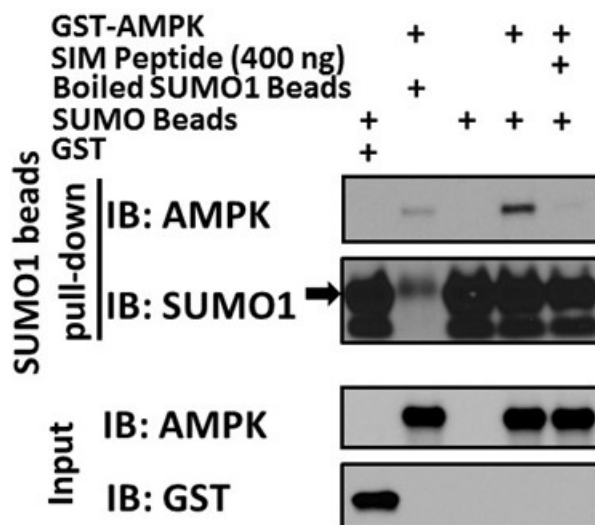


Figure 36. SUMO1 recognizes AMPK via a SUMO interacting motif

In vitro SUMO1 pulldown assay of GST-AMPKA1 recombinant protein with (A) varying PIASy SIM peptide concentrations, or (B) inclusion of boiled SUMO1 beads as control

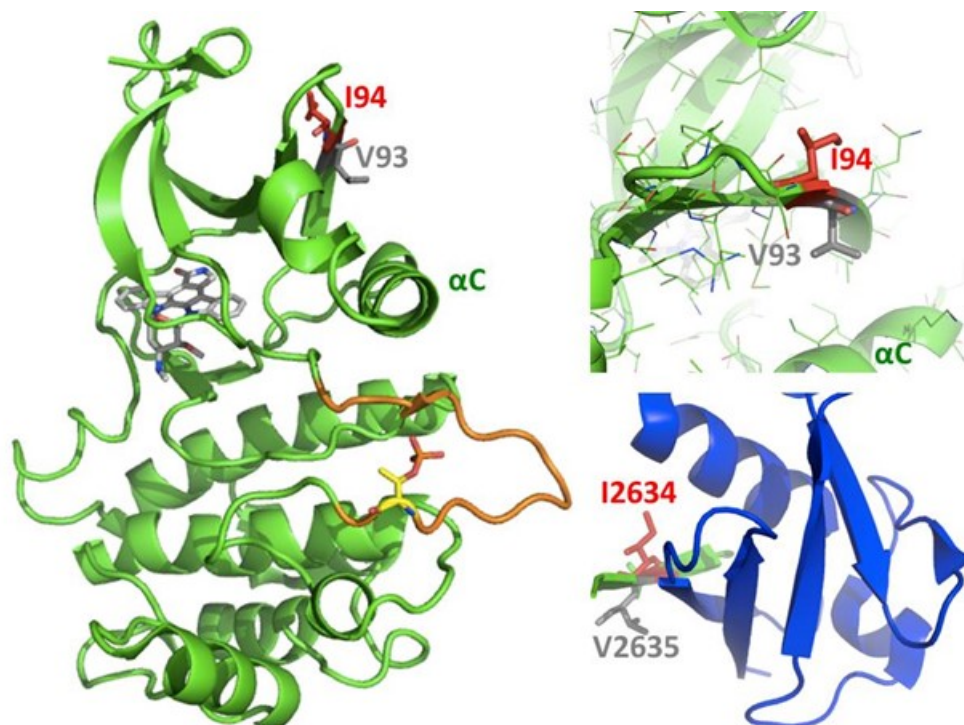
Identification of AMPK SIM

To further confirm our model, we sought to locate a possible SIM using the consensus motifs predicted by previous studies (Hecker et al., 2006). Various SIMs have been proposed, but the common theme in all the paradigms is the presence of a hydrophobic core that is often flanked by a cluster of negatively charged amino acids (Geiss-Friedlander and Melchior, 2007, Kerscher, 2007).

Approach: Using the crystal structure depicting the non-covalent recognition of SUMO1 by RanBP2 through β -strand interactions (taken from PDB 3UIP) as a structural basis, we then searched for potential SIMs in AMPK (Figure 37A).

Results: Using the interaction between SUMO1 and RanBP2 as our guide, we identified a conserved region in the N-terminal region of AMPK that conforms to the specifications of a SIM proposed by Minty et al. (Minty et al., 2000) (Figure 37B).

A



B

SIM Motif: h-h-X-(S)-(X)-(S/T)-a-a-a (Minty et al.)

h- hydrophobic amino acid, X- any amino acid, a- acidic amino acid

Human	77	QNLKLF	RHPHIIKLYQ	VISTPSD	IFMVM	EYVSGGELF	113
Mouse	77	QNLKLF	RHPHIIKLYQ	VISTPSD	IFMVM	EYVSGGELF	113
Rat	66	QNLKLF	RHPHIIKLYQ	VISTPSD	IFMVM	EYVSGGELF	102
Zebrafish	67	QNLKLF	RHPHIIKLYQ	VISTPTD	IFMVM	EYVSGGELF	103
Chicken	78	QNLKLF	RHPHIIKLYQ	VISTPTD	IFMVM	EYVSGGELF	114
Chimpanzee	77	QNLKLF	RHPHIIKLYQ	VISTPSD	IFMVM	EYVSGGELF	113
Rhesus	77	QNLKLF	RHPHIIKLYQ	VISTPSD	IFMVM	EYVSGGELF	113
Dog	76	QNLKLF	RHPHIIKLYQ	VISTPSD	IFMVM	EYVSGGELF	112

Figure 37. Identification of AMPK SIM

(A) The AMPK kinase domain is shown (green; taken from PDB 4CFH). Highlighted are the inhibitory staurosporin (white carbons), V93 (gray), I94 (orange), phosphorylated T172 (yellow carbons), and the activation loop (orange). Right panel, top: Zoom onto I94, showing that I94 is mostly solvent exposed, suggesting that its mutation to alanine is unlikely to compromise the 3D fold or stability of the AMPK kinase domain. Right panel, bottom: The non-covalent recognition of SUMO1 (blue) by RanBP2 (green – only the interacting part is shown) through β -strand interactions (taken from PDB 3UIP) was used as a structural basis for the search for potential SIMs in AMPK. The di-hydrophobic motif featuring a valine and isoleucine are shown. The AMPK N-terminal β -sheet offers a surface similar to a potential SIM (right panel, top). (B) A schematic of a conserved region in the N-terminal region of AMPK that conforms to the specifications of a previously proposed SIM (Minty et al., 2000).

Characterization of AMPK SIM

Following the identification of the region possibly responsible for the recognition and binding of LKB1 to AMPK, we designed experiments that would further validate our model.

Approach:

- Design and generate biotinylated wild-type and mutant AMPK SIM peptides (Figure 38A).
- Perform a competition assay and observe whether the wild-type AMPK SIM competed with GST-AMPK recombinant protein in the recognition and binding to SUMO1
- Conjugate the generated peptides to avidin beads to generate resin for a peptide pulldown assay. This will help determine whether the wild type AMPK SIM recognizes and bind to LKB1
- Mutate both hydrophobic residues (V93 and I94) to alanines in AMPK cDNA and check their binding affinities to SUMO1 and LKB1

Results: When we performed a competition assay using the biotinylated wild-type and mutant AMPK SIM peptides, we observed that the wild-type AMPK SIM peptides competed with GST-AMPK recombinant protein, thereby decreasing its affinity to SUMO1 (Figure 38B). In addition, when the peptide pulldown assay was performed using HEK293 cell lysates, the binding affinity of the mutant AMPK SIM peptides to endogenous LKB1 was markedly lower than that of the wild-type SIM peptide (Figure 38C). This further illustrated the importance of SUMOylation and, more specifically, the AMPK SIM motif sequence in regulating LKB1-AMPK interaction.

We further verified this model by mutating both hydrophobic residues (V93 and I94) to alanines in AMPK cDNA and checked their binding affinities to SUMO1 and LKB1. V93 and I94 are mostly solvent exposed, suggesting that their mutation to alanines is unlikely to compromise the 3D fold or stability of the AMPK kinase domain (Figure 37A). We found that the double SIM AMPK mutant (VI93-94AA) had a lower affinity to both SUMO1 and LKB1 in AMPK $\alpha^{-/-}$ mouse embryo fibroblast (MEF) cells than wild-type AMPK (Figure 39A).

Physiological relevance of AMPK SIM

To prove that AMPK SIM is physiologically relevant, we then checked whether the SIM mutant affected AMPK phosphorylation. We noted a consistent decrease in AMPK phosphorylation when the cells transfected with the AMPK SIM mutant were subjected to energy depletion (Figure 39B). Together, these data support the hypothesis that LKB1 is modified by SUMO1 at K178 during energy stress, thereby enabling the recognition of AMPK through its SIM, leading to AMPK activation.

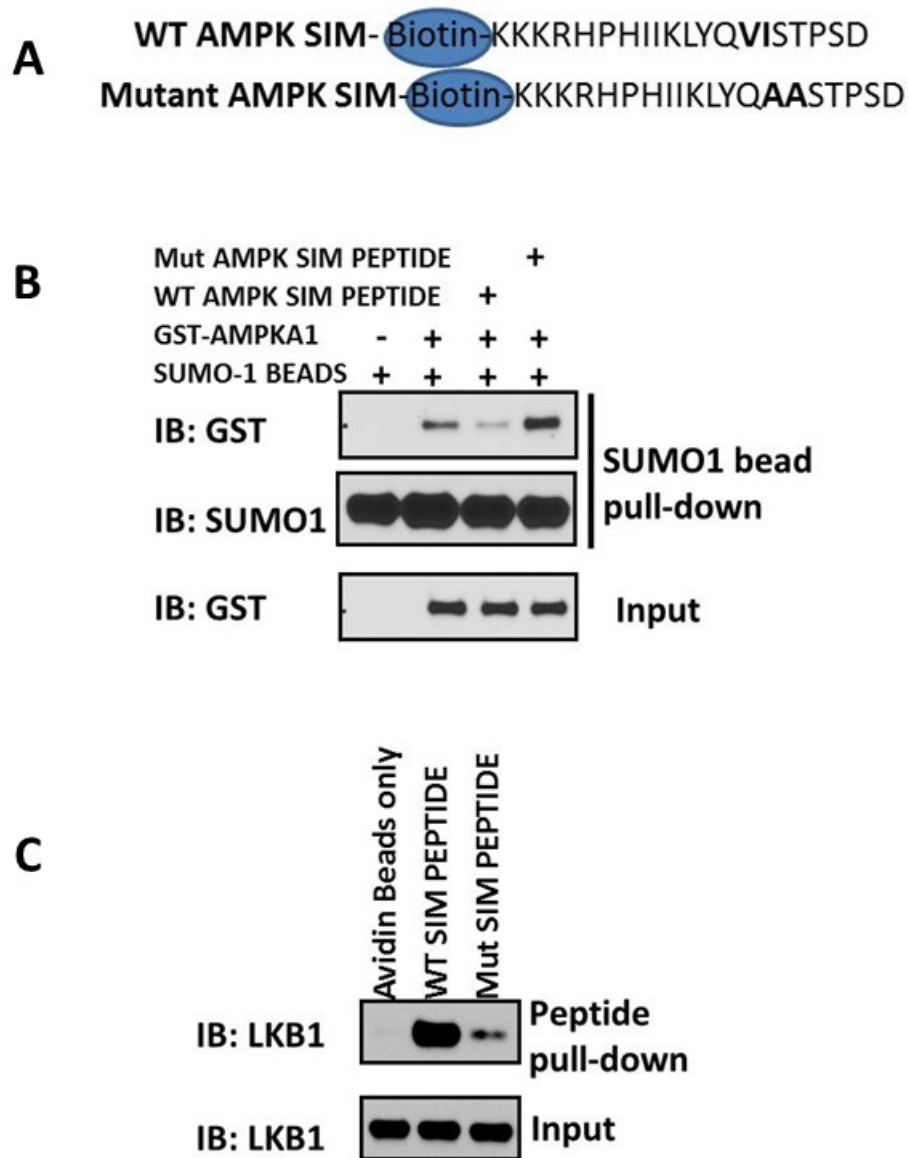


Figure 38. Characterization of AMPK SIM

(A) Amino acid sequence of the biotinylated wild-type and mutant AMPK SIM peptides that were generated (B) *In vitro* SUMO1 pulldown assay of GST-AMPKA1 recombinant protein in the presence of wild-type or mutant AMPK SIM peptides. (C) Peptide pulldown assay of biotinylated wild-type or mutant AMPK SIM peptides conjugated to avidin beads in HEK293 cell lysates.

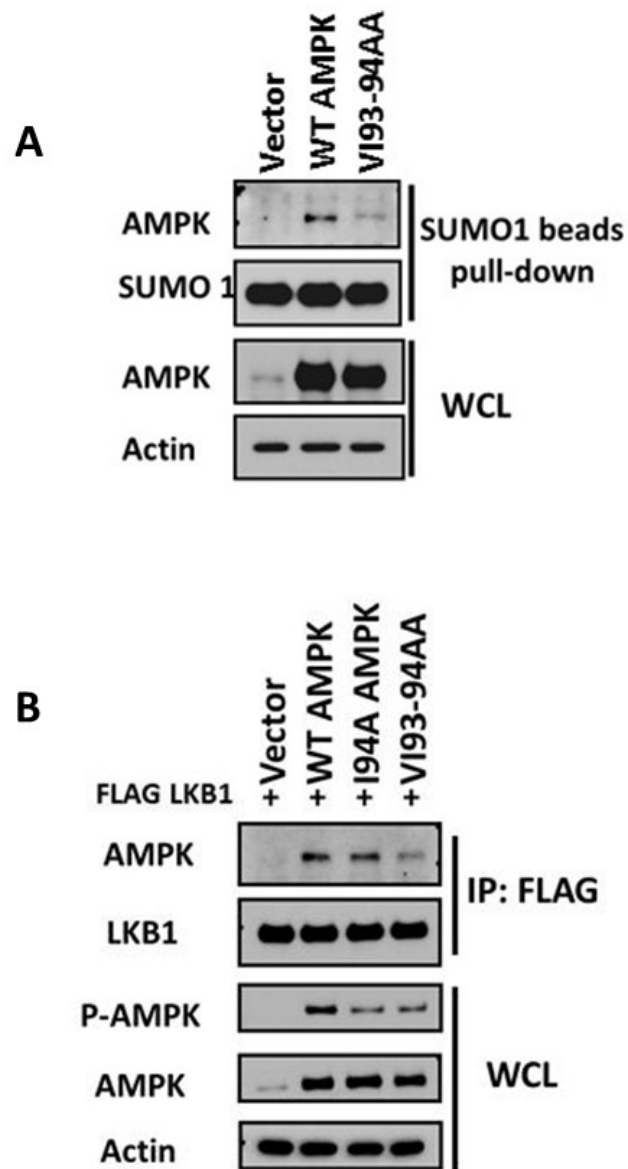


Figure 39. *In vivo* characterization of AMPK SIM and its physiological relevance

In vivo assay in AMPK α ^{-/-} MEF cells of binding between AMPK or AMPK SIM mutants and (A) SUMO1 and (B) LKB1 after 6 hours of metabolic stress. Whole-cell lysates were subjected to western blot using the indicated antibodies.

Conclusion

Protein SUMOylation can have three general non-exclusive consequences: 1) Occluding protein-protein interaction, 2) Change the conformation of a protein, or 3) augmenting protein-protein interaction through the recognition of the partner protein's SUMO interacting motif (Geiss-Friedlander and Melchior, 2007). Previously, we identified lysine 178 of LKB1 as the important SUMO1 site that is regulated during energy stress. In this chapter, we show that this site is essential in enhancing the LKB1-AMPK interaction. Furthermore, overexpression of SUMO protease SENP1 decreased LKB1-AMPK interaction. Accordingly, we illustrate that LKB1 SUMOylation is critical in recognizing the SIM of AMPK thereby facilitating its activation during energy stress. Our study identified a conserved region in the N-terminal region of AMPK that conforms to the specifications of a SIM (Figure 37). Further characterization of this region demonstrated that it is important in regulating LKB1-AMPK interaction. Indeed, mutation of the important hydrophobic residues important for SIM recognition impeded the activation of AMPK when the cells were subjected to energy stress (Figure 39). Therefore, our observations firmly demonstrate the importance of SUMOylation in regulating LKB1-AMPK interaction and activation of this vital homeostatic pathway during energy stress.

CHAPTER VII: LKB1 K178 SUMOylation is essential in the AMPK signaling pathway.

Introduction

The AMPK signaling pathway is responsible for making sure that the cell survives in conditions of energy deprivation. Pathways that consume energy are inhibited while catabolic pathways are activated. In the event that this pathway is deregulated or the cell fails to react appropriately, fateful measures are taken and the apoptotic pathway is activated. The means by which apoptosis is activated is indirectly via mitophagy. Mitophagy ensures effective clearance of damaged mitochondria (Melser et al., 2013). Defective mitochondria lead to the accumulation of ROS and this triggers apoptosis (Murphy, 2009). Our model suggests that during energy stress, LKB1 is SUMO1 modified and this leads to the recognition, binding and activation of AMPK. Therefore, it was necessary we understand or investigate the physiological consequences of deregulating SUMOylation or much more specifically, the SUMO1 modification of LKB1 at lysine 178. To this effect, we examined whether AMPK signaling is defective in the LKB1 K178R mutant, resulting in impaired mitochondrial function, and induction of apoptosis (Shackelford et al., 2013).

Approach:

- Stably express empty vector (pBABE), wild-type LKB1, LKB1 mutant (K178R), or kinase-dead LKB1 (K78I) in LKB1 null A549 cells. Treat the cells with vehicle control (Dulbecco modified eagle medium [DMEM]) or with 10 µg/mL of CAMKK inhibitor STO-609 alone or with 2 mM phenformin for 48 hours to induce metabolic stress. Check the downstream pathways regulated by AMPK
- Investigate the rate of cell death among the stable cells described above when treated with either with 10 µg/mL of CAMKK inhibitor STO-609 alone or with 2 mM phenformin for 48 hours
- Determine the status of mitophagy in the untreated stable cells using MitoTracker Red
- Examine the oxygen consumption rates of the stable cells to determine the status of their mitochondrial health

Results:

A549 cells stably transfected with empty vector (pBABE), wild-type LKB1, LKB1 K178R, or kinase-dead LKB1 (K78I) were treated with vehicle control (Dulbecco modified eagle medium [DMEM]) or with CAMKK inhibitor STO-609 alone or with phenformin for 48 hours to induce metabolic stress. Immunoblotting for phosphorylation of the downstream substrates of AMPK, ACC1 at Ser79, ULK1 at Ser555, and Raptor at Ser792, showed that both the cells expressing the kinase-dead mutant and those expressing the LKB1 K178R mutant were defective in AMPK signaling (Figure 40). We also confirm that the K178R mutant is unable to regulate energy stress through the measurement of intracellular ATP levels (Figure 41).

Apoptosis, as demonstrated by cleaved PARP and caspase-3 (Figure 40) and annexinV and 7AAD staining (Figure 42), was increased in the phenformin-treated cells expressing empty vector (pBABE), kinase-dead LKB1, or LKB1 K178R mutant, but not wild-type LKB1. Remarkably, the basal levels of activated AMPK were relatively high in these LKB1-null A549 cells. Inhibition of CAMKK led to a decrease in AMPK activation but did not induce apoptosis (Figure 42). This is consistent with findings that illustrate phenformin's potency in inducing death in LKB1-null cells (Shackelford et al., 2013).

Since the phosphorylation of ULK1 is required for mitophagy (Egan et al., 2011), we checked the mitochondrial content of these cells using MitoTracker Red staining. Consistent with our previous biochemical results, LKB1-null cells reconstituted with the wild-type LKB1 had less retention of the mitochondrial fluorescent dye than cells expressing the empty vector or kinase-dead or LKB1 K178R mutant (Figure 43). These findings illustrate that LKB1 K178 SUMOylation is essential for ensuring an effective mitochondrial clearance, thereby producing energy for the cell during metabolic stress.

To assess the mitochondrial health of these different cells, we also checked the oxygen consumption rates as a measure of mitochondrial respiration. As expected, A549-vector, A549-LKB1 dead-kinase, and A549-LKB1 K178R mutant cells had lower basal oxygen consumption rates than A549 cells transfected with wild-type LKB1 (Figures 44A and B). Taken together, these data confirm that SUMO1 modification of LKB1 K178 is indeed important in maintaining cellular energy balance during metabolic stress.

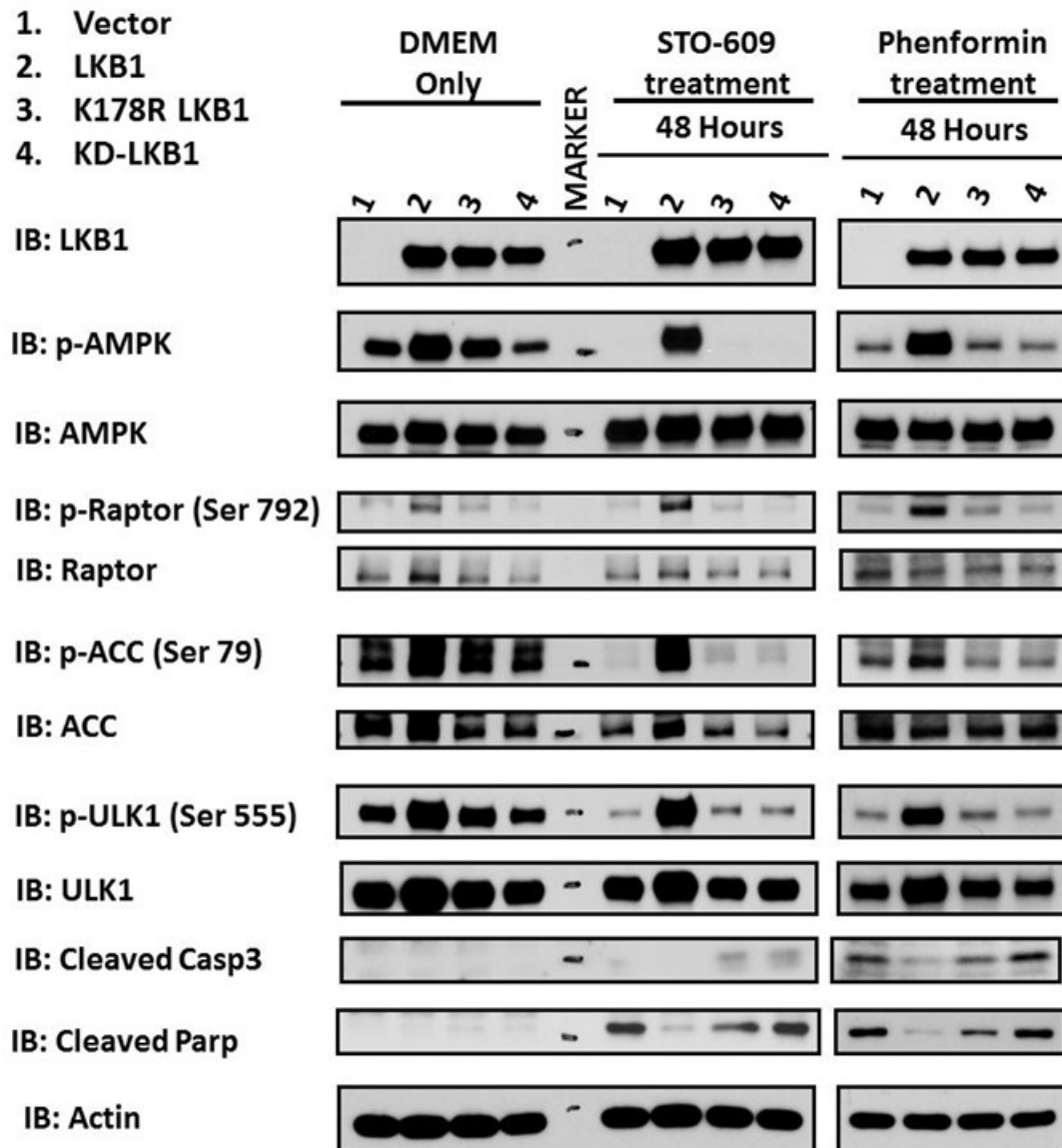


Figure 40. LKB1 K178 SUMOylation is important in regulating the activation of AMPK and its downstream targets during metabolic stress. A549 cells stably expressing empty vector (pBABE), wild-type LKB1, LKB1 mutant (K178R), or kinase-dead LKB1 (K78I) were treated with vehicle control (Dulbecco modified eagle medium [DMEM]) or with 10 μ g/mL of CAMKK inhibitor STO-609 alone or with 2 mM phenformin for 48 hours to induce metabolic stress. Cell lysates were subjected to western blot using the indicated antibodies.

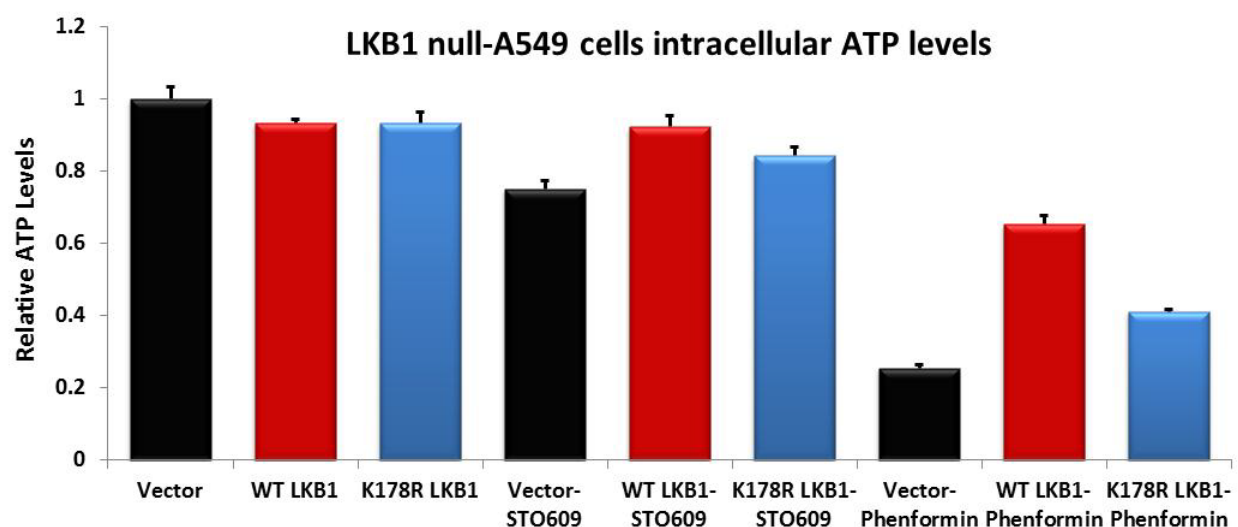


Figure 41. The role of LKB1 K178 SUMOylation in maintaining intracellular ATP levels during energy stress. A549 cells stably expressing empty vector (pBABE), wild-type LKB1, or LKB1 mutant (K178R) were treated with vehicle control (Dulbecco modified eagle medium [DMEM]) or with 10 μ g/mL of CAMKK inhibitor STO-609 alone or with 2 mM phenformin for 48 hours to induce metabolic stress. Intracellular ATP levels levels were evaluated in these cells. The experiment was run in triplicate and presented as the mean \pm S.D. (error bars).

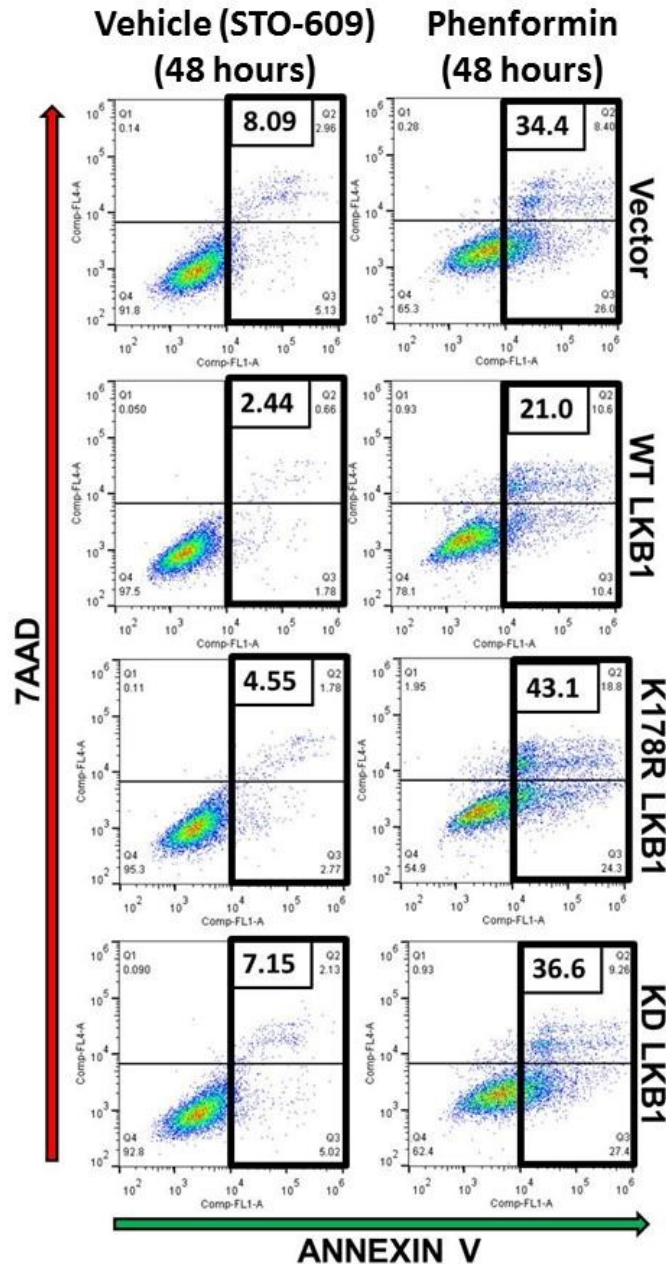


Figure 42. LKB1 K178 SUMOylation is important in preventing apoptosis during metabolic stress. Cells stably expressing empty vector (pBABE), wild-type LKB1, LKB1 mutant (K178R), or kinase-dead LKB1 (K78I) were stained with annexinV and 7AAD after a 48-hour treatment with vehicle (10 μ g/mL STO609) alone or with 2 mM phenformin and subjected to fluorescence-activated cell sorting (FACS) analysis.

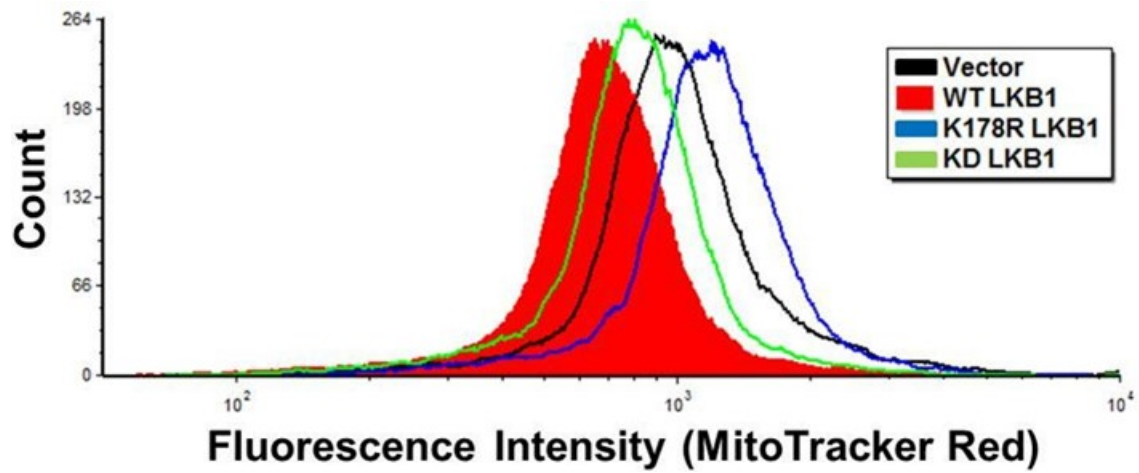


Figure 43. The role of LKB1 K178 SUMOylation in regulating mitophagy.

Cells stably expressing empty vector (pBABE), wild-type LKB1, LKB1 mutant (K178R), or kinase-dead LKB1 (K78I) were stained with MitoTracker Red and subjected to fluorescence-activated cell sorting (FACS) analysis.

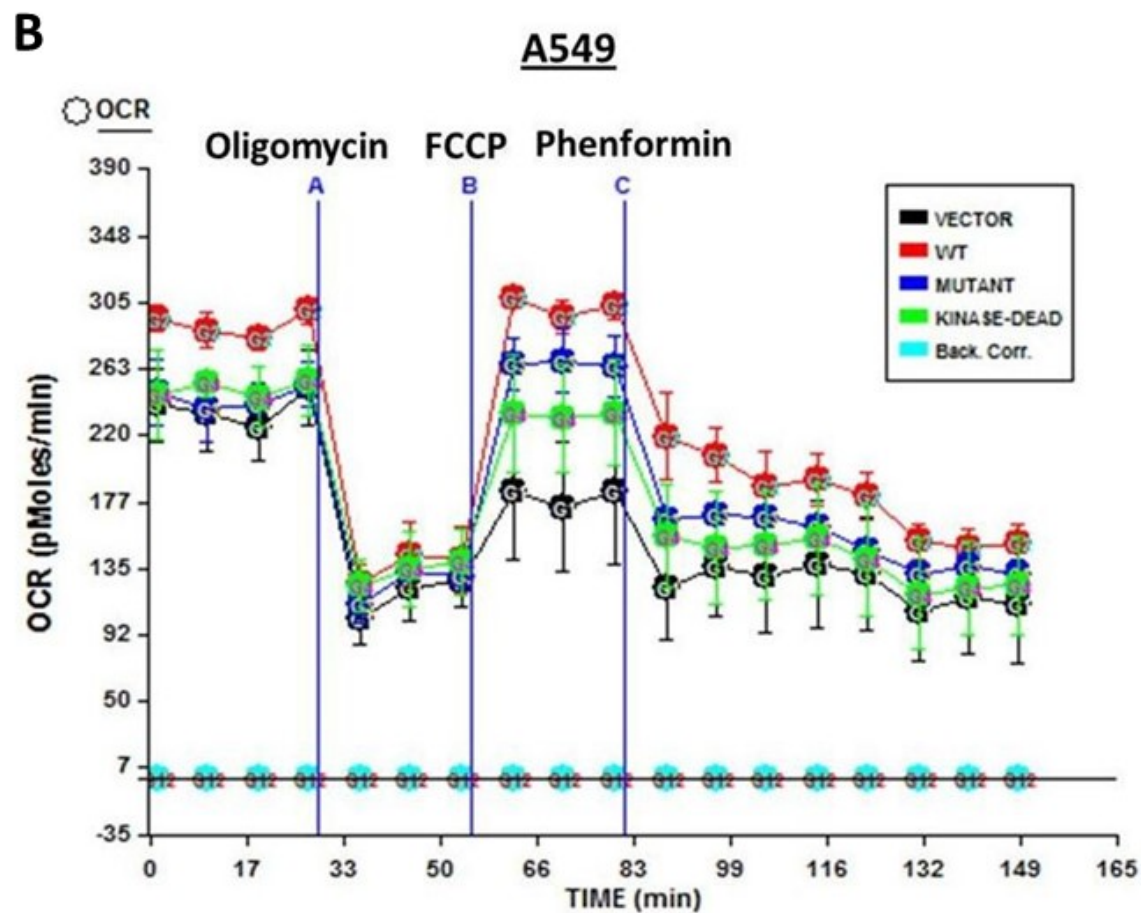
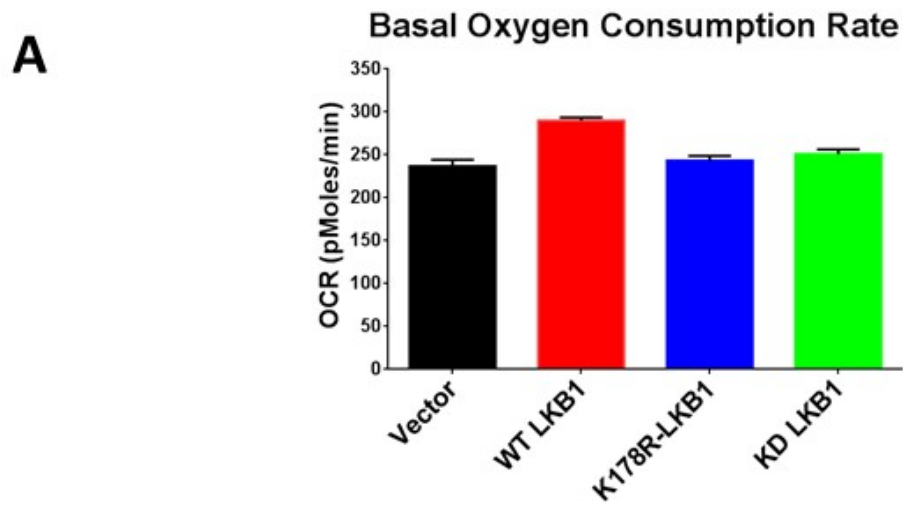


Figure 44. LKB1 K178 SUMOylation is important in regulating mitochondrial health

(A) Oxygen consumption rates (OCR) of cells stably expressing empty vector (pBABE), wild-type LKB1, LKB1 mutant (K178R), or kinase-dead LKB1 (K78I) were analyzed. (B) The metabolic profile of the same cells were assessed by sequentially treating them with oligomycin (1 μ M), FCCP (1 μ M), and phenformin (5 mM). These data were generated and analyzed using Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience). Error bars represent mean \pm S.E.M.

Conclusion

Our study has so far demonstrated that during energy stress, LKB1 is increasingly SUMO1 modified and this helps in the recognition, binding, and activation of AMPK. Therefore, it was imperative that we investigate and characterize the physiological relevance of LKB1 SUMOylation in maintaining energy balance. In this chapter, we show that the K178R SUMO mutant was incapable activating AMPK and its downstream targets during energy stress (Figure 40). AMPK is vital in the activation of the mitophagy inducer ULK1. We also demonstrate that mitophagy is altered when SUMOylation is deregulated (Figure 43). This leads to the accumulation of defective mitochondria, eventually leading to cell death due to increased ROS levels (Figure 42). The oxygen consumption rate of cells expressing the K178R SUMO mutant was also lower than the cells expressing wild type LKB1 (Figure 44).

The important observations made in this and previous chapters consequently validate the importance of SUMOylation in regulating energy homeostasis of a cell.

CHAPTER VIII: List of conclusions

List of Conclusions and model (Figure 45)

1. Characterization of LKB1 SUMOylation during energy stress

- 1.1 LKB1 is SUMOylated
- 1.2 SUMO1 modification of LKB1 increases during energy stress
- 1.3 During metabolic stress, LKB1 lysine 178 is a critical SUMO1 conjugation site

2. The role of SUMOylation in regulating LKB1-AMPK interaction

- 2.1 LKB1 K178R SUMO mutant has a decreased affinity to AMPK
- 2.2 SUMO1 is responsible for the increase in LKB1-AMPK interaction during energy stress
- 2.3 AMPK SUMO-interacting motif (SIM) regulates LKB1-AMPK interaction and consequently AMPK activation

3. The phenotypic readout when LKB1 SUMOylation is altered during metabolic stress

- 3.1 LKB1 K178R SUMO mutant has a defective AMPK signaling during energy stress
- 3.2 LKB1 lysine 178 SUMOylation is essential in ensuring effective mitochondrial clearance during metabolic stress
- 3.3 The oxygen consumption rates (a measure of mitochondrial respiration) is decreased in LKB1 K178R SUMO mutant-expressing cells

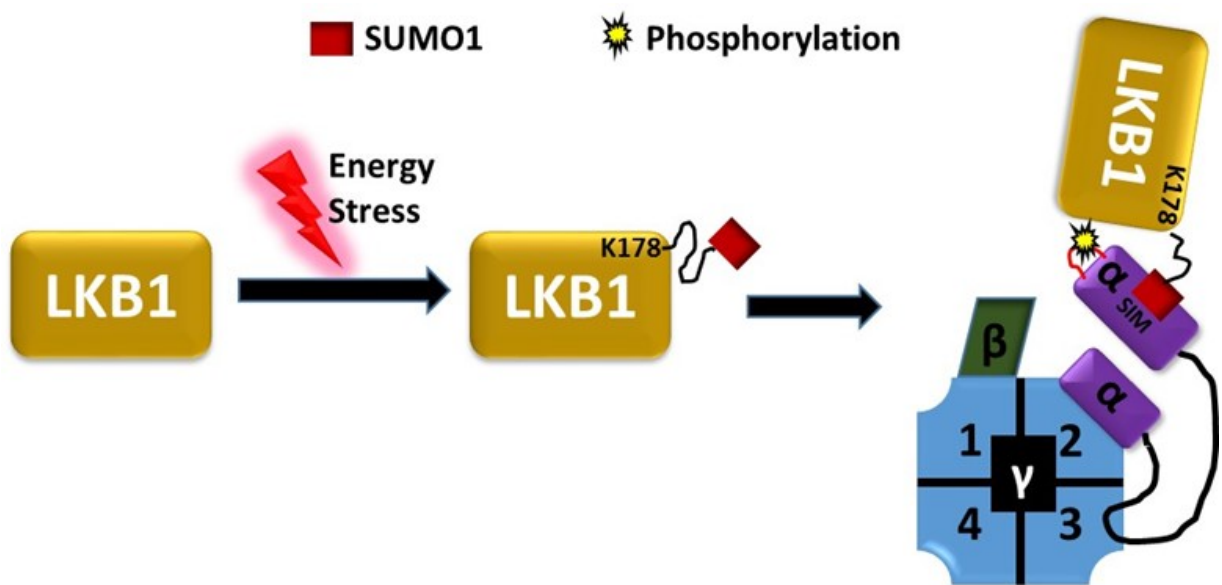


Figure 45. Proposed model illustrating the SIM mediated activation of AMPK.

During metabolic stress, SUMO1 modification of LKB1 lysine 178 is enhanced and is essential in promoting its interaction with AMPK via a SUMO-interacting motif (SIM). This increases AMPK activation consequently ensuring the cell maintains energy equilibrium. The LKB1 K178R SUMO mutant resulted in defective AMPK signaling and mitochondrial function, inducing death in energy-deprived cells.

CHAPTER IX: Discussion

Discussion

Immense strides have been made in the characterization of the SUMOylation pathway since its discovery around 20 years ago. Several seminal studies have catapulted it as an indispensable post-translational modification that is essential in diverse biological processes. For a while, the SUMO-targeted substrates remained elusive in the SUMOylation field. We now have a long list of substrates that are SUMOylated thanks to the recent proteomic efforts and strategies. Studies have demonstrated that SUMOylation can stabilize a protein, change its subcellular location, or even alter the protein's catalytic activity. It is also appreciated that at the molecular level, SUMOylation can: (1) inhibit the interaction between the target and its interacting partner; (2) enhance the interaction of protein partners through creation of a binding surface where the target would recognize the partner via a SUMO-interacting motif (SIM); or (3) change the conformation of the target, thereby altering its function (Geiss-Friedlander and Melchior, 2007, Wilkinson and Henley, 2010). With such essential functions, it's therefore no surprise that SUMOylation has emerged as an influential player in regulating cellular function.

Despite its dynamic nature, we now understand that SUMOylation is vital for proliferation (Dadke et al., 2007), normal heart function (Wang and Schwartz, 2010), DNA damage response (Jackson and Durocher, 2013), and also immune system development (Liu et al., 2004, Van Nguyen et al., 2012). More importantly, it's been shown that SUMOylation has a pivotal role at the helm of apt adaptation in the advent of cellular stress (Flotho and Melchior, 2013, Tempe et al., 2008). However, at the moment, there's contradictory information as to the standard response of SUMOylation to different stress agents. For example, oxidative, ethanol, heat or osmotic stress lead to increased global SUMO2/3 conjugation (Saitoh and Hinchey, 2000). On the other hand, the net SUMO1 conjugation remains unchanged. Interestingly, a study

by Ja Lee et al., demonstrated that overexpression of SUMO1 or SUMO2 made neuroblastoma cells more impervious to death mediated by glucose or oxygen deprivation (Lee et al., 2009). Conversely, knockdown of SUMO1 but not SUMO2 rendered the cells susceptible to death in the same stressful conditions. Despite all the conflicting observations, it is well appreciated that, this study and many others highlight the vital role SUMOylation plays in maintaining cellular homeostasis. The detailed mechanisms, nevertheless, remain largely unexplored. In addition, insights into its fundamental role in regulating metabolism are still in its nascent stages (Wilkinson and Henley, 2010). Our study hence elaborates on how SUMOylation influences or regulates metabolism and defines the SUMOylation changes that occur to maintain homeostasis in conditions of metabolic stress.

Biochemical and genetic studies have identified LKB1 as the major upstream kinase of AMPK, a crucial player involved in sensing energy stress and eliciting the correct adaptive responses to ensure cell survival (Hardie and Alessi, 2013). During energy stress, anabolic processes such as protein synthesis are inhibited, while processes like glycolysis that generate energy are activated. AMPK is also involved in regulating mitophagy (Egan et al., 2011). This process facilitates the effective elimination of defective mitochondria (Melser et al., 2013). Accumulation of malfunctioning mitochondria leads to increase in levels of reactive oxygen species, thereby initiating apoptosis (Murphy, 2009). This highlights the significance of LKB1 as an essential player involved in calibrating the cell's energy balance and regulation of cell survival.

A number of studies have described the influence of covalent post-translational modifications in governing LKB1 activity (Alessi et al., 2006, Lee et al., 2015, Lan et al., 2008). However, the role of SUMOylation in regulating LKB1 function was unknown. Our study, for

the first time, demonstrates that LKB1 is indeed SUMOylated. This created the important foundation upon which we could study and characterize SUMOylation's role in regulating LKB1 function and ultimately the cell's metabolic homeostasis. Owing to the multifaceted roles SUMOylation plays in the eukaryotic cell, we accordingly hypothesized that, during energy stress, SUMOylation regulates the LKB1-AMPK interaction and that this subsequently affects the kinase activity of AMPK.

Our first task was to identify the appropriate agent that would effectively cause an energetic imbalance in the cells. Many agents have been studied and characterized in defining their role in creating an energy deficit in cells. Glycolytic inhibitors such as 2-DG act by inhibiting enzymes involved in the catalysis of glucose to pyruvate that results in ATP generation (Wick et al., 1957). However, in normal cases, this process is almost an inadequate way of generating energy. Only cancer cells derive their energy using this process in a phenomenon known as the Warburg effect (Warburg et al., 1926, Warburg, 1956). The most efficient way of producing ATP to sustain the cell's normal processes is via the mitochondria. This is achieved via oxidation phosphorylation where ~36 mol ATP/mol glucose are produced per glucose molecule compared to the 2 mol ATP/mol glucose generated by the glycolytic pathway (Vander Heiden et al., 2009). As a result, deregulation of normal mitochondrial function could prove detrimental to the cell's energetic balance. Agents characterized to efficiently inhibit the mitochondria include biguanides such as phenformin or metformin. Biguanides inhibit mitochondria's complex I subsequently leading to a high intracellular AMP/ATP ratio (Dykens et al., 2008, El-Mir et al., 2000, Owen et al., 2000). However, there is a caveat when using metformin as it relies on the expression of Organic transporter 1 (OCT1) for its uptake, for that reason, limiting its efficacy to hepatocytes that highly express the transporter (Shu et al., 2007).

On the other hand, phenformin has a higher bioavailability and is 50 fold more potent (Owen et al., 2000, Dykens et al., 2008). In line with these studies, we also confirmed via the measurement of intracellular ATP in HEK293 cells that phenformin is more effective at reducing ATP (Figure 15). Based on our results and previous studies, phenformin therefore became our preferred choice of energy stress inducer in our experiments.

Interestingly, when cells were subjected to phenformin treatment, we observe a global decrease in SUMO1 and SUMO2/3 conjugated proteins. We also illustrate that this reduction is through the proteasome degradation pathway. This follows with the notion that the apparent way a cell can survive the energy deficit is by degrading proteins to generate ATP. Remarkably, we observe that the SUMO1 levels of LKB1 increase while its SUMO2/3 conjugation decreases. We posit that the pool of SUMO2/3-modified LKB1 is degraded, possibly through the SUMO targeted ubiquitin degradation process. Studies have shown that enzymes such as RNF4 specifically target SUMO2/3 paralogues and ubiquitinate them. This eventually leads to the degradation of specific proteins (Sun et al., 2007). Though much more work needs to be done to prove this hypothesis, it is safe to speculate that an enzyme such as RNF4 might be activated to degrade proteins in times where energy or ATP is required. This creates a much needed energy balance and prevents cell death. This could also highlight an additional example of the critical interplay of several post-translational modifications that ensure proper cell adaptation in the advent of stressful conditions.

We demonstrate in our study that the increase in SUMO1 conjugation of LKB1 during energy stress proves to be important in regulating AMPK activation. In situations where the SUMO protease SENP1 was overexpressed, we noted a decline in AMPK activation in both stressed and unstressed cells. Indeed, we demonstrate that SENP1 regulates the

autophosphorylation of LKB1 and subsequently its catalytic activity. Our data suggest that SUMOylation may directly or indirectly help maintain the catalytic activity of LKB1. Therefore, to some extent, our findings help explain how the constitutively active status of LKB1 is maintained. Also of note is that energy stress leads to the degradation of SUMO proteases SENP1 and SENP2. This also indicates that just like cellular energy, a SUMOylation balance has to be in check. We demonstrate that energy stress leads to a shift in the SUMOylation pathway that ensures maximal SUMOylation-mediated LKB1 activation allowing the appropriate cellular response to combat the stress. Our study hence establishes the important role SUMOylation plays in maintaining the cell's energy balance.

Upon discovering that the SUMO1 modification of LKB1 is upregulated during stress, we utilized a SUMOylation site prediction software (Ren et al., 2009) that took into consideration the different types (i.e., through consensus and non-consensus motifs) of SUMO conjugation. Though initially it was a general search, our true purpose was to find a SUMO1 specific site that is regulated by energy stress. Several sites were suggested but we selected ones with a high cutoff score. Through mutagenesis and characterization of the sites, lysine 178 proved to be the site that is important for SUMO1 conjugation during metabolic stress. Indeed, its SUMO1 modification status changed upon treatment with the energy stress inducer phenformin. Interestingly, lysine 178 is not conjugated by SUMO2/3, ubiquitin, or acetylation in conditions of metabolic stress. And even though it is close to the catalytic loop, it did not alter the autophosphorylation or catalytic activity of LKB1. Puzzlingly, the K178R LKB1 mutant was incapable of phosphorylating and activating AMPK when cells were subjected to energy stress. This proved to be a conundrum since we had demonstrated that the SUMO1 mutant is functional (associates and forms an active heterotrimeric with STRAD and MO25) and catalytically active

(can phosphorylate recombinant AMPK protein via an *in vitro* system), yet during stress the mutant is unable to activate AMPK.

As I stated before, at a molecular level, there are three outcomes to a SUMOylated protein. One of the consequences is to enhance protein-protein interaction through the recognition of its partner's SUMO interacting motif. This provided us a viable option or direction to look into and investigate what was preventing the K178R LKB1 SUMO1 mutant from activating AMPK during conditions of energy stress. Indeed, more analysis demonstrated that SUMOylation (by means of overexpressing SUMO protease SENP1 hence reducing global LKB1 SUMOylation) is critical for LKB1-AMPK interaction. More specifically, our findings also show that the K178R LKB1 mutant had a lower affinity to AMPK in conditions of metabolic stress. This observation, for the first time, posits the possible involvement of a SIM in regulating the manner the cell responds and adapts to stress.

Using the crystal structure depicting the non-covalent recognition of SUMO1 by RanBP2 as a structural basis, we identified a SIM in the N-terminal region of AMPK. Mutation of the hydrophobic residues necessary for SUMO1 interaction prevents LKB1 from recognizing and activating AMPK. Interestingly, in the AMPK crystal structure produced with a truncation in the carbohydrate-binding module (also called glycogen-binding domain) of the β subunit (PDB id 4CFH), the kinase N-lobe surface containing the SIM is completely solvent exposed, and the kinase domain is in its fully activated conformation (Xiao et al., 2011). Conversely, in the AMPK structure including the full-length carbohydrate-binding module, the “C-interacting helix” (residues 161-170) of the β subunit (4CFE) binds close to the SIM, contacting the catalytically important α C helix of the kinase domain. In this structure, the α C helix is in a catalytically inactive conformation, supposedly promoted by the C-interacting helix (Xiao et al.,

2013). The C-interacting helix has above-average B-factors in the crystal structure, suggesting increased mobility and hence a loose interaction. We therefore speculate that binding of SUMO1 to the SIM also promotes AMPK activation by displacing the C-interacting helix, and hence may allosterically activate AMPK by rearranging the α C helix, as seen in many other kinases (Endicott et al., 2012). Our study, as a result, illustrates for the first time that a SIM allows for the recognition and activation of AMPK by LKB1 during stress, ultimately ensuring cell survival.

To validate our model in the context of cellular function, we undertook several phenotypic experiments to establish that SUMOylation was critical in the energy metabolism pathway. During energy stress, AMPK activates several key proteins involved in recalibrating the metabolic balance in the cell. We illustrate that compared to the wild type LKB1, the K178R LKB1 SUMO mutant was incapable of activating or inhibiting downstream targets important for energy homeostasis. More specifically, the LKB1 null A549 cells expressing empty vector, LKB1 SUMO mutant or a kinase dead mutant had low mitophagy (as shown where low phosphorylation and activation of mitophagy-inducer ULK1 were observed), which leads to accumulation of defective mitochondria. MitoTracker red, a fluorescent dye that stains and quantifies mitochondria in live cells, confirmed that A549 cells containing the wild type LKB1 cDNA, had less mitochondrial content compared to the counterpart vector, LKB1 SUMO mutant or kinase dead mutant-expressing cells. However, their oxygen consumption or respiration rates were higher, indicating they had better mitochondrial health. The accrual of defective mitochondria in the mutant cells during energy stress led to an increase in ROS levels, thereby causing cell death. Altogether, we introduce and establish the essential role SUMOylation plays in regulating cellular energy balance.

Collectively, the findings in my thesis project put forward a model (Figure 45) in which energy stress triggers SUMO1 modification of LKB1 at lysine 178, leading to the recognition, binding and activation of its downstream target and energy sensor, AMPK. This significantly places SUMOylation front and center in the regulation of metabolic homeostasis thereby ensuring cellular health and survival.

CHAPTER X: Future directions

Future directions

My thesis work has identified an important role SUMO interacting motifs play in regulating protein-protein interaction. For the first time, we have illustrated that the SIM of AMPK is vital in augmenting LKB1-AMPK interaction thereby ensuring an apt response in the advent of energy deprivation, leading to cellular energy balance. As a result, our work opens up several avenues for future work.

Examine whether SIMs are involved in regulating the interaction between LKB1-STRAD-MO25 heterotrimeric complex and its other downstream targets

Over the years, LKB1 has emerged as an essential master kinase involved in activating several AMPK related kinases such as BRSK1, BRSK2, NUA1, NUA2, MARK1, MARK2, MARK3, MARK4, QSK, SIK1, SIK2, and SNRK (Lizcano et al., 2004) (Figure 46). These downstream targets are implicated in essential processes such as cell polarity, muscle growth and differentiation, and senescence to name a few. More details about the vital functions the LKB1 downstream targets play are illustrated in Figure 46. We therefore intend to examine whether SIMs are important in aiding LKB1 in recognizing these substrates, thereby activating them. We will undertake several strategies to identify the potential SIMs:

- Utilizing the same strategy described in my thesis, we intend to study the crystal structure depicting the non-covalent recognition of SUMO1 by RanBP2 to map out the possible SIMs in the LKB1 targets.
- We will use the AMPK SIM sequence we discovered to further streamline the search and investigate whether there are sequences that conform to ours.
- If the second strategy does not yield any results, we will scrutinize for sequences that conform to the predicted SIMs illustrated in figure 9.

All things considered, the identification and characterization of SIMs in these targets will not only open up a lot of opportunities of research but will highlight the important role SUMOylation plays in regulating critical processes that maintain cellular health and function.

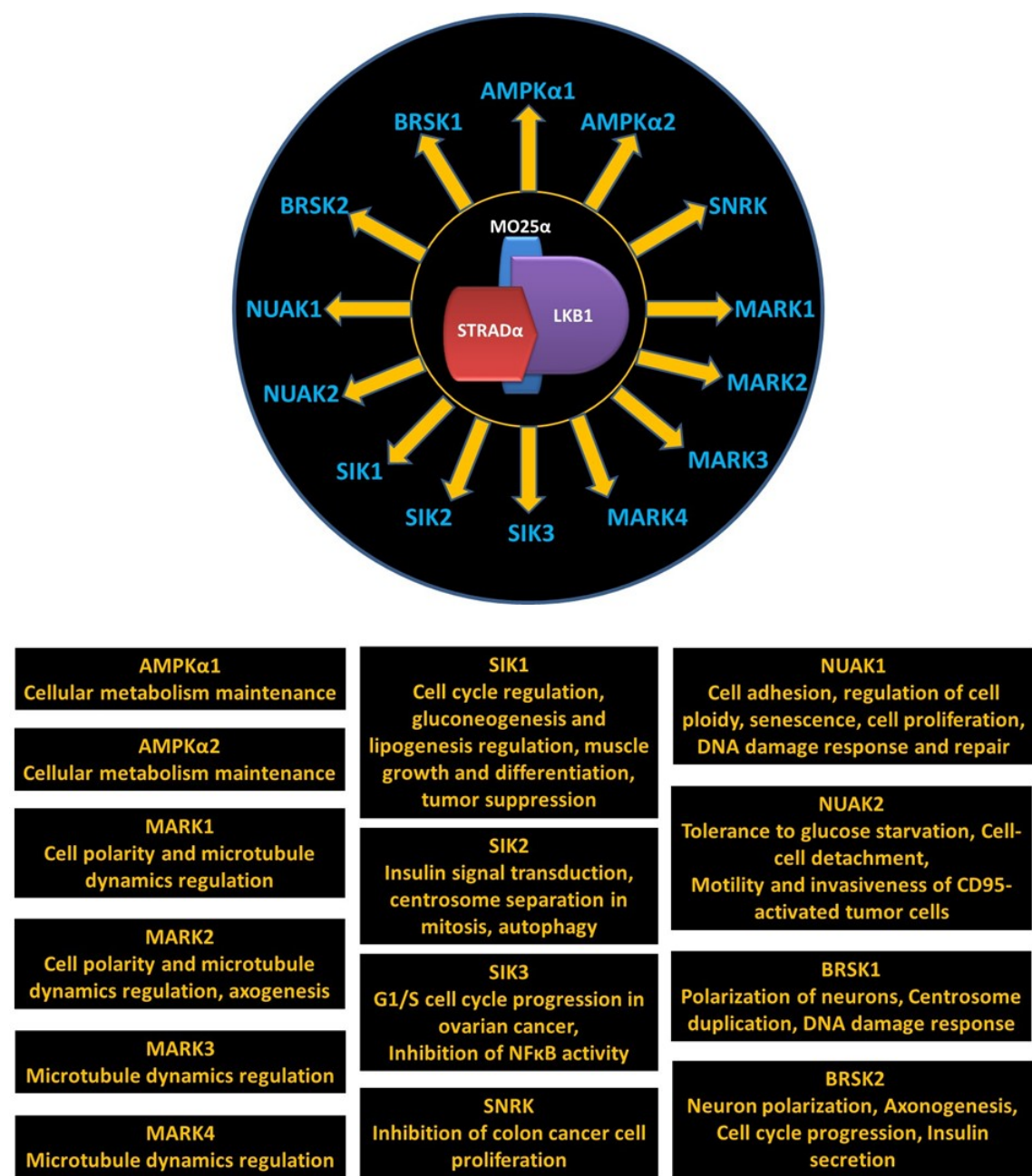


Figure 46. LKB1 is a master kinase that activates AMPK and 12 of its related kinases

A figure illustrating the kinases LKB1 activates and their respective functions.

Acknowledgements

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Vita

Joan Wairimu Ritho was born in Nakuru, Kenya on November 15th 1984 to two amazing and loving parents, Colonel Samuel Ritho Mwangi and Margaret Gatere Gakenia. She is an older sister to Captain Ronnie Mwangi Ritho and Andrew Gatere Ritho. She attended high school at Moi Forces Academy- Lanet from 1999-2002. This is where her interest and passion for cancer biology began. She moved to the United States of America, on her own, at the age of 18 in pursuit of a better education and life. Though the venture felt daunting, she was more excited about immersing into and exploring the unknown. She enrolled at Muscatine Community College, in 2003, and transferred her college credits to Simpson College in Indianola IA. There, she met two inspiring professors, Dr. Pat Singer and Dr. Timothy Steele, who would steer, push, and stimulate her passion for cancer research. She graduated Cum Laude from Simpson College in 2007 with a Bachelor of Arts degree in Biochemistry and a minor in Chemistry. In order to gain more scientific experience, she got a job as a research technician from 2007 to 2009 in Dr. Aravinda Chakravarti's lab at Johns Hopkins Medical Institute (McKusick-Nathans Institute of Genetic Medicine) - Baltimore, MD. In September of 2009, Joan began her Ph.D. training in Cancer Biology with the University of Texas Health Science Center at Houston and MD Anderson Cancer Center. She joined the lab of Dr. Edward Yeh in August 2011, where she investigated the role of SUMOylation in regulating LKB1 function. In May 2015, Joan was awarded the degree of Doctor of Philosophy in Cancer Biology.