IDENTIFICATION OF A NUCLEAR EXPORT SIGNAL IN THE CATALYTIC SUBUNIT OF AMP-ACTIVATED PROTEIN KINASE

by
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ABSTRACT

Nevzat Kazgan: IDENTIFICATION OF A NUCLEAR EXPORT SIGNAL IN THE CATALYTIC SUBUNIT OF AMP-ACTIVATED PROTEIN KINASE

(Under the direction of Jay E. Brenman)

AMP-activated protein kinase (AMPK) is an energy sensor that regulates both cellular and organismal energy levels through phosphorylation, leading to the activation or inhibition of target proteins. AMPK functions as a heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits. Until recently, research on AMPK was based on cell culture studies and the use of non-specific drugs. Since there are multiple isoforms of each subunit of AMPK, it was difficult to characterize AMPK's role due to genetic redundancy. In our lab, we identified the first null mutation of the AMPK α subunit in the fruit fly, *Drosophila Melanogaster*. Studying AMPK in fruit flies is somewhat ideal since all of its subunit functions and domains are conserved from flies to human and also since there are only one gene for each subunit of AMPK, overcoming the redundancy effect.

During my graduate study, we have characterized AMPK regulation in three distinct aspects. First, we identified AMPK's role in cell polarity and cell division. We demonstrated loss of AMPK activity causes loss of epithelial cell polarity and over proliferation under

energetic stress. Second, we characterized AMPK's role at the whole organism level. Reduced AMPK causes hypersensitivity to starvation conditions and this causes a shortened life span and higher locomoter activity under energetic stress. In addition, loss of AMPK function causes inability to process and store lipids efficiently. Third, we identified a highly-conserved nuclear export sequence (NES) at the very C-terminal end of AMPKα. We showed that the loss of this sequence leads to increased nuclear localization. A transgenic fly expressing a truncated copy of AMPKα does not rescue the lethality or neuronal phenotype of AMPKα null mutant flies. A truncated copy also shows a reduced phosphorylation rate -- proposing AMPK's phosphorylation and activation takes place in the cytoplasm.

To my mom and dad, my wife and rest of my family,
for their loving and constant support.

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As we all know, graduate school is not an easy road trip. It is a challenging and also a rewarding experience. I have been fortunate to be surrounded by people who have helped me in this challenging experience. Therefore, I must thank first and foremost to Jay, a mentor in every sense of the word. I have learned a lot from him. Thanks to my committee members, Franck, Jeff, Manzoor and Steve. They have been the best committee out there. Thanks to John and Rob for helping me through the stressful times of my last years. Thanks to Jrgang, who is a true friend and has been very helpful with my projects. Thanks to all current and former members of Brenman Lab for creating a happy environment for me. Thanks to my wife, Sumeyye, for being patient when I had to spend countless hours in the lab. I could not be where I am without her support. Thanks to my friend Murat for his support in making the right decisions.

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CHAPTER I

Background and Significance

Introduction

AMP-activated protein Kinase. All cells require energy to survive. They constantly take energy from the environment to sustain ATP levels. Cellular mechanisms, including catabolism, provide energy to the cell by converting ADP to ATP. Conversely, most cellular reactions use energy by breaking down ATP to ADP, or in some cases ATP to AMP (anabolism). What will be the deciding factor for a cell on the catabolism to anabolism ratio? Which cellular mechanisms/protein(s) will help cells to maintain energy equilibrium?

AMP-activated protein kinase (AMPK) is one of the major players sustaining that energy equilibrium from yeast to humans. AMPK was first discovered in cell extracts for its function of phosphorylating and inactivating the key enzymes of lipid biosynthesis (Haystead et al., 1989). In the budding yeast *Saccharomyces cerevisiae*, the mammalian AMPK orthologue SNF1 (Sucrose Non-Fermenting 1) was first discovered from a forward genetic screen where mutant yeast strains could not grow on a non-glucose containing media. (Celenza and Carlson, 1986). In yeast, SNF1 is essential for the switch from glucose to other carbon sources and for the switch from the fermentative/anaerobic to oxidative metabolism (Carlson, 1999). In almost every organism, including yeast, worm, fruit flies and all mammals, AMPK/SNF1 kinase plays an essential role in cellular responses to metabolic stresses.

AMPK, especially in mammals, is sensitive to the cellular AMP: ATP ratio and is activated by a high AMP level or by metabolic stresses that either stimulate ATP usage or interfere with ATP production. Stresses such as hypoxia and glucose deprivation will inhibit ATP production and biosynthetic pathways; on the other hand, growth and protein translation, fatty acid synthesis, activation of motor proteins, ion pumps or channels will increase ATP consumption.

How does AMPK sense the energy level in the cell and react to it? Under normal conditions, the cells will keep a low ADP:ATP ratio. An enzyme, adenylate kinase, will then catalyze the reaction of 2ADP ←→ ATP + AMP in favor of ADP synthesis and so the cellular AMP:ATP level will be lowered and AMPK will stay in an inactive form. When the cells are subject to metabolic stresses which interfere with the ATP synthesis or pathways that accelerate ATP consumption, then the ADP:ATP ratio will increase and this will cause Adenylate Kinase to increase the cellular AMP level and AMPK will become active. As a result, AMPK will turn-on catabolic pathways such as increasing fatty acid oxidation and glucose uptake and inhibit ATP consuming processes such as synthesis of proteins, fatty acids, cholesterol and glycogen. (Fig 1-1)

AMPK is a Serine/Threonine kinase. Kinases are well-known proteins which regulate other proteins and downstream targets by phosphorylating them and thereby inhibiting or increasing their activities. AMPK achieves its cellular energy sensor ability by phosphorylation of metabolic enzymes and transcription factors that regulate gene expression.

Figure 1-1

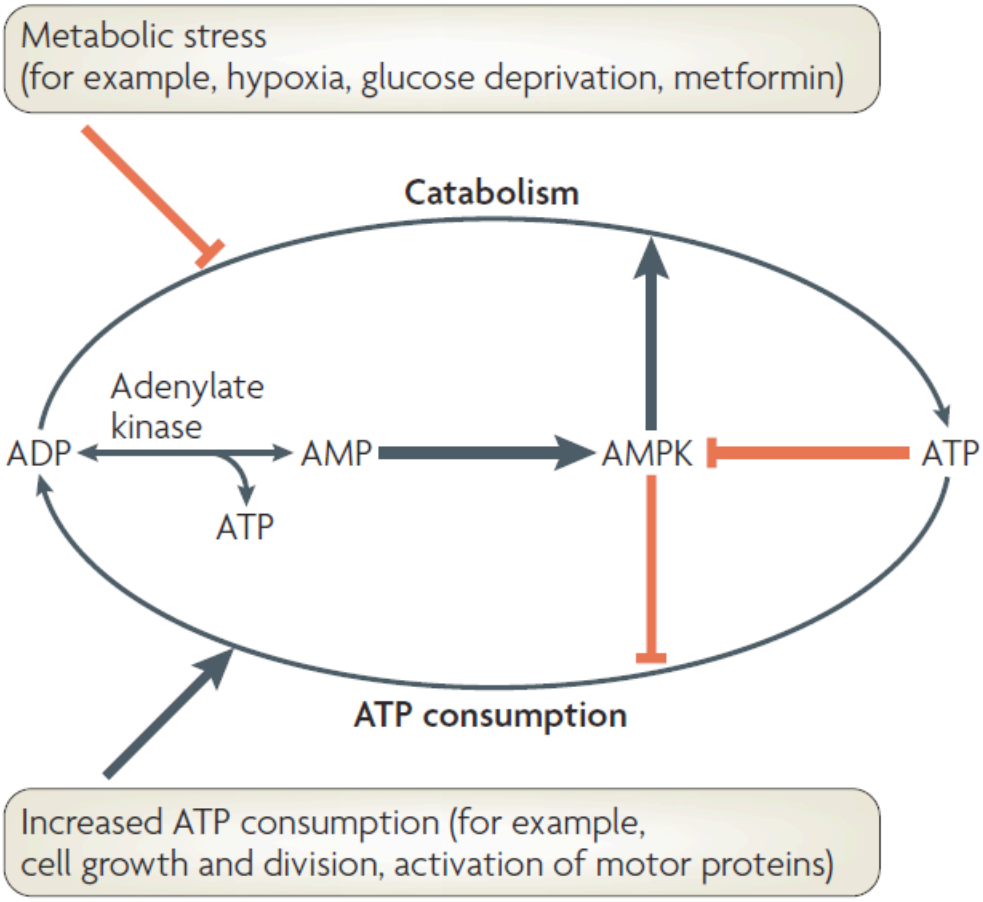

Figure from Hardie DG, Review, Nature Reviews, 2007

Fig 1-1. **AMPK is a cellular energy sensor.** Under normal conditions the cells maintain a high ratio of ATP: ADP**.** This will cause Adenylate kinase to generate high ADP levels and the cellular AMP:ATP level will be low. AMP-Activated protein kinase (AMPK) is therefore inactive. When the cells are subject to a metabolic stress that inhibits ATP synthesis, such as glucose deprivation or hypoxia, then Adenylate kinase will convert ADP to ATP. This will lead to an increased AMP level in the cell, and AMPK will become active. Once active, AMPK will balance the energy level in the cell by increasing catabolism and decreasing the rate of anabolism.

Does AMPK only sense cellular energy level or does it have any function at the organismal level? In recent years, it has been discovered that AMPK is a master regulator of food intake. It has been shown that fasting will increase the activity of an isoform of the catalytic subunit of AMPK (α2 subunit) in the hypothalamus. On the other hand, an increased level of insulin and glucose will decrease AMPK activity in the hypothalamus. Leptin is an adipose derived hormone which regulates food intake at the organismal level. Leptin will act on the receptors of the hypothalamus and cause a decrease in food intake. It has been shown that leptin will inhibit AMPK in the hypothalamus and this inhibition is the key for leptin's anorexigenic effects (Minokoshi et al., 2004).

AMPK subunits' structure and regulation. AMPK is a heterotrimeric protein complex which consists of catalytic α subunit and regulatory β and γ subunits. To be able to have an active AMPK complex, co-expression of all three subunits is required in mammalian cells (Woods et al., 1996).

The α subunit is the kinase and it includes a well-conserved N-terminal Ser/Thr kinase domain as well as a conserved Thr172 a. acid that must be phosphorylated for activation (Hawley et al., 1996). After being activated by an upstream regulator, the AMPK complex will bind to its substrates and transfer the phosphate from ATP to downstream targets (Woods et al., 1994). The region C-terminal to kinase domain includes an α helix domain which acts as an auto-inhibitory sequence (AIS). Constructs including this C-terminal region have been found to be 10-fold less active when expressed in bacteria (Pang et al., 2007). The AMPKα subunit also has a carboxy terminus domain which is characterized as the binding domain of the regulatory β and γ subunits (Iseli et al., 2005) . There are two α

subunit isoform genes (α1/α2) in mammals and only one (Snf1) in budding yeast and fruit fly, *Drosophila Melagonaster*.

The regulatory subunit β acts as a scaffolding protein where it's C-terminal domain bridges the α and γ subunits and forms the AMPK complex. The β subunit is proposed to have a central glycogen binding domain where the AMPK complex comes into association with glycogen in intact cells (Polekhina et al., 2003). This domain has been co-crystallized with β-cyclodextrin, a circular glucan of seven glucose units (Polekhina et al., 2005). A few hypotheses as to why AMPK would bind to glycogen exist 1) glycogen synthase is a known substrate of AMPK, so proximity might dictate the interaction, or 2) AMPK senses the cellular glycogen/energy level and reacts to it using its glycogen binding domain. There are two β subunit isoform genes (β1 and β2) which exist in mammals and three in the budding yeast, whereas fruit flies have only one β subunit.

The AMPKγ subunit includes 4 conserved 60 a. acid tandem repeats named after their discoverer Bateman as CBS motifs (first found in the enzyme *cystathionine* β *-synthase*) (Bateman, 1997) and variable N-terminal regions. Currently, the function of the N-terminal regions is not known. A short sequence immediately preceding this N-terminal region is involved in binding to the β subunit (Viana et al., 2007). On the other hand, the well-conserved 4 CBS motifs will act as pairs to form two Bateman domains which act as nucleotide binding sites. AMP and ATP will compete to bind these regions and regulate their activity level. Mutations in these regions will reduce AMP binding, suggesting they are binding sites for nucleotides. In addition, ATP will bind to these regions with a mutually exclusive manner to AMP but with five-fold lesser affinity than AMP binding, explaining how a low level of total cellular AMP can compete with a high level of ATP in the cell (Scott

et al., 2004). There are three AMPKγ subunit isoform genes (γ1, γ2, and γ3) which exist in mammals, whereas the budding yeast and fruit flies have only one γ subunit (Snf4).

Figure 1-2

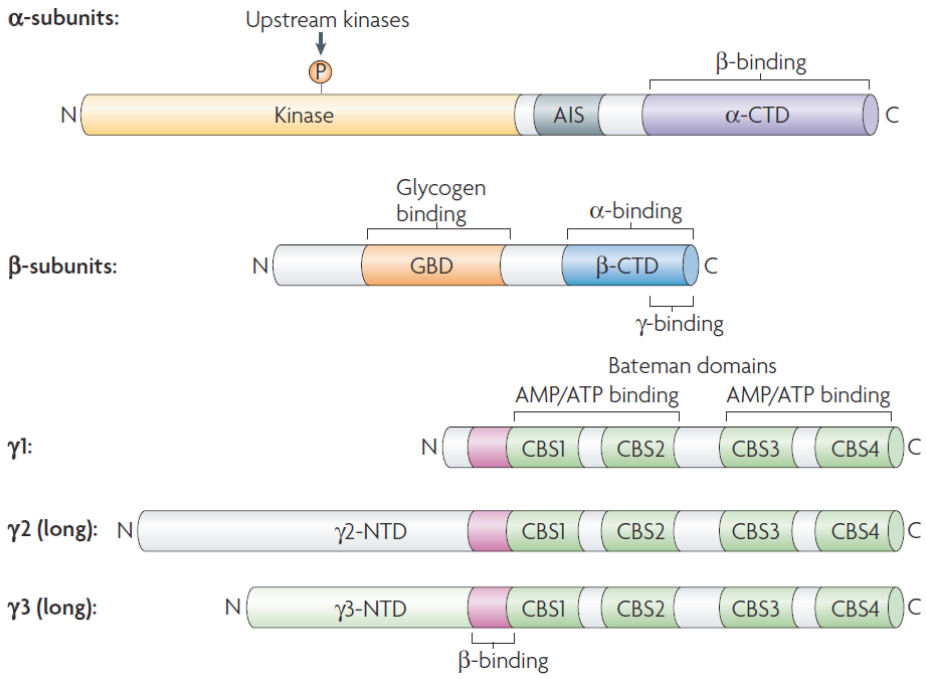

Figure from Hardie DG, Review, Nature Reviews, 2007

Figure1-2. AMPK subunits domain structure. The size of each subunit is drawn to scale and the same color domains consist of related sequences. Since the mammalian α1/ α2 and β1/ β2 are similar, only one example is drawn. The α domain consists of the kinase domain, including the upstream phosphorylation site and autoinhibitory sequence (AIS) and a C-terminal region for β and γ subunit binding. The β subunit includes a C-terminal α and γ subunit binding site and proposed central glycogen-binding domain. The γ subunits have a short region for β subunit binding and four conserved CBS motifs that bind as pair and form Bateman domains. AMP and ATP compete to bind these domains and regulate the activity of AMPK complex.

How is the activity of AMPK regulated? In mammalian cells, a high AMP level activates AMPK but this has not been demonstrated convincingly in lower eukaryotes like the budding yeast. In mammals, AMP activates AMPK by three distinct mechanisms. First, as is mentioned above, AMP binding to CBS domains in the γ subunit will activate the kinase domain in the α subunit. Second, AMP binding will cause Thr172, a residue in the kinase domain necessary for activation, to be phosphorylated by an upstream regulator (Hawley et al., 2003). Third, AMP binding will inhibit the dephosphorylation of Thr172 by the protein phosphatases PP2A and PP2C (Davies et al., 1995). There is also recent work suggesting AMP, rather than increasing both the phosphorylation of Thr172 and inhibiting the effects of protein phosphatases at the same time, is most essential for keeping the AMPK complex active (Sanders et al., 2007). In any case, all of these mechanisms combined cause more than a 1000-fold increase in the kinase activity (Suter et al., 2006). All of the activation effects of AMP are antagonized by AT, thus, proving AMPK acts as a cellular energy sensor.

AMPK activity regulation by the upstream kinases. AMPK must be phosphorylated at Thr172 by an upstream regulator to become active. In yeast, three upstream kinases (Sak1, Elm1 and Tos3) have been demonstrated to phosphorylate AMPK by *in vitro* and *in vivo* studies (Hong et al., 2003; Sutherland et al., 2003). In mammalian systems, there are no orthologue of the yeast upstream kinases but there are three kinases that are closest in sequence to the yeast kinases. One of them is LKB1 which has been identified as a major upstream regulatory kinase for AMPK and activates AMPK with the help of the regulatory subunits, STRAD and Mo25 (Woods et al., 2003). LKB1 is a tumour suppressor gene with mutations in this gene leading to an inherited human cancer, Peutz-Jegher Syndrome (Alessi et al., 2006). Although LKB1 must be bound to two regulatory subunits to

be functionally active, its kinase activity is constitutive and independent of the AMP level in the cell (Lizcano et al., 2004).

In cells lacking LKB1, like HeLa cells (tumor cells lacking LKB1), there is still some AMPK activity and Thr172 phosphorylation can be increased by adding Ca^{+2} to the medium. It is identified now that under those conditions, AMPK will be phosphorylated by an additional upstream kinase, Ca/Calmodulin-dependent protein kinase kinase (CaMKK) (Hurley et al., 2005). The ability of CaMKK to activate AMPK indicates that under certain paradigms where increased cytosolic Ca^{+2} is present, AMPK might be activated without a requirement for a change in AMP level. On the other hand, increasing cytosolic Ca^{+2} often increases ATP-demand, i.e., Ca^{+2} has to be actively pumped back out of the cytosol. Furthermore, CaMKK is only expressed in certain sub-types of cells, especially neurons, but LKB1 is expressed ubiquitously.

Activators of AMPK. Since metabolic stresses diminish ATP levels, they activate AMPK regardless of their cause. One of these stresses is exercise, which will activate AMPK depending on the intensity level (Winder and Hardie, 1996). It will cause this effect not only in skeletal muscle, but also in liver and adipose tissue (Kelly et al., 2004). It is well known that exercise reduces the risk of developing many diseases including Type 2 diabetes and obesity, and AMPK may play a role preventing these diseases. This hypothesis was confirmed by the discovery that the Type II diabetes drug metformin indirectly activates AMPK (Zhou et al., 2001).

Furthermore, stresses such as glucose deprivation, hypoxia, oxidative stress and ischemia all increase the AMP:ATP ratio and lead to activation of AMPK (Barnes et al., 2002).

In recent years, hormones that are secreted by the adipose tissue have been discovered, and these hormones regulate processes such as glucose and lipid metabolism, whole body weight regulation, and etc. (Friedman and Halaas, 1998). Leptin is one of these hormones; regulating food intake and body weight. Leptin will increase fatty acid oxidation and glucose uptake (Kamohara et al., 1997) in non-adipose tissues and will prevent them from a functional impairment known as "lipotoxicity" (Minokoshi et al., 2002). Although many of leptin's downstream effects are through activation of the Jak-Stat signaling pathway (Rosenblum CI, Endocrinology, 1996), in recent years leptin has been found to phosphorylate and activate AMPKα2 in skeletal muscle (Minokoshi et al., 2002). Adiponectin is another molecule that is secreted by adipocytes to increase glucose uptake and fatty acid oxidation, and thereby inhibit gluconeogenesis (Goldstein and Scalia, 2004). Adiponectin will regulate these effects by stimulating AMPK phosphorylation in skeletal muscle and liver since blocking AMPK activity by a dominant-negative mutant of AMPK also blocks these effects (Yamauchi et al., 2002).

Downstream targets of AMPK and its effects on cellular mechanisms. AMPK was first discovered in mammalian cells as the kinase that phosphorylated, and thereby inactivated, the two main enzymes of lipid biosynthesis, acetyl-CoA carboxylase (ACC), which regulates fatty acid synthesis, and 3-hydroxy-3-methylglutaryl-CoA reductase (3-HMG-CoA reductase), which regulates cholesterol synthesis (Haystead et al., 1989). The direct downstream targets of AMPK which have been identified to date include more than 20 proteins. In each case, either a Ser or Thr residue is phosphorylated, i.e., a classic serine/threonine kinase (Weekes et al., 1993).

In addition, there are numerous downstream targets of AMPK which have been identified in various tissues and through *in vitro* studies using the AMPK activator 5-aminoimidazole-4-carboxamide (AICA) riboside.

Regulation of lipid metabolism. Using AICA riboside, a nucleoside which when taken into cells is converted into the AMP analogue ZMP, it has been shown that increased AMPK activity causes phosphorylation and inactivation of acetyl-CoA carboxylase and HMG-CoA reductase activity in hepatocytes. Inactivation of these enzymes specifically halts fatty acid and sterol synthesis (Corton et al., 1995). Furthermore, in skeletal muscle AMPK will phosphorylate ACC and inhibit the enzyme. The level of malonyl-CoA will decrease causing an inhibition of fatty acid synthesis. This set of events will lead to a stimulation of fatty acid oxidation and thereby catabolism (Merrill et al., 1997).

Regulation of glucose metabolism. AMPK will regulate glucose metabolism by stimulating glucose uptake through translocation of the glucose transporter Glut4 to the plasma membrane in skeletal muscle (Kurth-Kraczek et al., 1999). Once the glucose is accumulated in the muscles, AMPK will phosphorylate and inhibit glycogen synthase. The glycolysis rate will then increase (Jorgensen et al., 2004).

In the liver under fasting conditions, glucagon will stimulate glucose release from the liver by stimulating transcription of gluconeogenesis genes. When cellular ATP levels are low, AMPK will be activated and attenuate gluconeogenesis by phosphorylating and inhibiting the nuclear translocation of TORC2 (Koo et al., 2005). Monocytes, immune system cells, mostly function in the damaged tissue area under hypoxic conditions. To be able to function properly, monocytes use glycolysis as their energy source. AMPK has been

shown to increase the level of glycolysis in monocytes by phosphorylating inducible 6-phosphofructo-2-kinase (iPFK-2) under hypoxic conditions (Marsin et al., 2002).

The summary of AMPK's role in glucose and lipid metabolism can be found in Figure 1-3.

Figure 1-3

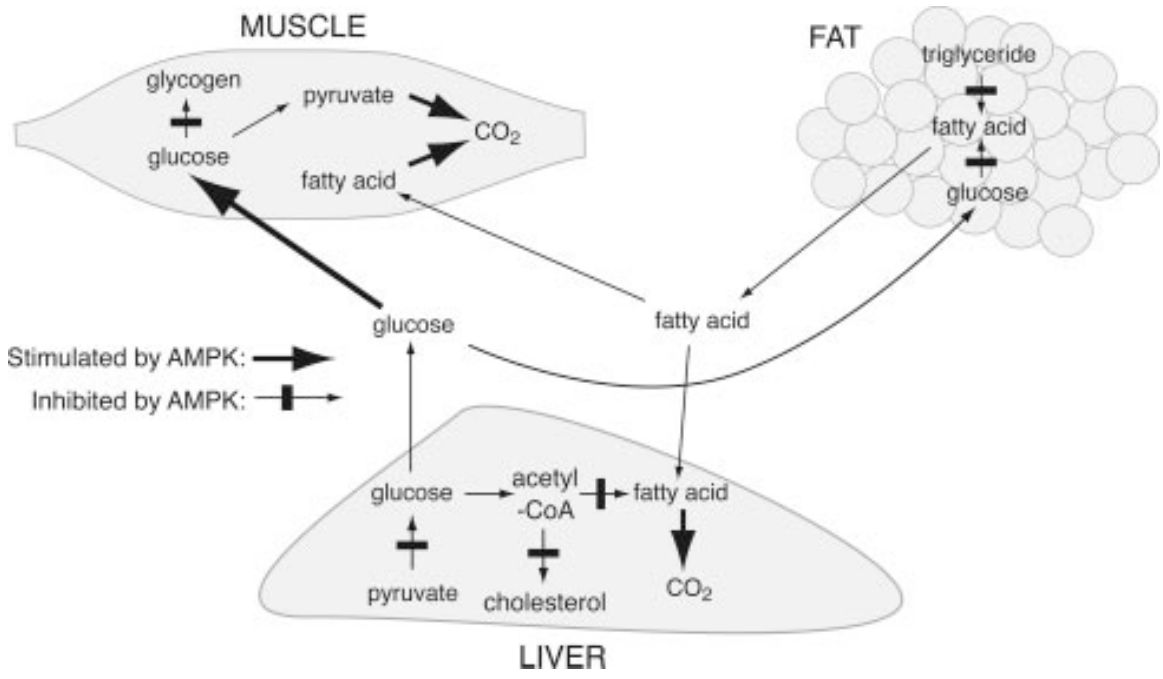

Figure from Towler and Hardie, Review, Circulation Research, 2007

Figure 1-3. AMPK activation effects glucose and lipid metabolism in liver, muscle and adipose tissue. The thick arrows show pathways stimulated by AMPK. Thos inhibited are shown by thin arrows with thick bars across them. Pyruvate oxidation through AMPK is mediated by upregulation of mitochondrial biogenesis. Fatty acid oxidation through AMPK is mediated by phosphorylation of ACC2 and increased fatty acid entry into mitochondria, in addition to upregulation of mitochondrial biogenesis.

Regulation of growth and cell division. AMPK also regulates cell growth and proliferation processes since these pathways require immense amounts of energy. In mouse embryonic fibroblasts, AMPK acts as a cellular energy sensor and the deprivation of glucose will cause activation of AMPK. This will cause phosphorylation of p53 and entry into G1-S cell cycle arrest (Jones et al., 2005). AMPK activated by AICA ribose inhibits the growth of cancer cells by increasing expression of p53 and other cyclin dependent kinases (Rattan et al., 2005).

Under energetic stress, AMPK will inhibit protein synthesis by inhibiting the activity of mTOR, a regulator of the initiation steps of protein synthesis (Proud, 2004). mTor is regulated by the TSC1-TSC2 complex and the Ras–related G protein, Rheb. AMPK will achieve its goal through phosphorylation and activation of the TSC1-TSC2 heterodimer. When AMPK phosphorylates TSC1-TSC2, the complex will inhibit Rheb by its GTP-ase activity. Rheb will not then be able to activate mTor (Inoki et al., 2003). On the other hand, insulin-like growth factor 1 (IGF1) will activate Akt. Akt will phosphorylate the TSC1-TSC2 complex at different sites. This will prevent Rheb inhibition and mTor will be activated (Inoki et al., 2002). Furthermore, hepatocytes are the main tissue in liver that are responsible for protein synthesis and storage. Incubating hepatocytes under low oxygen conditions cause AMPK activation and phosphorylation of elongation factor (eEF2), leading to the inhibition of its activity. This effect leads to inhibition of protein synthesis (Horman et al., 2002).

In summary, in mammals AMPK activation decreases the expression of genes involved in lipogenesis and gluconeogenesis in liver. As mentioned above, this will increase glucose transport and fatty acid synthesis in muscle cells. In addition to phosphorylation of many targets, AMPK will also achieve some of these functions by regulating transcription

factors. For instance, in muscle cells mitochondrial biogenesis is essential for adaptation to chronic energy deprivation. AMPK has been shown to increase mitochondrial biogenesis under such stresses. AMPK will increase activation of the transcription factor PPARγ (peroxisome proliferator activated receptor γ) co-activator 1-α (PGC1-α), a key regulator of mitochondrial biogenesis (Zong et al., 2002). A summary of all these and other downstream targets of AMPK can be found in Table 1-1.

Table 1-1

Protein Target	Site	Effect on Protein Function	Pathway	Tissue	Effect on Pathway
Lipid metabolism					
ACC-1	S80	↓ Activity	Fatty acid synthesis	All cells?	↓ Fatty acid synthesis
ACC-2	S221	↓ Activity	Fatty acid oxidation	Muscle, liver	↑ Fatty acid oxidation
HMG-CoA reductase	S872	↓ Activity	Isoprenoid synthesis	Liver	↓ Cholesterol synthesis
Hormone-sensitive lipase	S554	↓ Activity	Lipolysis	Adipose tissue	↓ Lipolysis
Carbohydrate metabolism					
Glycogen synthase	S8	↓ Activity	Glycogen synthesis	Muscle	↓ Glycogen synthesis
6-Phosphofructo-2-kinase					
Cardiac isoform	S466	↑ Activity	Regulation of glycolysis	Heart	↑ Glycolysis
Inducible isoform	S461	↑ Activity	Regulation of glycolysis	Monocytes, macrophages	↑ Glycolysis
Cell signaling					
Endothelial NO synthase	S1177	↑ Activity	NO production	Endothelial cells	↑ NO, increased blood flow?
TSC2 (tuberin)	S1387	↑ Rheb-GAP	Regulation of TOR	All cells?	↓ Cell growth, protein synthesis
Insulin receptor substrate-1	S794	↑ PI3K binding	Insulin signaling	All cells?	↑ Insulin signaling?
AS160	?	↓ Rab-GAP?	GLUT4 trafficking	Muscle	↑ Glucose uptake
Transcription					
p300	S89	↓ Interaction	Gene expression	All cells?	↓ Transcription by nuclear receptors
HNF4-α	S313	↓ DNA binding; ↑ degradation	Gene expression	Liver, others	↓ Transcription
ChREBP	S568	↓ DNA binding	Gene expression	Liver	↓ Transcription, L-pyruvate kinase gene
TORC2	S171	↑ Cytoplasmic localization	Gene expression	Liver	↓ Transcription, gluconeogenic genes
Ion transport/ion balance					
CFTR	?	↓ Channel opening	Ion transport, fluid secretion	Airway, gut, epithelium	↓ Ion transport

Table from Hardie DG, Review, Nature Reviews, 2007

Table 1-1. Protein targets proposed to be phosphorylated by AMPK.

AMPK's role in diseases

AMPK and obesity, diabetes and metabolic syndrome. Type II diabetes is caused by increased glucose in the plasma due to insulin resistance with obesity known to increase the risk of developing Type II diabetes. Since the activation of AMPK decreases gluconeogenesis in the liver and increases glucose uptake by muscle and other tissues, the activation of AMPK might help to decrease plasma glucose levels. In addition, insulin resistance causes accumulation of fatty acids in the liver and muscle even though these tissues don't have high amounts of mitochondrial oxidative capacity (Lowell and Shulman, 2005). Since AMPK increases fatty acid oxidation and increase mitochondrial biogenesis and decreases fatty acid synthesis, AMPK might decrease the high level of fatty acids in those tissues.

AMPK activation can reverse the risk of developing metabolic syndrome since it can decrease high levels of glucose and fatty acids in plasma and other tissues -- decreasing the risk of obesity. Consistent with these observations two of the main Type II diabetes drugs, e.g., metformin, are shown to indirectly activate AMPK *in vitro* (Fryer et al., 2002; Zhou et al., 2001). Later, it was demonstrated in the liver that metformin lowers plasma glucose levels entirely through the LKB1/AMPK pathway (Shaw et al., 2005).

In addition, exercise is an effective way of preventing insulin resistance and obesity and some of these effects are through the activity of AMPK. Furthermore, the use of the AMPK activator, A-769662, has led to: the reversal of insulin resistance and glucose intolerance, lower plasma lipid and free fatty acids levels, and reduced body weight in ob/ob obese mice (Cool et al., 2006).

AMPK and Cancer. LKB1 is major upstream regulator of AMPK and a tumor suppressor gene. LKB1 mutations cause Peutz-Jeghers syndrome where the loss of LKB1 leads to polyp formation in the intestine (Hemminki et al., 1998). AMPK is upstream of the cell growth and cell division protein mTor and therefore regulates cell proliferation and autophagy. Activation of AMPK in cancer cells causes growth arrest in these cells through activation of p53 and cyclin-dependent kinase inhibitors (Rattan et al., 2005). Furthermore, a number of studies point out both obesity and Type II diabetes are associated with an increased risk of cancer and mortality (Dossus and Kaaks, 2008). AMPK's protective effect from obesity and Type II diabetes could help protect from tumor onset. On the other hand, AMPK has a role in cell survival under energetic stress. p27 is a cell cycle regulator (Massague, 2004) which also regulates cell survival through autophagy (Wu et al., 2006). AMPK is known to regulate p27 phosphorylation and its stability; leading to cell survival under metabolic stress instead of cell-death (Liang et al., 2007). This could very well explain why tumor cells persist under metabolic stress conditions and their resistance to chemotherapy. Further information is needed to define AMPK's role in cell growth/division and in cell survival and cancer biology.

AMPK and heart disease. As mentioned above, AMPKγ subunits contain 4 CBS motifs that form two Bateman domains where AMP and ATP bind and regulate the AMPK complex. The AMPKγ subunit has received much more attention since some hereditary dominant mutations of AMPKγ2 have been shown to lead to heart disease in humans (Arad et al., 2007). Two of these mutations R531Q and R384T are not inherited and cause death due to heart failure and/or respiratory problems during the first few months of infancy in humans (Burwinkel et al., 2005). Most of these mutations cause ventricular pre-excitation, a

condition called Wolff-Parkinson White syndrome (WPW Syndrome). The other symptoms include: cardiac hypertrophy, excessive cardiac glycogen, preexcitation, and supraventricular arrhythmias. These dominant mutations are either in the CBS motifs or at the linker domains between the CBS motifs and demonstrated to inhibit AMP-binding and lead to reduced AMPK activity (Daniel and Carling, 2002).

AMPK identified from a forward genetic screen. In the Brenman lab, a forward genetic screen was performed using *Drosophila Melagonaster* as an assay organism. To perform this assay, the Gal4-UAS system has been used to express actin fused to GFP in the peripheral nervous system (PNS) neurons. Using an alkylating agent, EMS, random mutations were introduced into the fly genome and then characterized when the mutations were homozygous lethality. For the homozygous lethal mutations, any mutation displaying a morphological defect in the dendrites, axons and/or filopodia of the PNS were retained and characterized further. Through this method, 13 different mutations were isolated (Medina et al., 2006). Two were chosen to study in greater detail, one was a gene termed *capulet* on the 2^{nd} chromosome, and the other was located on the X chromosome, later discovered to be the AMP-Activated Protein Kinase α subunit. There were two different alleles isolated that were both mutation of AMPKα with both giving rise to the same phenotype in the PNS neurons. One of the alleles caused an amino acid substitution, S211L, which was located within the conserved region of the kinase domain. The other isolated mutation caused an amino acid change, Q295STOP that leads to a truncation. The phenotype and lethality of the flies were rescued either by reintroducing a wild-type copy of the gene or through the reintroduction of a duplication that carries the wild-type copy of AMPKα (Mirouse et al., 2007).

AMPK is highly conserved among organisms. It is essential to know that the gene of study is conserved among species, especially when you are studying an invertebrate, *Drosophila Melagonaster*, as your assay system. After identifying the first AMPKα mutation in fruit flies, conservation among organisms was checked. The AMPKα kinase domain is highly conserved between yeast to *Drosophila* (72%) and between *Drosophila* to humans (96%)(Lee et al., 2007). The function of AMPK as a cellular energy sensor, phosphorylation by upstream kinases including LKB1, and the regulatory subunits β and γ are all conserved from yeast to fruit flies to humans.

Rationale of Studies. Most of the research on AMPK is based on *in vitro* studies of isolated cultured cells and the use of non-specific drugs. Due to the genetic redundancy for each subunit of AMPK in mice, *in vivo* functions in multicellular animals have been difficult to identify. Mouse mutants of either AMPKα1 or AMPKα2 are viable and have no obvious defects while the double knockout of the AMPK*α* mouse dies in early stages (~embryonic 9.5)(personal communication B. Viollet). For these reasons, studying AMPK function genetically in *Drosophila* is an excellent approach. It should also be noted there is no functionally redundancy for any of the three AMPK subunits, i.e., one gene for every subunit of AMPK in fruit flies. In *Drosophila* the AMPK complex has not been characterized to date, other than some *in vitro* studies on S2 cells. In addition, there is also a lack of detailed localization studies for AMPK. With numerous genetic and cell biological reagents we have, and that we can generate, we should be able to better understand the *in vivo* function of AMPK in *Drosophila*. For instance, by generating transgenic flies, it is easier to express the wild-type/mutant version of a gene in a temporal and spatial expression pattern.

References

Alessi, D.R., Sakamoto, K., and Bayascas, J.R. (2006). LKB1-dependent signaling pathways. Annu Rev Biochem *75*, 137-163.

Arad, M., Seidman, C.E., and Seidman, J.G. (2007). AMP-activated protein kinase in the heart: role during health and disease. Circ Res *100*, 474-488.

Barnes, K., Ingram, J.C., Porras, O.H., Barros, L.F., Hudson, E.R., Fryer, L.G., Foufelle, F., Carling, D., Hardie, D.G., and Baldwin, S.A. (2002). Activation of GLUT1 by metabolic and osmotic stress: potential involvement of AMP-activated protein kinase (AMPK). J Cell Sci *115*, 2433-2442.

Bateman, A. (1997). The structure of a domain common to archaebacteria and the homocystinuria disease protein. Trends Biochem Sci *22*, 12-13.

Burwinkel, B., Scott, J.W., Buhrer, C., van Landeghem, F.K., Cox, G.F., Wilson, C.J., Grahame Hardie, D., and Kilimann, M.W. (2005). Fatal congenital heart glycogenosis caused by a recurrent activating R531Q mutation in the gamma 2-subunit of AMP-activated protein kinase (PRKAG2), not by phosphorylase kinase deficiency. Am J Hum Genet *76*, 1034-1049.

Carlson, M. (1999). Glucose repression in yeast. Curr Opin Microbiol *2*, 202-207.

Celenza, J.L., and Carlson, M. (1986). A yeast gene that is essential for release from glucose repression encodes a protein kinase. Science *233*, 1175-1180.

Cool, B., Zinker, B., Chiou, W., Kifle, L., Cao, N., Perham, M., Dickinson, R., Adler, A., Gagne, G., Iyengar, R.*, et al.* (2006). Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. Cell Metab *3*, 403-416.

Corton, J.M., Gillespie, J.G., Hawley, S.A., and Hardie, D.G. (1995). 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? Eur J Biochem *229*, 558-565.

Daniel, T., and Carling, D. (2002). Functional analysis of mutations in the gamma 2 subunit of AMP-activated protein kinase associated with cardiac hypertrophy and Wolff-Parkinson-White syndrome. J Biol Chem *277*, 51017-51024.

Davies, S.P., Helps, N.R., Cohen, P.T., and Hardie, D.G. (1995). 5'-AMP inhibits dephosphorylation, as well as promoting phosphorylation, of the AMP-activated protein kinase. Studies using bacterially expressed human protein phosphatase-2C alpha and native bovine protein phosphatase-2AC. FEBS Lett *377*, 421-425.

Dossus, L., and Kaaks, R. (2008). Nutrition, metabolic factors and cancer risk. Best Pract Res Clin Endocrinol Metab *22*, 551-571.

Friedman, J.M., and Halaas, J.L. (1998). Leptin and the regulation of body weight in mammals. Nature *395*, 763-770.

Fryer, L.G., Parbu-Patel, A., and Carling, D. (2002). The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. J Biol Chem *277*, 25226-25232.

Goldstein, B.J., and Scalia, R. (2004). Adiponectin: A novel adipokine linking adipocytes and vascular function. J Clin Endocrinol Metab *89*, 2563-2568.

Hawley, S.A., Boudeau, J., Reid, J.L., Mustard, K.J., Udd, L., Makela, T.P., Alessi, D.R., and Hardie, D.G. (2003). Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol *2*, 28.

Hawley, S.A., Davison, M., Woods, A., Davies, S.P., Beri, R.K., Carling, D., and Hardie, D.G. (1996). Characterization of the AMP-activated protein kinase kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. J Biol Chem *271*, 27879-27887.

Haystead, T.A., Sim, A.T., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P., and Hardie, D.G. (1989). Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature *337*, 78-81.

Hemminki, A., Markie, D., Tomlinson, I., Avizienyte, E., Roth, S., Loukola, A., Bignell, G., Warren, W., Aminoff, M., Hoglund, P.*, et al.* (1998). A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. Nature *391*, 184-187.

Hong, S.P., Leiper, F.C., Woods, A., Carling, D., and Carlson, M. (2003). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. Proc Natl Acad Sci U S A *100*, 8839-8843.

Horman, S., Browne, G., Krause, U., Patel, J., Vertommen, D., Bertrand, L., Lavoinne, A., Hue, L., Proud, C., and Rider, M. (2002). Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. Curr Biol *12*, 1419-1423.

Hurley, R.L., Anderson, K.A., Franzone, J.M., Kemp, B.E., Means, A.R., and Witters, L.A. (2005). The Ca2+/calmodulin-dependent protein kinase kinases are AMP-activated protein kinase kinases. J Biol Chem *280*, 29060-29066.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol *4*, 648-657.

Inoki, K., Zhu, T., and Guan, K.L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577-590.

Iseli, T.J., Walter, M., van Denderen, B.J., Katsis, F., Witters, L.A., Kemp, B.E., Michell, B.J., and Stapleton, D. (2005). AMP-activated protein kinase beta subunit tethers alpha and gamma subunits via its C-terminal sequence (186-270). J Biol Chem *280*, 13395-13400.

Jones, R.G., Plas, D.R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M.J., and Thompson, C.B. (2005). AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. Mol Cell *18*, 283-293.

Jorgensen, S.B., Nielsen, J.N., Birk, J.B., Olsen, G.S., Viollet, B., Andreelli, F., Schjerling, P., Vaulont, S., Hardie, D.G., Hansen, B.F*., et al.* (2004). The alpha2-5'AMP-activated protein kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive to glucose loading. Diabetes *53*, 3074-3081.

Kamohara, S., Burcelin, R., Halaas, J.L., Friedman, J.M., and Charron, M.J. (1997). Acute stimulation of glucose metabolism in mice by leptin treatment. Nature *389*, 374-377.

Kelly, M., Keller, C., Avilucea, P.R., Keller, P., Luo, Z., Xiang, X., Giralt, M., Hidalgo, J., Saha, A.K., Pedersen, B.K*., et al.* (2004). AMPK activity is diminished in tissues of IL-6 knockout mice: the effect of exercise. Biochem Biophys Res Commun *320*, 449-454.

Koo, S.H., Flechner, L., Qi, L., Zhang, X., Screaton, R.A., Jeffries, S., Hedrick, S., Xu, W., Boussouar, F., Brindle, P*., et al.* (2005). The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. Nature *437*, 1109-1111.

Kurth-Kraczek, E.J., Hirshman, M.F., Goodyear, L.J., and Winder, W.W. (1999). 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes *48*, 1667-1671.

Lee, J.H., Koh, H., Kim, M., Kim, Y., Lee, S.Y., Karess, R.E., Lee, S.H., Shong, M., Kim, J.M., Kim, J*., et al.* (2007). Energy-dependent regulation of cell structure by AMP-activated protein kinase. Nature *447*, 1017-1020.

Liang, J., Shao, S.H., Xu, Z.X., Hennessy, B., Ding, Z., Larrea, M., Kondo, S., Dumont, D.J., Gutterman, J.U., Walker, C.L*., et al.* (2007). The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol *9*, 218-224.

Lizcano, J.M., Goransson, O., Toth, R., Deak, M., Morrice, N.A., Boudeau, J., Hawley, S.A., Udd, L., Makela, T.P., Hardie, D.G*., et al.* (2004). LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. EMBO J *23*, 833-843.

Lowell, B.B., and Shulman, G.I. (2005). Mitochondrial dysfunction and type 2 diabetes. Science *307*, 384-387.

Marsin, A.S., Bouzin, C., Bertrand, L., and Hue, L. (2002). The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase. J Biol Chem *277*, 30778-30783.

Massague, J. (2004). G1 cell-cycle control and cancer. Nature *432*, 298-306.

Medina, P.M., Swick, L.L., Andersen, R., Blalock, Z., and Brenman, J.E. (2006). A novel forward genetic screen for identifying mutations affecting larval neuronal dendrite development in Drosophila melanogaster. Genetics *172*, 2325-2335.

Merrill, G.F., Kurth, E.J., Hardie, D.G., and Winder, W.W. (1997). AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am J Physiol *273*, E1107-1112.

Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y.B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferre, P., Birnbaum, M.J*., et al.* (2004). AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature *428*, 569-574.

Minokoshi, Y., Kim, Y.B., Peroni, O.D., Fryer, L.G., Muller, C., Carling, D., and Kahn, B.B. (2002). Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature *415*, 339-343.

Mirouse, V., Swick, L.L., Kazgan, N., St Johnston, D., and Brenman, J.E. (2007). LKB1 and AMPK maintain epithelial cell polarity under energetic stress. J Cell Biol *177*, 387-392.

Pang, T., Xiong, B., Li, J.Y., Qiu, B.Y., Jin, G.Z., Shen, J.K., and Li, J. (2007). Conserved alpha-helix acts as autoinhibitory sequence in AMP-activated protein kinase alpha subunits. J Biol Chem *282*, 495-506.

Polekhina, G., Gupta, A., Michell, B.J., van Denderen, B., Murthy, S., Feil, S.C., Jennings, I.G., Campbell, D.J., Witters, L.A., Parker, M.W*., et al.* (2003). AMPK beta subunit targets metabolic stress sensing to glycogen. Curr Biol *13*, 867-871.

Polekhina, G., Gupta, A., van Denderen, B.J., Feil, S.C., Kemp, B.E., Stapleton, D., and Parker, M.W. (2005). Structural basis for glycogen recognition by AMP-activated protein kinase. Structure *13*, 1453-1462.

Proud, C.G. (2004). Role of mTOR signalling in the control of translation initiation and elongation by nutrients. Curr Top Microbiol Immunol *279*, 215-244.

Rattan, R., Giri, S., Singh, A.K., and Singh, I. (2005). 5-Aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside inhibits cancer cell proliferation in vitro and in vivo via AMP-activated protein kinase. J Biol Chem *280*, 39582-39593.

Sanders, M.J., Grondin, P.O., Hegarty, B.D., Snowden, M.A., and Carling, D. (2007). Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. Biochem J *403*, 139-148.

Scott, J.W., Hawley, S.A., Green, K.A., Anis, M., Stewart, G., Scullion, G.A., Norman, D.G., and Hardie, D.G. (2004). CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. J Clin Invest *113*, 274-284.

Shaw, R.J., Lamia, K.A., Vasquez, D., Koo, S.H., Bardeesy, N., Depinho, R.A., Montminy, M., and Cantley, L.C. (2005). The kinase LKB1 mediates glucose homeostasis in liver and therapeutic effects of metformin. Science *310*, 1642-1646.

Suter, M., Riek, U., Tuerk, R., Schlattner, U., Wallimann, T., and Neumann, D. (2006). Dissecting the role of 5'-AMP for allosteric stimulation, activation, and deactivation of AMP-activated protein kinase. J Biol Chem *281*, 32207-32216.

Sutherland, C.M., Hawley, S.A., McCartney, R.R., Leech, A., Stark, M.J., Schmidt, M.C., and Hardie, D.G. (2003). Elm1p is one of three upstream kinases for the Saccharomyces cerevisiae SNF1 complex. Curr Biol *13*, 1299-1305.

Viana, R., Towler, M.C., Pan, D.A., Carling, D., Viollet, B., Hardie, D.G., and Sanz, P. (2007). A conserved sequence immediately N-terminal to the Bateman domains in AMP-activated protein kinase gamma subunits is required for the interaction with the beta subunits. J Biol Chem *282*, 16117-16125.

Weekes, J., Ball, K.L., Caudwell, F.B., and Hardie, D.G. (1993). Specificity determinants for the AMP-activated protein kinase and its plant homologue analysed using synthetic peptides. FEBS Lett *334*, 335-339.

Winder, W.W., and Hardie, D.G. (1996). Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. Am J Physiol *270*, E299-304.

Woods, A., Johnstone, S.R., Dickerson, K., Leiper, F.C., Fryer, L.G., Neumann, D., Schlattner, U., Wallimann, T., Carlson, M., and Carling, D. (2003). LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr Biol *13*, 2004-2008.

Woods, A., Munday, M.R., Scott, J., Yang, X., Carlson, M., and Carling, D. (1994). Yeast SNF1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase in vivo. J Biol Chem *269*, 19509-19515.

Woods, A., Salt, I., Scott, J., Hardie, D.G., and Carling, D. (1996). The alpha1 and alpha2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity in vitro. FEBS Lett *397*, 347-351.

Wu, F.Y., Wang, S.E., Sanders, M.E., Shin, I., Rojo, F., Baselga, J., and Arteaga, C.L. (2006). Reduction of cytosolic p27(Kip1) inhibits cancer cell motility, survival, and tumorigenicity. Cancer Res *66*, 2162-2172.

Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K.*, et al.* (2002). Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med *8*, 1288-1295.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N.*, et al.* (2001). Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest *108*, 1167-1174.

Zong, H., Ren, J.M., Young, L.H., Pypaert, M., Mu, J., Birnbaum, M.J., and Shulman, G.I. (2002). AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. Proc Natl Acad Sci U S A *99*, 15983-15987.

Preface

In this chapter, I helped in the characterization of AMPKα null mutants. In addition, I generated the transgenic fly that overexpresses the constitutively active (T184D) version of AMPKα. We mapped the lines to the chromosome and crossed them to *lkb1* null mutant flies. Overexpression of this transgene rescued the loss of epithelial polarity due to the *lkb1* null mutant.

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CHAPTER II

LKB1 and AMPK maintain epithelial cell polarity under energetic stress

Abstract

LKB1 is mutated in both familial and spontaneous tumours, and acts as a master kinase that activates the PAR-1 polarity kinase, and the Adenosine 5'monophosphate-activated kinase, AMPK. This has led to the hypothesis that LKB1 acts as a tumour suppressor because it is required to maintain cell polarity and growth control through PAR-1 and AMPK respectively. However, the genetic analysis of LKB1-AMPK signaling in vertebrates has been complicated by the existence of multiple redundant AMPK subunits. Here, we describe the identification of mutations in the single *Drosophila* AMPK catalytic subunit, AMPKα. Surprisingly, *ampk*α mutant epithelial cells lose their polarity and over-proliferate under energetic stress. LKB1 is required *in vivo* for AMPK activation and *lkb1* mutations cause similar energetic stress-dependent phenotypes to *ampk*α mutations. Furthermore, *lkb1* phenotypes are rescued by a phosphomimetic version of AMPKα. Thus, LKB1 signals through AMPK to coordinate epithelial polarity and proliferation with cellular energy status, and this might underlie the tumour suppressor function of LKB1.

Introduction

LKB1 is a serine threonine kinase that is mutated in most cases of Peutz Jeghers Syndrome, an autosomal dominant disorder in which patients develop benign harmatomas and a high frequency of malignant tumours (Alessi et al., 2006). Furthermore, *LKB1* is also mutated in some sporadic cancers, such as 30% of lung adenocarcinomas, and the expression of the kinase is also down-regulated in a significant proportion of breast cancers (Sanchez-Cespedes et al., 2002). In both cases, tumours associated with LKB1 mutations usually derive from epithelial tissue. LKB1 is a master kinase that can potentially activate a number of downstream kinases by phosphorylating a conserved threonine in their activation loops (Lizcano et al., 2004). Two of these kinases have been extensively characterized: PAR-1/MARKs (microtubule affinity-regulating kinases) and AMPK (AMP-activated protein kinase). PAR-1 regulates cell polarity in numerous cell types and organisms (Benton and St Johnston, 2003; Bohm et al., 1997; Cohen et al., 2004; Kemphues et al., 1988; Shulman et al., 2000). AMPK acts as a cellular energy sensor, since it is activated by AMP, which accumulates when ATP levels are low(Kahn et al., 2005). AMPK then mediates the cellular response to energetic stress by activating energy-producing activities, while inhibiting energy-consuming ones, such as translation and proliferation. LKB1 regulates both cell polarity and cell growth and division in cell culture and *in vivo* (Baas et al., 2004; Kemphues et al., 1988; Martin and St Johnston, 2003; Narbonne and Roy, 2006; Tiainen et al., 1999). One hypothesis envisions LKB1-signaling mediated through PAR-1 regulating cell polarity while LKB1-signaling through AMPK could control cell growth and proliferation. However, recent cell culture experiments suggest that AMPK also plays a role in the polarization of MDCK cells by promoting tight-junction assembly (Zhang et al., 2006) (Zheng and Cantley,

2007). Here, we show that LKB1 and AMPK are required to maintain epithelial polarity and integrity under energy-limiting conditions in *Drosophila*. These results therefore provide a potential mechanism to coordinate the regulation of cell polarity and proliferation with energy conditions within a multi-cellular animal.

Materials and methods

Mutant characterization. An EMS mutagenesis screen on the X-chromosome was performed as previously described (Medina et al., 2006). Early second instar larvae were visually screened for dendritic defects using fluorescent microscopy. The *ampkα* mutants were mapped to approximately 150 kb on the X-chromosome using a molecularly defined deficiency, *Df(1)Exel6227*, an undefined deficiency, *Df(1)AD11*, and a duplication of the Y-chromosome, *Dp(1;Y) / Df(1)svr*. Predicted coding regions for genes in the region were sequenced using PCR amplicons made from mutant genomic DNA and one gene (*AMPKα*; *CG3051*; NM_057965) was discovered that had significant mutations in all three alleles.

Construction of *AMPKα* transgenes. The wild type *AMPKα* transgene was cloned into the *pUAST* vector (Brand and Perrimon, 1993) as an *EcoRI-BglII* fragment of an EST, corresponding to *AMPKα-RA* transcript (www.flybase.org). The mCherry-AMPKα fusion protein was made using an mCherry construct (kindly provided by Roger Tsien, University of California, San Diego) at the amino-terminus fused in-frame to *AMPKα* into the *pUAST* vector. The phosphomimetic-activated form of AMPKα (AMPKα T184D) was made by PCR-based site directed mutagenesis converting base C549 to G549. The transgenes were introduced into a w^{1118} stock by P-element-mediated transformation.

Fly stocks and crosses. *AMPKα* alleles were recombined with *FRT101* for mitotic recombination. Other mutant stocks used are *FRT82B,* lkb^{4A4-2} and *FRT82B, tend.* *UAS:Cherry-AMPKα* and *UAS:GFP-LKB1* were expressed in follicle cells using the *Cy2-Gal4* driver. Flip out experiments were performed by crossing *UAS:Cherry-AMPKα* and *UAS:AMPKα-T184D* to *y, w, hs:Flp; tub-FRT-cc-FRT-Gal4, UAS:GFP* and heat-shocking pupae. For rescue experiments two independent stocks were established and crossed together:

w; *UAS:AMPKα-T184D/CyO; FRT82B, Ubi:nlsGFP* and *y*, *w*, *hs:flp; da:Gal4, FRT82B,lkb1*$^{4A4-2}$ */TM3,Sb.*

Starvation conditions and clone induction. Adult flies were placed in vials containing "normal" Drosophila food media (5% glucose, 5% yeast extract, 3.5% wheat flour, agar 0.8%), energetic starvation medium (1% yeast extract, 3.5% wheat flour , agar 0.8%), or specific nutrient starvation medium (5% glucose, agar 0.8%). Clones were induced by heat-shocking adult females at 37°C for 2 hours on two consecutive days. Females were dissected 2 days after the last heat-shock.

Staining and imaging procedures. Immunofluorescence on ovaries was performed using standard procedures. Primary antibodies were used as follows: rat anti-*D*ECad (1/1000) (Oda et al., 1994); mouse anti-Crb (cq4) (1/50, Developmental Studies Hybridoma Bank); Guinea pig anti-Cora (1/2000) (Fehon et al., 1994)*;* rabbit anti-aPKC (1/500, Sigma); rat anti-Baz (1/500) (Wodarz et al., 1999), mouse anti Dlg (1/50, Developmental Studies Hybridoma Bank); rabbit anti-Dg (1/1000) (Deng et al., 2003), rabbit anti- phospoT385-AMPK (1/100, Cell Signaling). Actin staining was performed with Rhodamine-conjugated phalloidin (Molecular Probes, Oregon). Second instar larvae were dissected in 4% paraformaldehyde as previously described (Medina et al., 2006). Secondary antibodies coupled with Cy5 (anti-rabbit and anti-guinea pig) or Texas-Red (anti-mouse and anti-rabbit)(Jackson ImmunoResearch, 1/500). Images of follicle cells were done on a Biorad Radiance 2000 confocal microscope with a Nikon Plan Fluor 40X (NA 1.3) objective using Lasersharp software. Live animal images of dendrite morphology were acquired using a Zeiss LSM 510 confocal microscope by exciting the 488-nm argon-line to excite GFP. Larvae were covered in a glycerol solution at 22°C and gently cover-slipped (22X50mm,

Fisher Scientific Pittsburgh, PA) to restrict movement, but not cause bursting of the body wall. Images were taken using a Pan-Neofluar 40X/1.3 oil immersion lens with a 2µm optical slice and Zeiss LSM Imaging software. Images were sized and cropped with Adobe Photoshop (San Jose, CA), and placed into Adobe Illustrator (San Jose, CA) for labels and arrangement.

Results and Discussion

AMPK contains three protein subunits - α, β, and γ - that form a heterotrimer. The α-subunit (AMPKα) encodes a highly conserved serine-threonine kinase, while the other subunits are regulatory. From a *Drosophila* forward genetic screen for mutants affecting larval neuronal dendrite development (Medina et al., 2006), we identified several lethal mutations in *AMPKα*. The EMS mutants, *ampkα* ^{1} and *ampkα*^{2}, contain a single amino acid change (S211L (completely conserved)) and a pre-mature stop codon (Q295 STOP), respectively, while *ampkα* ^{3} has a 16 base pair deletion creating a stop codon (Y141 STOP) (Fig. 2-1A). All *ampkα* mutants, homozygous or in trans with a deletion covering the locus, displayed a completely penetrant and nearly identical phenotype, with greatly enlarged plasma membrane domains in dendrites, but not in axonal compartments (Fig. 2-1C, and data not shown). In addition, *ampkα*^{1} and *ampkα*^{3} could be rescued to viability with either a chromosomal duplication carrying a Wild-type *ampkα* allele, a wild type AMPKα transgene, or a transgene that is tagged with the red fluorescent protein mCherry (Fig. 2-1D and Methods). The requirement for *ampkα* is cell-autonomous, since transgene expression within only neurons rescues the phenotype (Fig. 2-1D). These mutations therefore represent the first knockouts of the single AMPKα catalytic subunit in the *Drosophila* genome, and allow the genetic analysis of AMPK function *in vivo*.

Figure 2-1

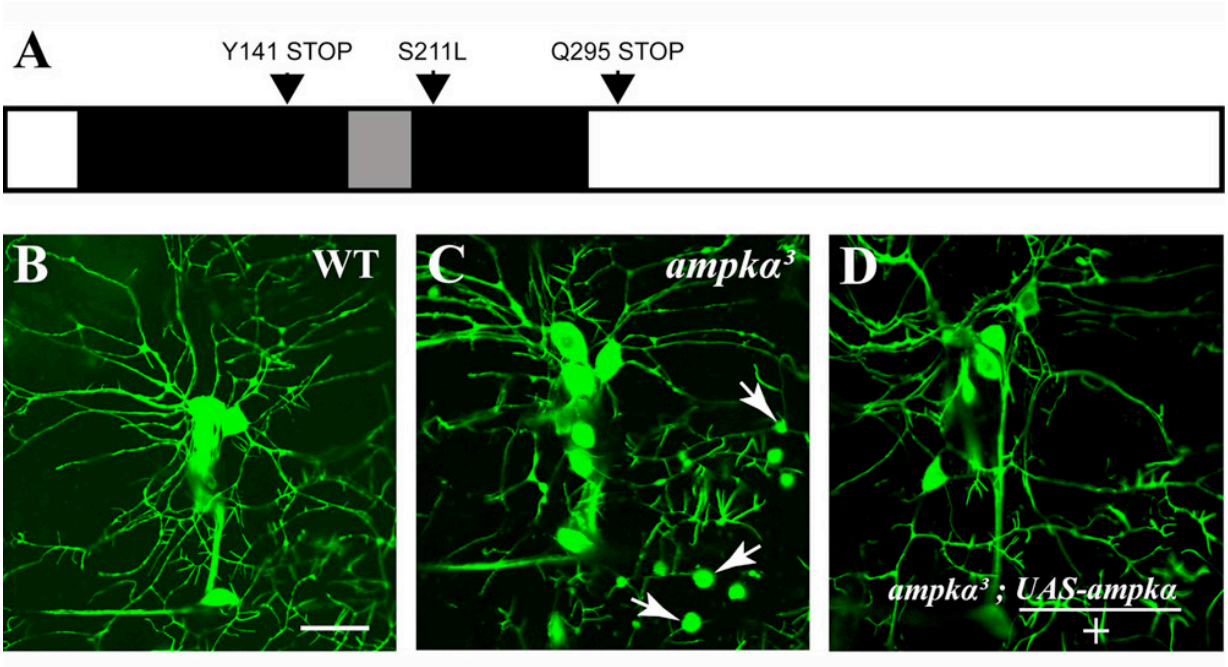


Figure 2-1: Identification of mutations within the single *Drosophila ampkα* gene.

A) Schematic domain representation of AMPKα and corresponding genetic lesions in mutants. The Serine-Threonine kinase domain (black, amino acids 39-280) and T-Loop (grey, amino acids 167-194) are shown with the sites of mutations, S211L, Q295STOP and Y141STOP for *ampkα*^{1} *ampkα*^{2} *and ampkα*^{3}, respectively. **(B)** Representative image of wild type da neurons expressing an *Actin::GFP*-fusion transgene in a 2nd instar larva. **(C)** *ampkα* mutants display enlarged plasma membrane domains (arrows) in sensory neuron dendrites but not axons. **(D)** A wild type *ampkα* transgene expressed autonomously within da neurons completely rescues the dendrite phenotype. **(B-D)** Background genotypes are *w; Gal4109(2)80, UAS-actin::GFP*. Anterior: at left and dorsal at top; Scale bars, 20 μm.

While *ampkα* mutants display a strong phenotype in larval neuronal dendrites, no phenotype was observed in early larval *lkb1* mutants (data not shown), probably due to the large maternal contribution of this protein. In order to explore the relationship between AMPKα and LKB1 function without the confounding issues caused by the differing maternal contributions of each protein, we chose to examine follicle cells of the *Drosophila* ovary. The follicle cells that surround the oocyte have a typical epithelial architecture with a highly polarized actin cytoskeleton, in which the apical surface is marked by dense actin bundles in the apical microvilli, the lateral cortex is covered by a thin actin mesh, and the basal side contains a prominent network of parallel actin stress fibers. This polarized organization of actin typifies many epithelia, including the main mammalian tissue culture model for polarized epithelial cells, MDCK cells (Fievet et al., 2004). We did not observe any actin phenotypes in *ampkα*^{3} mutant follicle cells using standard detection procedures (Fig. 2-2A). Since AMPK is maximally activated under low cellular energy levels, we also tested the influence of energy stress by strongly reducing the availability of sugar in the *Drosophila* culture medium. Under these conditions *ampkα*^{3} mutant cells display a strong actin phenotype (Fig. 2-2A). The density of basal stress fibers is strongly reduced, whereas the amount of apical F-actin increases. This phenotype is highly penetrant under these starvation conditions (98%, n=49) and is also observed with the two other alleles of *ampkα.*

Since this phenotype reflects a disruption of the apical-basal polarity of the actin cytsokeleton, we examined other polarity markers within these cells. *ampkα* mutant clones induced in adult flies fed with high sugar diets did not show any polarity phenotypes, consistent with the absence of an actin phenotype under these conditions (Fig. 2-2A). Under energetic starvation conditions, however, *ampkα* mutant cells show a fully penetrant loss of

polarity. Apical markers, such as atypical Protein kinase C (aPKC) and Crumbs (Crb) lose their cortical localization completely and appear to be down-regulated, as do the lateral markers Discs-large (Dlg) and Coracle (Cora) (Fig. 2-2A). In contrast, Dystroglycan (Dg), which is normally enriched at the basal cortex, extends into the lateral domain, and occasionally even reaches the apical membrane (Fig. 2-2A). This suggests that the phenotype represents an expansion of the basal domain at the expense of the lateral and apical domains.

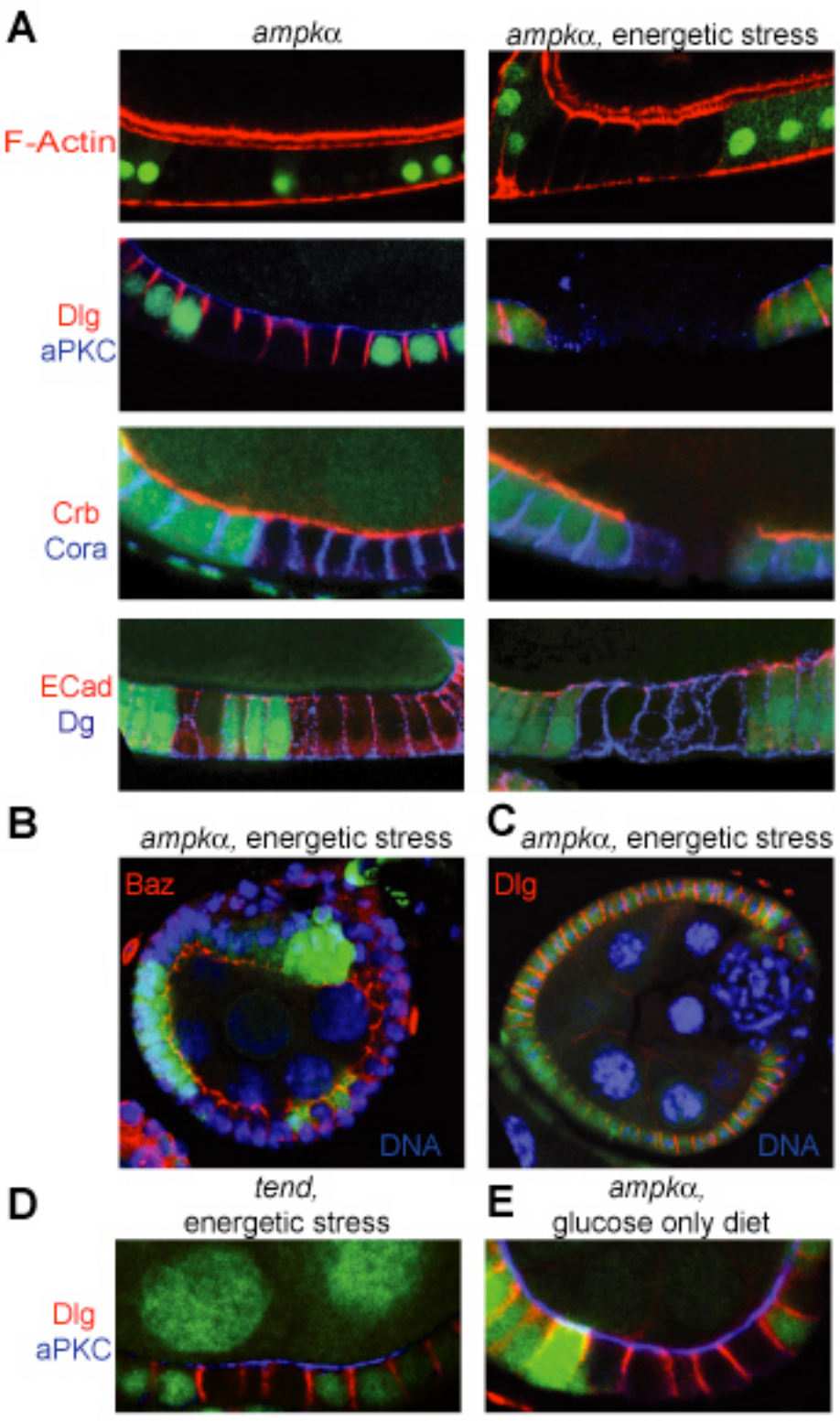
Although most aspects of apical-basal polarity are completely disrupted in *ampkα* mutant clones under energetic stress, E-Cadherin (ECad) is usually still enriched at the adherens junctions, suggesting that the altered polarity is not a secondary consequence of a loss of intercellular adhesion. The sub-apical localization of Bazooka (Baz) with Cadherin is also maintained in most cases (Fig. 2-2B). This indicates that Bazooka is not in a complex with aPKC in columnar follicle cells, but is instead associated with the adherens junctions, as has recently been described in the *Drosophila* embryo and in neuroepithelial cells of the zebra fish neural tube (Afonso and Henrique, 2006; Harris and Peifer, 2005).

A significant proportion of *ampkα* mutant clones show a more severe phenotype, in which the cells round up and lose their epithelial organization to form multiple layers of cells (Fig. 2-2B). In these cases, Baz is now also absent from the cell cortex. Finally, larger mutant clones, particularly at the anterior or the posterior of the egg chamber, show a complete loss of epithelial organisation and over-proliferate to form small tumour-like growths (Fig. 2-2C).

As one proposed function for AMPK is to sense and maintain cellular ATP levels, the polarity phenotype observed under starvation conditions could be due to low cellular ATP concentrations. To test this hypothesis, we examined cells mutant for *tenured* (*tend*). Tend encodes a mitochondrial cytochrome oxidase subunit and mutants therefore have reduced

intracellular ATP concentrations to levels sufficient to maintain cell survival and growth but not cell division (Mandal et al., 2005). This cell cycle block is believed to require AMPK activation. In agreement with a role for Tenured in cell cycle progression, we did not observed *tend* clones bigger than four to six cells under energetic starvation conditions (Fig. 2-2D). In contrast to *ampkα* mutant cells, however, *tend* mutant cells showed no polarity defects, ruling out the possibility that the *ampkα* phenotype is a secondary effect of low ATP levels. We also tested the effect of specific nutrient starvation, by feeding flies only glucose, but these conditions did not induce any polarity phenotypes in *ampkα* mutant cells (Fig. 2-2E). Thus, AMPKα is specifically required to maintain epithelial polarity and growth control under conditions of energetic stress.

Figure 2-2


A
ampkα
ampkα, energetic stress
F-Actin
Dlg
aPKC
Crb
Cora
ECad
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ampkα, energetic stress
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ampkα, energetic stress
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tend,
energetic stress
Dlg
aPKC
E
ampkα,
glucose only diet

Figure 2-2: *ampkα* is required to maintain epithelial polarity under energetic stress.

A) *ampkα*^{3} mutant follicle cell clones under normal (left) or energetic stress conditions (right). Mutant cells are marked by the absence of GFP (green). Markers are indicated to the left; F-Actin (red), Discs large (Dlg, red,), atypical Protein Kinase C (aPKC, blue), Crumbs (Crb, red), Coracle (Cora, blue), *D*Ecadherin (ECad, red) and Dystroglycan (Dg, blue). The apical domain is at the top and faces the oocyte, which also contains a layer of cortical actin (top panels).

B) *ampkα*^{3} clone stained for DNA (blue) and Bazooka (Baz, red). Baz is usually present at the apical domain of mutant cells, but is lost in the most severely-affected clones that lose their epithelial organization and form multiple layers.

C) *ampkα* ^{3} clone stained for DNA (blue) and Discs Large (Dlg, red). Large *ampkα* ^{3} clones at the anterior or posterior poles of the egg chamber over-proliferate to form tumour-like structures composed of unpolarized cells.

D) *tenured (tend)* mutant cells stained for aPKC (blue) and Dlg (red) . Cells maintain normal epithelial polarity.

E) *ampkα* ^{3} clone from a female fed on a glucose only diet stained for aPKC (blue) and Dlg (red). These conditions of protein and lipid starvation do not affect the polarity of *ampkα* ^{3} mutant cells.

The stage (st) of the egg chamber is indicated on each picture.

Since our results indicate that *ampkα* plays a role in epithelial polarity, we assessed whether the localization of the protein itself is polarized. We also examined LKB1 localization, as it is a potential regulator of AMPK. Transgenic wild type fusion proteins for both AMPKα and LKB1 rescue lethal null mutants to viability, and should therefore mimic the localizations of the endogenous proteins. LKB1-GFP is mainly found at the apical and lateral cortex of the follicle cells, and is absent from the basal domain (Fig. 2-3A). This basal exclusion is surprising, as cortical localization of LKB1 requires its membrane targeting by prenylation of a conserved CAAX motif (Martin and St Johnston, 2003). This suggests that the lipid composition of the basal domain is different from the rest of the plasma membrane and/or that LKB1 post-translational modifications are asymmetrically controlled. In contrast, mCherry-AMPKα does not show any enrichment or asymmetric localization at the plasma membrane, and is found distributed throughout the cytoplasm, but absent from the nucleus (Fig. 2-3A). The localization of LKB1 suggests that AMPK could be activated specifically at the apical and lateral cortices of the cells. To test this hypothesis, we used an antibody against the LKB1 phosphorylation site of AMPK (Phospho-T184). The immunostaining is reduced to background levels in both *ampkα* and *lkb1*mutant clones. This confirms the specificity of the antibody and indicates that LKB1 is the principle AMPK kinase in these cells (Fig. 2-3B). In wild-type cells, PhosphoT184-AMPK is found diffusely in the cytoplasm (Fig. 2-3B). The effect of AMPK on apical-basal polarity is therefore not related to a polarized distribution of the kinase or its localized activation by LKB1.

Figure 2-3

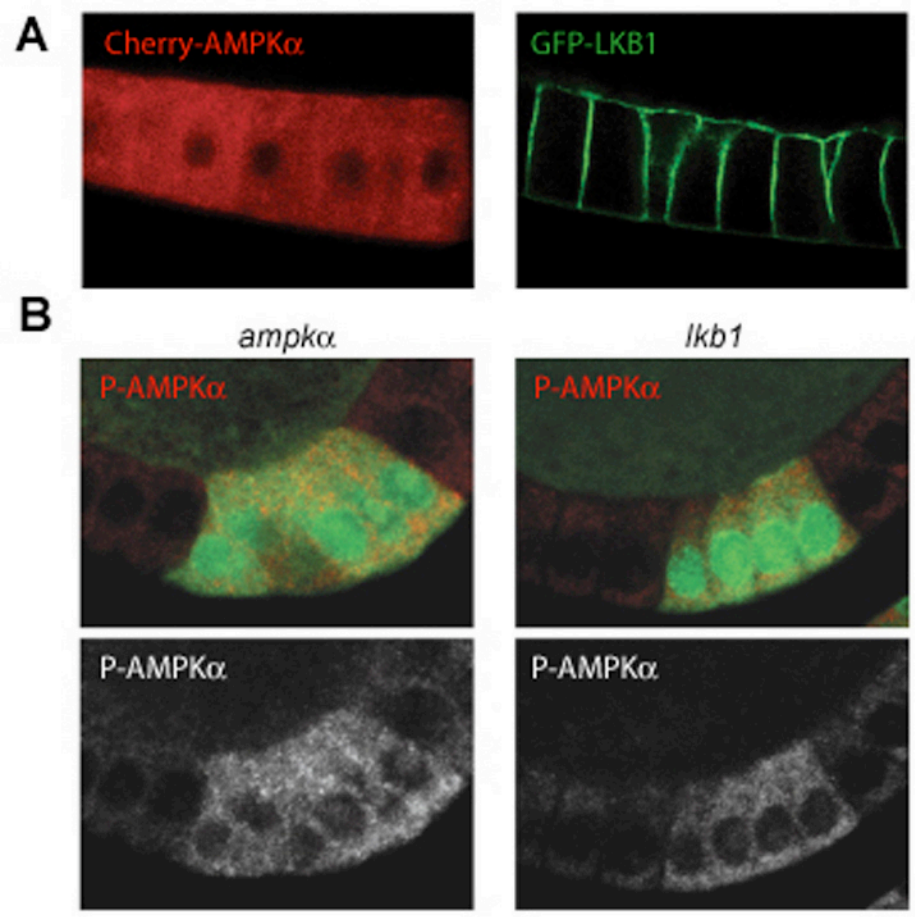

Figure 2-3: AMPKα activation is not polarized.

A) Follicle cells expressing Cherry-AMPKα (left) and GFP-LKB1 (right).

B) Wild-type follicle cells (green) adjacent to *ampk*α^{3} (left) or *lkb1*$^{4A4\text{-}2}$ (right) mutant cells marked by the loss of GFP stained for PhosphoT184-AMPKα (red, top panels; white, bottom).

Since LKB1 activates AMPK, we wondered if similar phenotypes could be observed in *lkb1* mutant cells. *lkb1* clones can lead to severe polarity defects in follicle cells in normally fed flies (Martin and St Johnston, 2003). However, these defects are observed only in large clones that are induced before the follicular epithelium forms from the somatic stem cells, and small *lkb1* mutant clones, induced after the formation of the epithelium, have no effect on follicle cell polarity or the organization of the actin cytoskeleton (n=24) (Fig. 2-4A). This suggests that LKB1 is required for the establishment of epithelial polarity in well-fed flies, but not for its maintenance, as is the case for PAR-1 (Doerflinger et al., 2003). In contrast, under conditions of glucose starvation, small *lkb1* clones that were induced after the formation of the follicular epithelium show a fully penetrant polarity phenotype (100%, n=21). Under these conditions, we observed a loss of the polarized localization of Dlg, aPKC, Crb and Cora (Fig. 2-4A). However, Baz distribution is usually not affected by *lkb1* loss of function (data not shown). Dg extends laterally and occasionally localizes to the apical domain (Fig. 2-4A). The actin cytoskeleton is also disturbed with more F-actin apically and a decreased density of stress fibers on the basal side. Finally, large *lkb1* clones lose their epithelial organization completely and over-proliferate to form small neoplasms (Fig. 2-4B and C). Thus, *lkb1* mutant cells exhibit identical phenotypes to *ampkα* mutant cells under low energy conditions.

Figure 2-4

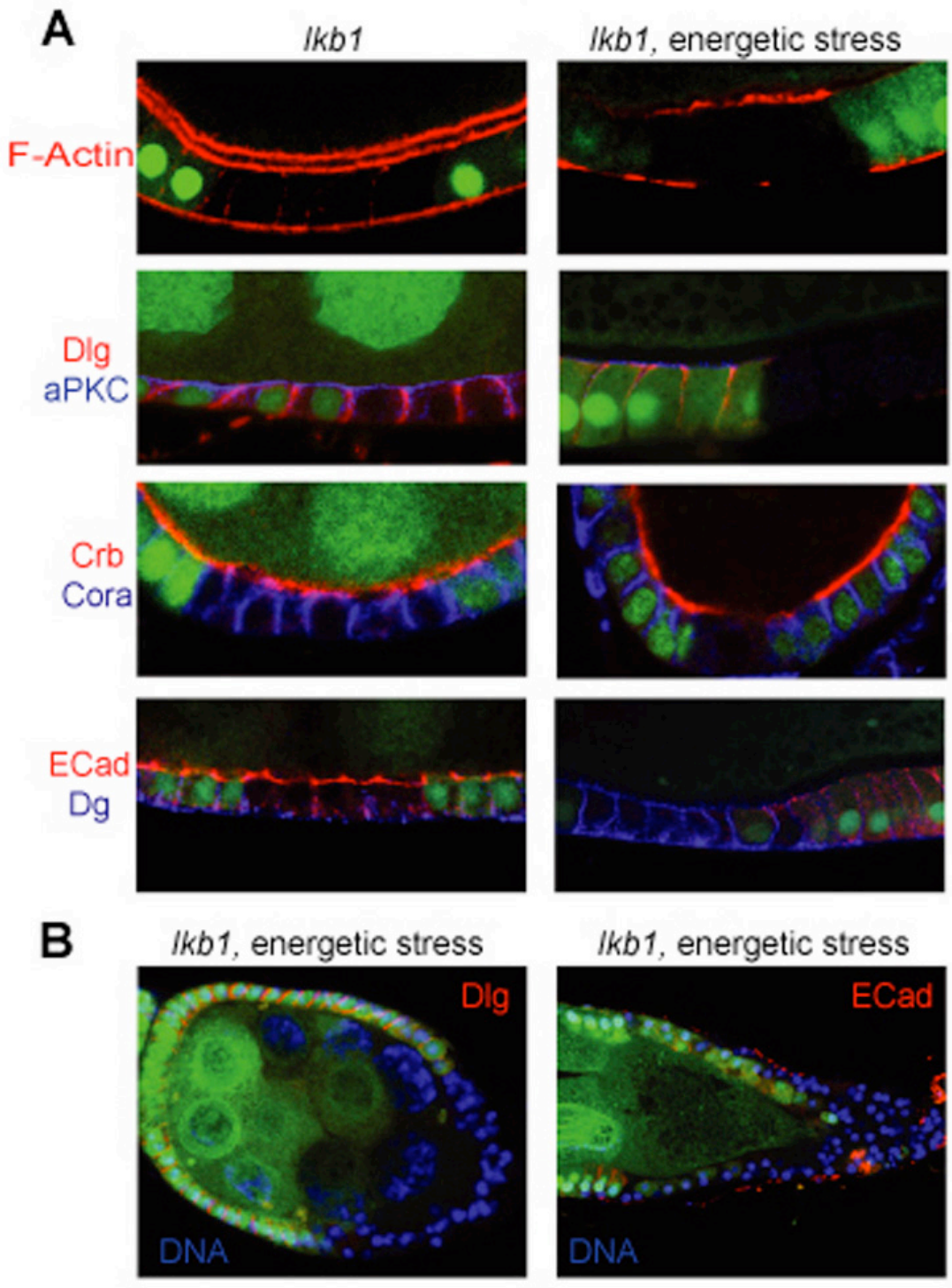
A
lkb1
lkb1, energetic stress
F-Actin
Dlg
aPKC
Crb
Cora
ECad
Dg
B
lkb1, energetic stress
lkb1, energetic stress
Dlg
DNA
ECad
DNA

Figure 2-4: LKB1 is required to maintain epithelial polarity under energetic starvation conditions.

A) *lkb1*^{4A} mutant follicle cell clones under normal (left) or energetic stress conditions (right). Mutant cells are marked by the absence of GFP (green) and visualized with the same markers (at left) as described in Figure 2-2.

B) *lkb1* ^{4} clone stained for DNA (blue) and Discs Large (red, left panel) or *D*ECad (red, right panel). Large mutant clones at the anterior or posterior poles of the egg chamber over-proliferate to form tumour-like aggregates of unpolarized cells.

Since *lkb1* and *ampkα* mutant clones lead to very similar polarity defects and LKB1 phosphorylates AMPKα, we wondered if a constitutively active form of AMPKα could rescue the *lkb1* phenotype. We therefore generated transgenic lines carrying a *UAS-AMPKα* construct, in which Threonine184 is replaced by an Aspartate, which should mimic the activating phosphorylation of this site by LKB1 (Lizcano et al., 2004). The expression of the *AMPKα-T184D* transgene in *lkb1* mutant clones fully rescues their starvation-dependent polarity and over-proliferation phenotypes (n=37), whereas the Gal4 driver alone has no effect (Fig. 2-5). Furthermore, AMPKα-T184D-expressing mutant clones also have a normal actin cytoskeleton (100%, n=13) (Fig. 2-5). Thus, the phosphomimetic version of AMPK*α* completely rescues the *lkb1* mutant phenotype under conditions of energetic stress.

Figure 2-5

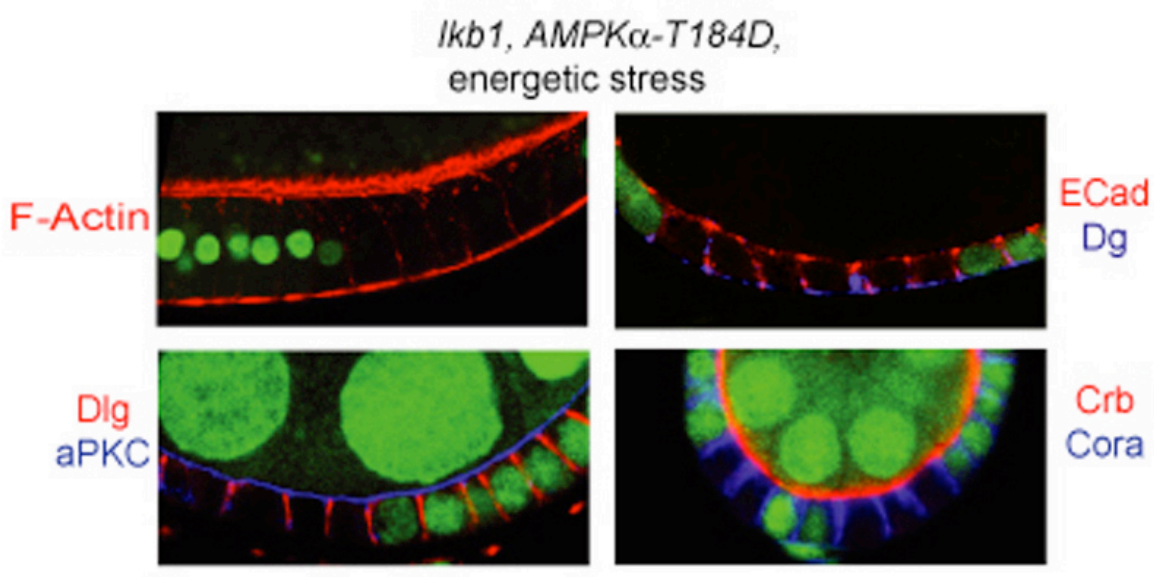

Figure 2-5: The AMPKα-T184D phosphomimetic transgene rescues the starvation dependent *lkb1* phenotypes. *lkb1* mutant cells marked by the absence of GFP (green) expressing the *UAS*-AMPKα-T184D transgene. The expression of the phosphomimetic *AMPKα* transgene (T184D) rescues the *lkb1* energetic stress dependent phenotypes as indicated by the normal distribution of various polarity markers (indicated to the left or right of the panels). Top: F-actin and DECad (red); DG (blue). Bottom: Dlg and Crb (red) aPKC and Cora (blue).

The recovery of null mutations in ampkα has allowed the first *in vivo* analysis of AMPK function in a multicellular organism, which has revealed an unexpected role for the kinase in the maintenance of epithelial polarity under conditions of energetic stress. This implies that at least one of the pathways that normally maintain cell polarity cannot function when cellular energy levels are too low, and that AMPK activation compensates for this defect. Therefore, this new function of AMPK may underlie that it is a priority for epithelial cells to maintain their polarity even when they are under stress conditions.

A surprising feature of the *ampkα* polarity phenotype is that it has opposite effects on the actin cytoskeleton and the cortical polarity cues. In mildly affected clones, basal actin is strongly reduced, with a corresponding increase in the amount of apical actin. In contrast, mutant clones show an expansion of the basal markers into the lateral and apical regions, and a loss of lateral and apical markers. Thus, the effects on actin may be independent of other polarity defects, suggesting that AMPK acts though different pathways to regulate actin and cortical polarity in opposite ways.

It is unclear how AMPK regulates the actin cytoskeleton, but it is possible that it acts on only one side of the cell and that the reciprocal changes on the other are caused by a change in the concentration of free G-actin or an actin nucleator, as has been shown for *abl* mutants during cellularization (Grevengoed et al., 2003). For example, loss of AMPK could increase actin polymerization apically, thereby depleting the pool of free actin that can polymerize basally. Alternatively, *ampkα* mutants may prevent the formation of basal actin stress fibers, and thus increase the concentration of free actin, which enhances apical actin polymerization.

The cortical polarity defects of *ampkα* mutant clones also suggest a reciprocal relationship between the basal and apical/lateral membrane domains, since the basal domain, marked by Dystroglycan, is dramatically expanded, whereas the determinants for the lateral domain (Discs large) and the apical domain (aPKC and Crumbs) disappear from the cortex. This suggests that there is some form of mutual antagonism between the basal and lateral domains that maintains a sharp boundary between them, as has been described for apical and lateral domains through the inhibitory phosphorylation of Bazooka (PAR-3) by lateral PAR-1, and of PAR-1 by apical aPKC (Benton and St Johnston, 2003; Suzuki et al., 2004). If this model is correct, AMPK could be required to restrict the extent of the basal domain, with the expansion of this domain in *ampkα* mutants leading to the exclusion of lateral and apical markers. Indeed, the over-expression of Dystroglycan has been found to cause a similar loss of apical and lateral markers to that seen in *ampkα* clones (Deng et al., 2003). Alternatively, AMPK could be necessary to maintain the localization of the apical and lateral determinants, which in turn prevent the basal domain from extending into these regions.

Mutations in *AMPK* not only disrupt the polarity of the follicle cell epithelium, but also cause the cells to over-proliferate, giving rise to a tumorous phenotype. One possible explanation for this phenotype is that it is caused by the mislocalization and down-regulation of Dlg. Dlg is a member of a class of tumour suppressors in *Drosophila* that also includes Lgl and Scribble, and follicle cell clones mutant for any of these genes over-proliferate to form invasive tumours that are similar to those formed by *ampkα* and *lkb1* clones under low energy conditions (Bilder and Perrimon, 2000; Goode et al., 2005; Hariharan and Bilder, 2006). Furthermore, the tumour suppressor function of these proteins is probably conserved in humans, since Scribble restricts proliferation by repressing the G1/S transition and is a

target of the papilloma virus E6 oncoprotein (Nagasaka et al., 2006; Takizawa et al., 2006). This may account for the observation that AMPK is required to trigger the G1/S checkpoint under conditions of energetic stress (Mandal et al., 2005). However, it has also been shown in mammals that AMPK activates TSC2 to repress the Insulin/TOR pathway, and therefore functions as a tumour suppressor that inhibits cell growth and division (Inoki et al., 2005; Inoki et al., 2003). Loss of this repression might therefore provide an alternative explanation for the overgrowth of *ampkα* mutant clones.

Although the molecular pathways involved remain to be elucidated, our results demonstrate that *ampkα* mutant cells lose their polarity under low energy conditions and over-proliferate to give rise to tumour-like growths. The activation of AMPK depends on its phosphorylation by LKB1, and loss of LKB1 produces an identical tumorous phenotype. Thus, the novel functions of AMPK reported here may provide a basis for the tumour suppressor function of LKB1.

References

Afonso, C., and D. Henrique. 2006. PAR3 acts as a molecular organizer to define the apical domain of chick neuroepithelial cells. *J Cell Sci*. 119:4293-304.

Alessi, D.R., K. Sakamoto, and J.R. Bayascas. 2006. Lkb1-dependent signaling pathways. *Annu Rev Biochem*. 75:137-63.

Baas, A.F., J. Kuipers, N.N. van der Wel, E. Batlle, H.K. Koerten, P.J. Peters, and H.C. Clevers. 2004. Complete polarization of single intestinal epithelial cells upon activation of LKB1 by STRAD. *Cell*. 116:457-66.

Benton, R., and D. St Johnston. 2003. Drosophila PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. *Cell*. 115:691-704.

Bilder, D., and N. Perrimon. 2000. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature*. 403:676-80.

Bohm, H., V. Brinkmann, M. Drab, A. Henske, and T.V. Kurzchalia. 1997. Mammalian homologues of C. elegans PAR-1 are asymmetrically localized in epithelial cells and may influence their polarity. *Curr Biol*. 7:603-6.

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 118:401-15.

Cohen, D., P.J. Brennwald, E. Rodriguez-Boulan, and A. Musch. 2004. Mammalian PAR-1 determines epithelial lumen polarity by organizing the microtubule cytoskeleton. *J Cell Biol*. 164:717-27.

Deng, W.M., M. Schneider, R. Frock, C. Castillejo-Lopez, E.A. Gaman, S. Baumgartner, and H. Ruohola-Baker. 2003. Dystroglycan is required for polarizing the epithelial cells and the oocyte in Drosophila. *Development*. 130:173-84.

Doerflinger, H., R. Benton, J.M. Shulman, and D. St Johnston. 2003. The role of PAR-1 in regulating the polarised microtubule cytoskeleton in the Drosophila follicular epithelium. *Development*. 130:3965-75.

Fehon, R.G., I.A. Dawson, and S. Artavanis-Tsakonas. 1994. A Drosophila homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the coracle gene. *Development*. 120:545-57.

Fievet, B.T., A. Gautreau, C. Roy, L. Del Maestro, P. Mangeat, D. Louvard, and M. Arpin. 2004. Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin. *J Cell Biol*. 164:653-9.

Goode, S., J. Wei, and S. Kishore. 2005. Novel spatiotemporal patterns of epithelial tumor invasion in Drosophila discs large egg chambers. *Dev Dyn*. 232:855-64.

Grevengoed, E.E., D.T. Fox, J. Gates, and M. Peifer. 2003. Balancing different types of actin polymerization at distinct sites: roles for Abelson kinase and Enabled. *J Cell Biol*. 163:1267-79.

Hariharan, I.K., and D. Bilder. 2006. Regulation of imaginal disc growth by tumor-suppressor genes in Drosophila. *Annu Rev Genet*. 40:335-61.

Harris, T.J., and M. Peifer. 2005. The positioning and segregation of apical cues during epithelial polarity establishment in Drosophila. *J Cell Biol*. 170:813-23.

Inoki, K., M.N. Corradetti, and K.L. Guan. 2005. Dysregulation of the TSC-mTOR pathway in human disease. *Nat Genet*. 37:19-24.

Inoki, K., T. Zhu, and K.L. Guan. 2003. TSC2 mediates cellular energy response to control cell growth and survival. *Cell*. 115:577-90.

Kahn, B.B., T. Alquier, D. Carling, and D.G. Hardie. 2005. AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab*. 1:15-25.

Kemphues, K.J., J.R. Priess, D.G. Morton, and N.S. Cheng. 1988. Identification of genes required for cytoplasmic localization in early C. elegans embryos. *Cell*. 52:311-20.

Lizcano, J.M., O. Goransson, R. Toth, M. Deak, N.A. Morrice, J. Boudeau, S.A. Hawley, L. Udd, T.P. Makela, D.G. Hardie, and D.R. Alessi. 2004. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *Embo J*. 23:833-43.

Mandal, S., P. Guptan, E. Owusu-Ansah, and U. Banerjee. 2005. Mitochondrial regulation of cell cycle progression during development as revealed by the tenured mutation in Drosophila. *Dev Cell*. 9:843-54.

Martin, S.G., and D. St Johnston. 2003. A role for Drosophila LKB1 in anterior-posterior axis formation and epithelial polarity. *Nature*. 421:379-84.

Medina, P.M., L.L. Swick, R. Andersen, Z. Blalock, and J.E. Brenman. 2006. A novel forward genetic screen for identifying mutations affecting larval neuronal dendrite development in Drosophila melanogaster. *Genetics*. 172:2325-35.

Nagasaka, K., S. Nakagawa, T. Yano, S. Takizawa, Y. Matsumoto, T. Tsuruga, K. Nakagawa, T. Minaguchi, K. Oda, O. Hiraike-Wada, H. Ooishi, T. Yasugi, and Y. Taketani. 2006. Human homolog of Drosophila tumor suppressor Scribble negatively

regulates cell-cycle progression from G1 to S phase by localizing at the basolateral membrane in epithelial cells. *Cancer Sci*. 97:1217-25.

Narbonne, P., and R. Roy. 2006. Inhibition of germline proliferation during C. elegans dauer development requires PTEN, LKB1 and AMPK signalling. *Development*. 133:611-9.

Oda, H., T. Uemura, Y. Harada, Y. Iwai, and M. Takeichi. 1994. A Drosophila homolog of cadherin associated with armadillo and essential for embryonic cell-cell adhesion. *Dev Biol*. 165:716-26.

Sanchez-Cespedes, M., P. Parrella, M. Esteller, S. Nomoto, B. Trink, J.M. Engles, W.H. Westra, J.G. Herman, and D. Sidransky. 2002. Inactivation of LKB1/STK11 is a common event in adenocarcinomas of the lung. *Cancer Res*. 62:3659-62.

Shulman, J.M., R. Benton, and D. St Johnston. 2000. The Drosophila homolog of C. elegans PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. *Cell*. 101:377-88.

Suzuki, A., M. Hirata, K. Kamimura, R. Maniwa, T. Yamanaka, K. Mizuno, M. Kishikawa, H. Hirose, Y. Amano, N. Izumi, Y. Miwa, and S. Ohno. 2004. aPKC acts upstream of PAR-1b in both the establishment and maintenance of mammalian epithelial polarity. *Curr Biol*. 14:1425-35.

Takizawa, S., K. Nagasaka, S. Nakagawa, T. Yano, K. Nakagawa, T. Yasugi, T. Takeuchi, T. Kanda, J.M. Huibregtse, T. Akiyama, and Y. Taketani. 2006. Human scribble, a novel tumor suppressor identified as a target of high-risk HPV E6 for ubiquitin-mediated degradation, interacts with adenomatous polyposis coli. *Genes Cells*. 11:453-64.

Tiainen, M., A. Ylikorkala, and T.P. Makela. 1999. Growth suppression by Lkb1 is mediated by a G(1) cell cycle arrest. *Proc Natl Acad Sci U S A*. 96:9248-51.

Wodarz, A., A. Ramrath, U. Kuchinke, and E. Knust. 1999. Bazooka provides an apical cue for Inscuteable localization in Drosophila neuroblasts. *Nature*. 402:544-7.

Zhang, L., J. Li, L.H. Young, and M.J. Caplan. 2006. AMP-activated protein kinase regulates the assembly of epithelial tight junctions. *Proc Natl Acad Sci U S A*. 103:17272-7.

Zheng, B., and L.C. Cantley. 2007. Regulation of epithelial tight junction assembly and disassembly by AMP-activated protein kinase. *Proc Natl Acad Sci U S A*. 104:819-22.

Preface

In this chapter, I generated the transgenic flies that overexpress the dominant negative copy of AMPKα. Then, we did initial studies of hypersensitivity to starvation stress. I performed the initial studies of lipid accumulation in the liver-like oenocytes cells (Gutierrez et al.,2007). I performed the characterization of the neuronal phenotypes of the dominant negative AMPKα and RNAi γ constructs.

Citation:

Erik C. Johnson, Nevzat Kazgan, Colin A. Bretz, Lawrence J. Forsberg, Clare E. Hector, Ryan J. Worthen, and Jay E. Brenman. Altered starvation behavior and reduced energy stores caused by reduced AMPK function in *Drosophila.*

CHAPTER III

Altered starvation behavior and reduced energy stores caused by reduced AMPK function in *Drosophila Melagonaster*

Abstract

Organisms must utilize multiple mechanisms to maintain energetic homeostasis in the face of limited nutrient availability. One mechanism involves activation of the heterotrimeric AMP-activated protein kinase (AMPK), a cell-autonomous sensor to energetic changes regulated by ATP to AMP ratios. We examined the phenotypic consequences of reduced AMPK function, both through RNAi knockdown of the gamma subunit (AMPKγ) and through expression of a dominant negative alpha (AMPKα) variant in *Drosophila melanogaster*. Reduced AMPK signaling causes hypersensitivity to starvation conditions as measured by altered lifespan and locomotor activity. Locomotor levels in flies with reduced AMPK function were lower during unstressed conditions, but significantly higher than wild type during starvation-induced hyperactivity, an adaptive response to encourage foraging. Unexpectedly, total dietary intake was greater in animals with reduced AMPK function yet total triglyceride levels were lower. AMPK mutant animals displayed starvation-like lipid accumulation patterns in metabolically key liver-like cells, oenocytes, even under fed conditions, consistent with a cellular starvation phenotype. Measurements of O_2 consumption reveal that metabolic rates were greater in animals with reduced AMPK function. Lastly, rapamycin treatment reduces the starvation sensitivity of animals with reduced AMPK function. Collectively, these results are consistent with models that AMPK shifts energy

usage away from expenditures into a conservation mode during nutrient-limited conditions at a cellular level. The highly conserved AMPK subunits throughout the Metazoa, suggest these findings may provide significant insight into phenotypic outcomes of pharmacologically manipulating AMPK function in humans.

Introduction

The precise mechanisms of how organisms maintain energetic homeostasis in light of dynamic food availability remains largely unknown. A molecule thought to represent a central cellular mechanism mediating energy allocation is the AMP-activated protein kinase, by virtue of its activation by the end product of ATP hydrolysis (Winder and Thomson, 2007). Activation of AMPK by AMP leads to the phosphorylation of Acetyl CoA carboxylase (ACC) and the Peroxisome Proliferator Activated Receptor (PPAR), which has the net biological effect of diverting energy away from cellular processes that require energy and towards a conservation or energy production mode (Long and Zierath, 2006). The functional AMP-activated protein kinase (AMPK) is a heterotrimer consisting of a catalytic alpha (α), a regulatory gamma (γ), and a scaffolding beta subunit (β) (Riek *et al*., 2008). In mammals, multiple genes encode each of the subunits, which form different heterotrimeric complexes (Birk and Wojtaszewski, 2006), so despite intense scrutiny of the roles of AMPK in mammals, the complete organismal context of AMPK function remains poorly understood. In *Drosophila*, a single gene encodes each subunit, and clear homologs of each subunit are present and display 60%, 62%, and 62% identity with human α, β, and γ subunits, respectively. *Drosophila* AMPK is highly similar to mammalian AMPK, as it is formed via a heterotrimeric complex, is activated by AMP, and has many of the same targets, including acetyl-CoA carboxylase (ACC) (Pan and Hardie, 2002).

Genetic modulation of AMPK activity in various model systems suggests that AMPK may represent an underlying mechanism to increase longevity as a consequence of dietary restriction (Greer and Brunet, 2009), and also that AMPK activity may be responsible for the benefits of exercise (Narkar *et al*., 2008). Additionally, pharmacological agents

targeting AMPK activity have been developed as therapeutics for treatment of human pathologies associated with diabetes (Viollet *et al*., 2009).

We report here that attenuated AMPK function in *Drosophila* leads to a series of behavioral and metabolic phenotypes demonstrating these animals behave as though they are experiencing chronic starvation. Specifically, animals deficient in AMPK signaling show a hypersensitivity to starvation conditions, altered activity levels, and differential lipid quantities and distributions. These phenotypes further demonstrate a pivotal role of the AMPK molecule in the organization of behavioral and physiological responses to nutrient-limitations, and that such responses appear to be highly conserved throughout the Metazoa.

Experimental Procedures

***Drosophila* cultures and stocks.** The AMPK^{K57A} transgene was generated by a mutagenic PCR strategy and cloned into the UAS-vector for embryo transformation. The UAS-AMPK-α WT transgene was previously described (Mirouse *et al*., 2007). Other lines that were employed in this study were Ubi-GAL4, Act5C-GAL4, hsp70-GAL4, 109 (80) GAL4, UAS-alpha RNAi, UAS-gamma RNAi, UAS-LSD2-GFP and the UAS-dcr 2. The UAS-dcr2 and UAS-LSD2-GFP lines were kind contributions from Dr. Paul Taghert and Ronald Kuhnlein respectively. All other lines, with the exception of the Ubiq-GAL4 and AMPK alpha transgenes were procured from the Bloomington Stock center (Bloomington, IN). All flies were maintained in an incubator maintained at 25° C and under a 12:12 LD cycle. Flies were cultured on a standard molasses-malt-cornmeal-agar-yeast medium and housed in uncrowded conditions. All transgenes were backcrossed to the w^{1118} background. For hsp70-GAL4 lines, we raised and maintained animals at 19° C for all assays (uninduced), and induction was carried out by placing animals at 30° C for 1 hr twice daily for three days, and then assaying animals at 25°C.

Lifespan Measurements. Measurements of lifespan have been widely employed in *Drosophila* as a metric of stress sensitivity (*e.g*, Broughton *et al*., 2005; Hector *et al*., 2009). We placed thirty 3 to 5 day old mated flies (males and females housed separately) in vials with a two percent agar solution to starve the animals. We assessed percent survival of at least three replicate vials twice daily. For each vial, we assessed the median survival for the treatment and data were pooled to estimate a mean median survival and then employed a one-way ANOVA with post-hoc Tukey's comparison for differences between genotypes/treatments.

Locomotor measurements. Locomotor activity was monitored with a TriKinetics Locomotor Monitor (Waltham, MA) on the aggregate population of thirty 3-5 day old flies (Zhao *et al*., 2010). Flies were housed in a 12:12 LD cycle for three days prior to the experiments. Flies were transferred to a vial containing starvation or normal medium at ZT0. Total beam counts were monitored continuously through an automated system. For analysis of the starvation-induced component, we chose to examine only the first 12 hours of locomotor activity, as significant numbers of AMPK deficient flies would die after 12 hours and confound interpretation. We determined the amount of activity during starvation relative to the activity of fed conditions for the same time period.

Triglyceride and Oil Red O Measurements. Total triglyceride levels were assessed through use of a Serum Total Triglyceride Kit (Sigma, St. Louis) according to manufacturer's instructions and as previously described (Zhao *et al.*, 2010). Thirty flies were weighed, and homogenized completely in 0.5 mL 100mM Tris-HCl (pH 7.4), 1 NaCl, 1% TritonX-100 buffer. Homogenates were centrifuged for 2 minutes at room temperature at 13000 x g to collect particulates. One hundred microliters of supernatant was transferred to a 96 well plate and an equal volume of working triglyceride reagent was added to each well. Triplicate wells were performed for each sample and three replicate samples of flies were run for each treatment condition. Absorbance at 510 nm wavelength was measured on a Perkin Elmer Victor III multilabel microplate reader. Absorbance values were compared to standards and normalized by fly weight. Homogeneity of slopes test was performed following linear regression on normalized triglyceride levels to compare different genotypes following starvation conditions. Oil Red O staining was done as described (Gutierrez *et al*., 2007). Briefly, wild-type and AMPK alpha null larvae were dissected in 4%

paraformaldehyde in PBS to expose the oenocytes, and then fixed for 10 minutes. After rinsing twice with water, lipid droplets were stained with a 0.06% Oil Red O solution in 60% isopropanol for 30 minutes at room temperature, and then rinsed three times with water. Stained larvae were then mounted in glycerol and visualized on a light microscope.

Feeding Assays. To assess food intake, we employed the CAFE assay (Ja *et al*., 2007), which quantifies dietary intake using a volumetric capillary feeder. We placed three 3-5 day old adult flies in the CAFE chamber and monitored the dietary intake of either 5% sucrose/5% yeast extract or 5% sucrose media. Red food coloring was added to each diet to facilitate measurements. Mineral oil was placed over the top of the capillary to reduce evaporation. Animals were placed in a 12:12 LD cycle at ZT0 (lights on). Humidity was controlled by placing the chamber over a water source. Animals were then transferred the following day and capillary tubes were marked and measured. We also determined the amount of diet that was lost to evaporation by placing a capillary feeder in a chamber without flies and this value was subtracted from the values determined for the feeding experiments. Males and females were housed in separate chambers. Differences in dietary intake were assessed using a Two-Way ANOVA. A complementary group of experiments employed variations in a two-choice assay (Al-Anzi *et al*., 2006). This assay was developed to assess aversion to various stimuli and relies on scoring of an impregnated dye in the food and subsequent scoring of abdominal color. High numbers of scored individuals are predicated on a period of starvation, so we modified the number of scored individuals on a single dye in the food with variations in food access.

For rapamycin (LC Laboratories, Woburn MA) feeding, 200 adult *Drosophila* were put in cages over standard cornmeal-yeast media containing vehicle (ethanol) or 1μM

rapamycin to lay eggs overnight. Larvae were scored (n>200 for all conditions) on days 5 and 7 after egg laying and scored for the absence or presence of the green fluorescence of the FM7i balancer. Mutant survival percentages were normalized to their survival at 5 days without rapamycin (but vehicle alone). For adult rapamycin feeding, adult animals were placed on instant Carolina medium containing 100 uM rapamycin for three days prior to starvation assays.

Oxygen Consumption measurements. A calibrated 50uL micropipette was flame bent to fashion a micromanometer. Whatman filter paper was saturated in a KOH solution, to act as a CO_2 scavenger, and placed in the bottom of a 15 mL polypropylene tube. Loose cotton was placed over the KOH-saturated filter paper, and 10 flies were loaded into the chamber which was then sealed with wax. Water was delivered to the end of the micromanometer and given ten minutes to equalize. After this time period, the position of the meniscus was marked and re-marked following a ten minute period for three replicates of ten minutes to estimate an average reading. Measurements were taken on two genotypes differing in AMPK function at the same time; since manometric readings may be influenced by external factors such as changes in barometric pressures, and these grouped readings were repeated six times were analyzed for statistical significance using a nested ANOVA (GraphPad, San Diego).

Results

Altered AMPK function causes abbreviated lifespan during starvation. To investigate the roles of AMPK in the maintenance of metabolic homeostasis in adults, we relied on two experimental strategies to alter AMPK activity. The first approach was to generate a dominant negative construct (K57A), in which the catalytic domain of the alpha subunit was altered to inactivate the ATP binding domain, as suggested by a number of studies in mammalian systems (Woods *et al.*, 2000; Nagata *et al.*, 2009). As a complementary approach, we genetically introduced a double-stranded RNA species targeting the gamma subunit (γRNAi). A null mutation in the alpha subunit of AMPK has been shown to cause lethality in late larval stages (Mirouse *et al.*, 2007).

Expression of the K57A variant with the Act5C-GAL4 driver completely phenocopies the lethality associated with the null mutant, as did specific RNAi constructs targeting the gamma subunits (data not shown). Thus, employing this ubiquitous and strong driver to express elements aimed at reducing AMPK expression or function results in a phenocopy of the lethality caused by a null mutant. Furthermore, the larval lethality caused by the null mutant was not rescued by expression of this dominant negative construct, but was rescued by a wild-type alpha transgene (data not shown). In early larval stages, the null mutant causes aberrant morphology of the Class IV multi-dendritic neurons (Mirouse *et al.*, 2007). Using the 109(2)80-GAL4 driver, we specifically expressed the K57A alpha variant in these sensory neurons, which results in similar morphological defects (Figure 3-1A-F), indicating that this element is effective at genetically reducing AMPK function in a manner similar to loss of function mutations. Consequently, we refer to this variant as the $AMPK^{DN}$ for its dominant negative function. In parallel, we evaluated expression of the AMPKα

subunit in animals expressing either an RNAi targeting the γ (γRNAi) or α (αRNAi) subunits, and observed a significant reduction in the α protein levels in these two genotypes but not in animals possessing the GAL4 alone or in animals expressing an RNAi targeting a different gene (Figure 3-2).

Figure 3-1

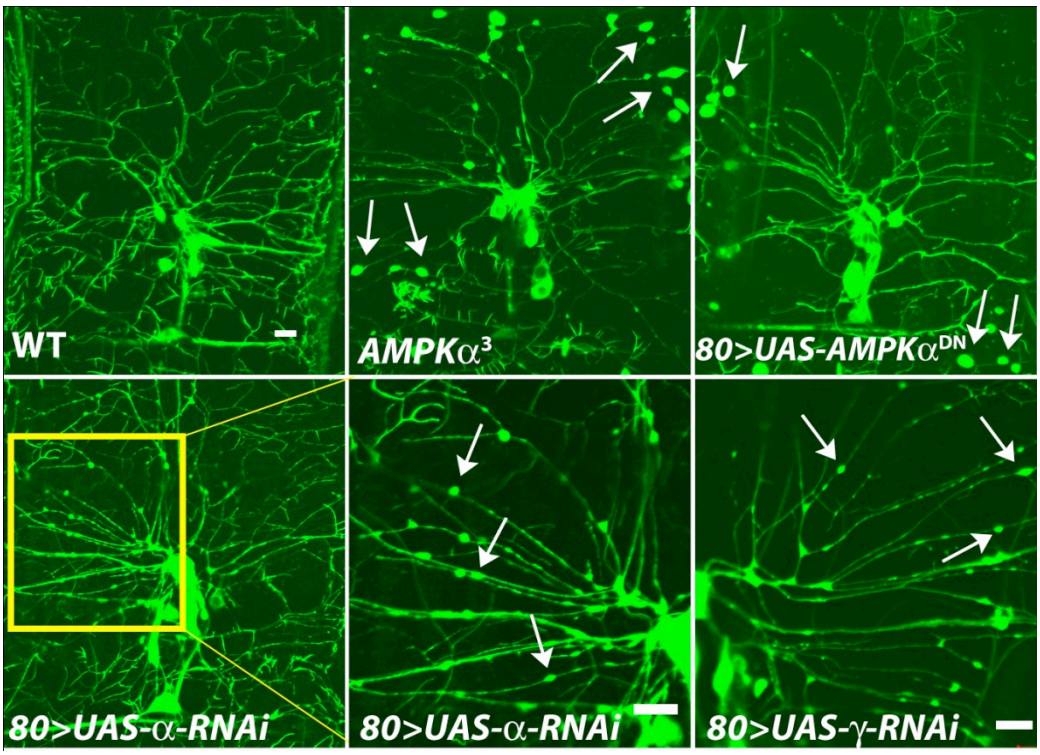

Figure 3-1. Expressing either dominant negative AMPKα or RNAi targeting α or γ subunits phenocopies a null allele of the AMPK α subunit. Representative confocal images from animals specifically expressing an actin-GFP construct in class IV multi-dendritic sensory neurons in larval *Drosophila*, with different levels of AMPK function. (**A)** Normal dendritic morphology of animals with wild-type AMPK. (**B**) Dendritic morphology in animals with an AMPKα subunit null mutation (Mirouse *et al.*, 2007): note the large varicosities on neuronal dendrites. (**C**) Animals expressing the dominant negative alpha subunit variant which phenocopies the null mutant, showing the same abnormal varicosities (white arrows). (Scale bar = 10μm) (**D**) Animals expressing an RNAi targeting the alpha subunit also phenocopies the null mutant, but with smaller varicosities (**E**) Inset of D. **F**. Animals expressing RNAi targeting the gamma subunit also, but more weakly, phenocopies the null mutant (A-E) 22°C (F) 29°C.

Figure 3-2

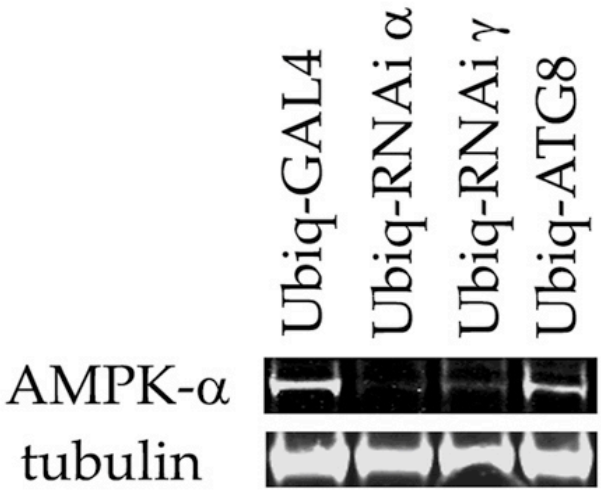

Figure 3-2. Reduced expression of the alpha subunit via expression of RNAi elements. Western blot for the AMPK alpha subunit from lysates from different genotypes, some expressing RNAi for either the alpha (second lane) or the gamma subunit (third lane). Compared to animals possessing the driver alone (first lane) or an unrelated gene (ATG8) (fourth lane), there was significantly less expression of the alpha subunit standardized to loading control (tubulin).

Having validated that these genetic strategies were producing phenotypes consistent with reduced AMPK activity, we then asked whether attenuated AMPK function would alter sensitivity to metabolic challenges. We tested the progeny of a UAS-AMPK^{DN} under the control of a Ubiquitin-GAL4 (Ubiq) driver for survival during starvation, and found a significant reduction of lifespan as compared to a similar cross using a wild-type alpha sequence (which only differs in one amino acid) or to parental stocks (Figure 3-3A, C). This driver recapitulates the global expression pattern of the ubiquitin gene, but is not as strong as the Act5C-GAL4 driver (Schulz *et al.*, 2001). Reduced viability during adult starvation was also evident in animals expressing an RNAi line specifically targeting the gamma subunit also under the control of the Ubiq driver (Figure 3-3B, D). We also assessed aging in these animals and found a significant reduction in lifespan under nutrient-rich conditions (Figure 3-4), although reduced AMPK function did not significantly impact lifespan for younger cohorts used for starvation studies.

Given the developmental defects exhibited in animals lacking functional AMPK, we performed experiments to exclude developmental defects that could be caused by impaired AMPK function as a contributor to starvation sensitivity in adults. We therefore employed the hsp70-GAL4 element, which allows temporal control through stage-specific induction by heat-shock and thus avoid any developmental disturbances caused by earlier transgene expression. Comparison of the median survival of animals experiencing different heat-shock-induced AMPK impairment in adult stages still demonstrated significant reduction in starvation survival (Figure 3-3E-F).

Figure 3-3

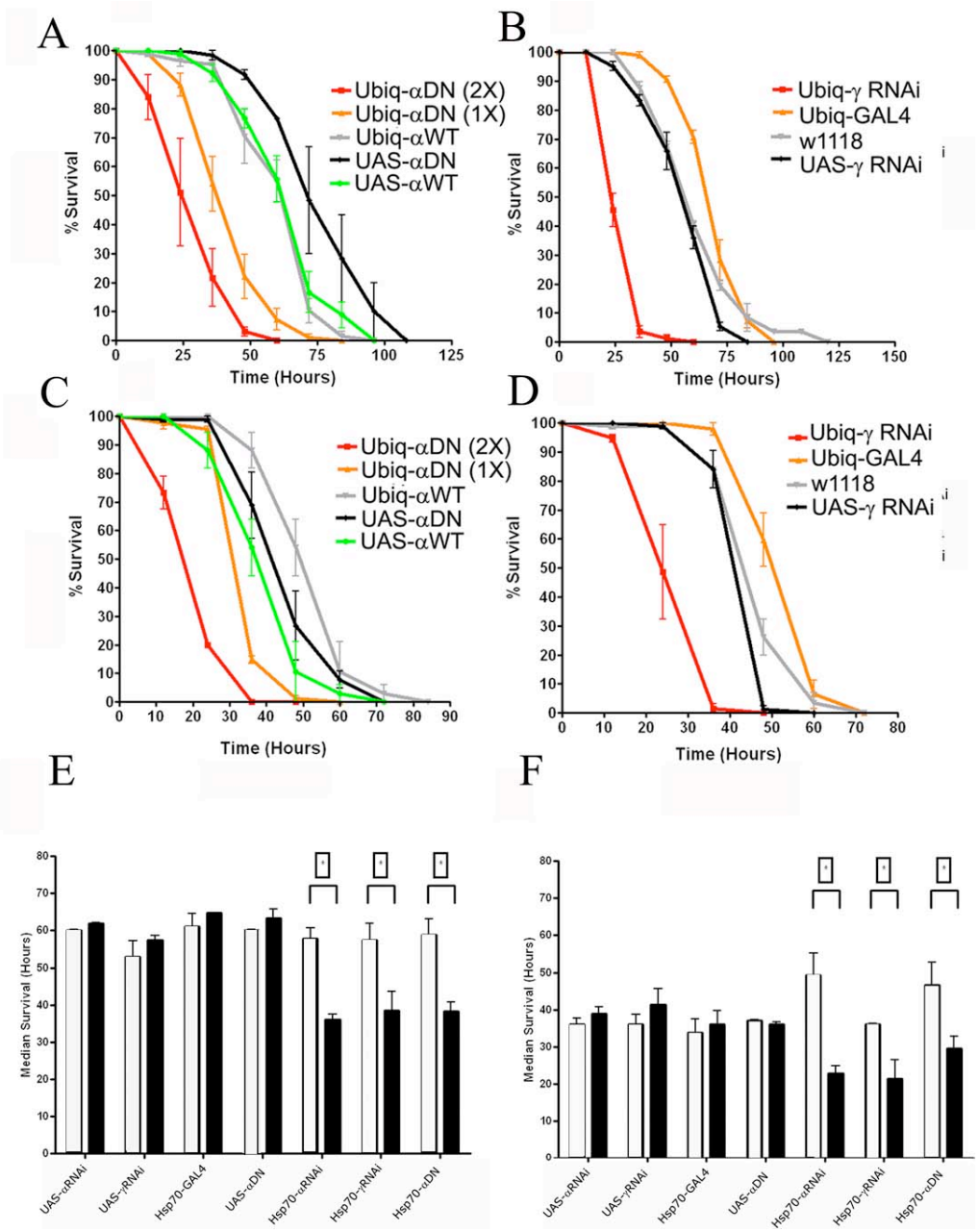

Figure 3-3. Reduction of AMPK function causes a reduced lifespan under starvation.

Survival curves from adult females **(A)** and males **(C)** expressing either one or two copies of the $AMPK^{DN}$ transgenes under the control of the Ubiq-GAL4 driver. Expression of these transgenes reduced lifespan compared to the parental controls or animals expressing a wild-type α subunit. Survival curves of adult females **(B)** and males **(D)** expressing an RNAi element targeting the gamma subunit. The lifespan of these animals was significantly shorter than the parental lines or the w^{1118} genetic background control ($p < 0.05$, ANOVA). **(E)** Female and **(F)** male adult induction of the AMPK-interfering transgenes significantly reduces median lifespan (* indicate significant differences between non-induced (unfilled bars) and induced (filled bars) median survival ($p<0.05$, two-tailed T-test)).

Figure 3-4:

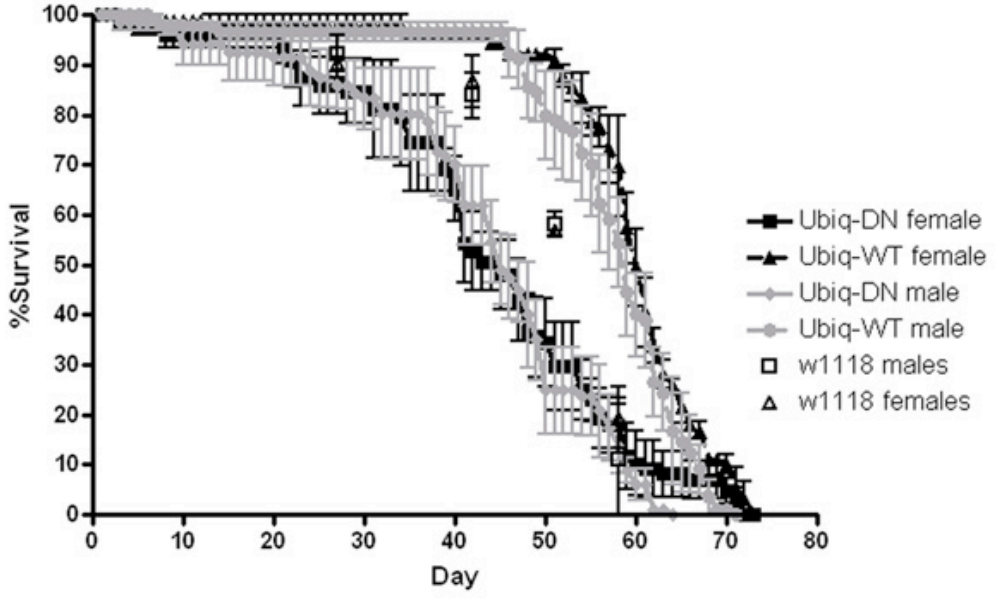

Figure 3-4. Aging is significantly impacted by altered AMPK function. We measured percent survival daily from males and females either expressing the dominant negative or wild type α subunit under normal fed conditions. Overexpression of the α subunit leads to increased longevity as compared to w^{1118} (the genetic background) and in contrast expression of the dominant negative α element leads to significant reduction in longevity.

Reduced AMPK causes changes in locomotor activity. Starvation induces hyperactivity in *Drosophila* and other Metazoa, and is believed to be an adaptive response, as elevated activity promotes increased foraging (Isabel *et al.*, 2005; Lee and Park, 2005). Given the profound nature of the sensitivity to starvation in animals with reduced AMPK function, we hypothesized that a potential mechanism of such sensitivity may be a greater level of hyperactivity. The rationale is that accelerated activity would have the net effect of more rapid exhaustion of energy stores. We first evaluated normal locomotor activity levels in animals with reduced and wild-type AMPK. Animals expressing the dominant negative AMPK α transgene or γ RNAi had significantly lower amounts of locomotion during normal, unstressed conditions as compared to animals expressing a wild-type AMPK α subunit or to w^{1118} genetic background controls (Fig 3-5A) (ANOVA – Tukey posthoc comparison $P< .001$). However, while wild-type animals show a steady increase in activity levels as a function of starvation duration, animals expressing the dominant negative AMPK transgene or the gamma RNAi element displayed instantaneous elevated activity levels (Figure 3-5B, C). Notably, this pattern of locomotor activity was not evident in any of the parental lines.

Figure 3-5

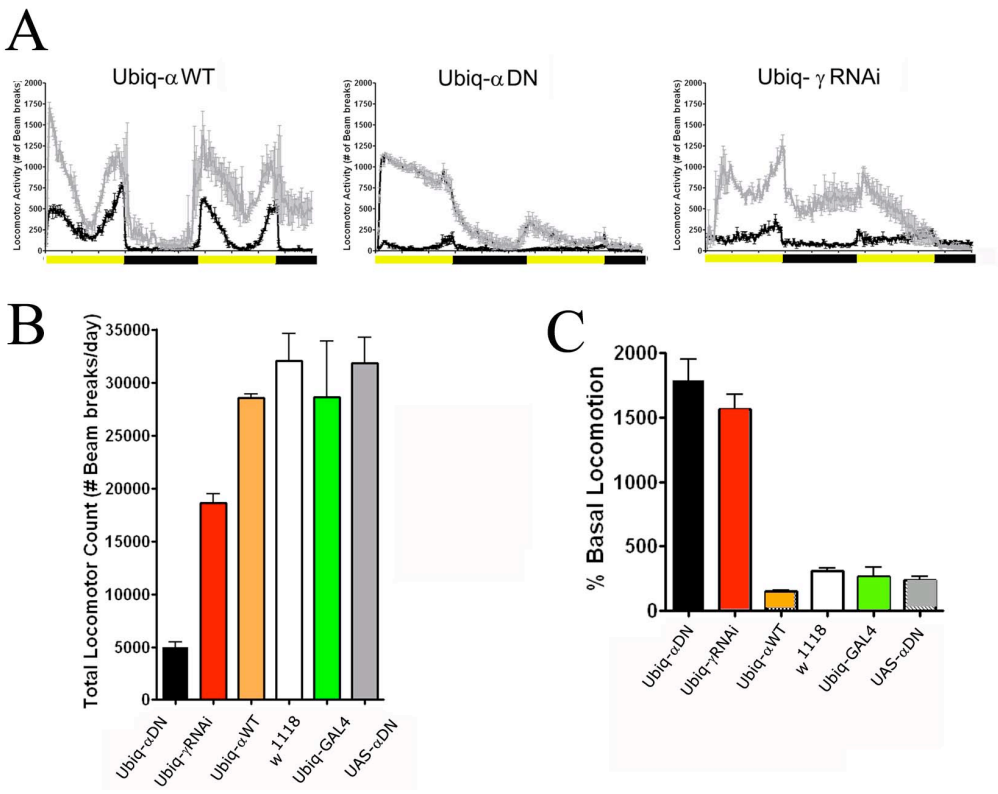

Figure 3-5. Locomotor activity and starvation-induced hyperactivity is altered as a consequence of reduced AMPK signaling. Locomotor activity measured during fed (black line) and starved conditions (gray line) in female animals (**A**) expressing the wild-type α subunit (**left**), the dominant negative α transgene (**middle**), and the γ RNAi element (**right**). Quantification of total locomotion during fed conditions (**B**) and the magnitude of starvation-induced hyperactivity (**C**). Measurements of locomotor activity were initiated at ZT0 (lights on) and flies were maintained in a 12:12 LD (light-dark) cycle (shaded and dark bars). (B) Expression of either the α dominant negative or the γ RNAi element lead to reduced locomotor activity during the fed state (ANOVA, $P< 0.05$). (C) Both the γ RNAi element and the α dominant negative variant caused greater levels of starvation-induced activity relative to basal locomotion (ANOVA, $P<0.05$).

Reduced AMPK causes hyperphagia. Since the loss of AMPK function causes exaggerated responses to starvation, we speculated that this may be caused by reduced food intake. Further rationale comes from mammalian studies where AMPK activity has been shown to regulate the expression of orexigenic transmitters (Claret *et al.*, 2007). To test this hypothesis, we measured total dietary intake employing the CAFE assay (Ja *et al.*, 2007). Total daily food intake was significantly increased in animals expressing the $AMPK^{DN}$ or AMPK gamma RNAi variant and this increase was evident, independent of nutritional value (Figure 3-6A). Specifically, in both males and females, total dietary intake was nearly twice that of animals with wild-type AMPK function ($P < 0.0001$, $F = 29.09$, Two-Way ANOVA). This implicates that not only are animals with reduced AMPK function actively feeding, but that they also display hyperphagia. This hyperphagia might be caused by a persistent starvation signal/phenotype. To test whether loss of AMPK leads to altered hunger-driven feeding behavior, we scored individuals for incorporation of blue dyed food in the abdomens. A variation of this assay was originally developed to assess whether compounds were aversive or appetitive, and require a period of starvation to achieve significant numbers of individuals that feed enough to facilitate scoring (Al-Anzi *et al.*, 2006). We reasoned that if animals deficient in AMPK were consistently starved, then higher rates of scored individuals would be attained without prior starvation conditions. Consistent with these predictions, we found that animals expressing the dominant negative AMPK construct incorporated the dye at a higher level (62.2 %) compared to animals with wild-type AMPK function (37.7 %). Notably, comparable numbers of individuals were scored following a period of starvation, independent of genotype (88.5 % for $AMPK^{DN}$ and 91.8 % for $AMPK^{WT}$) (Figure 3-6B).

Figure 3-6

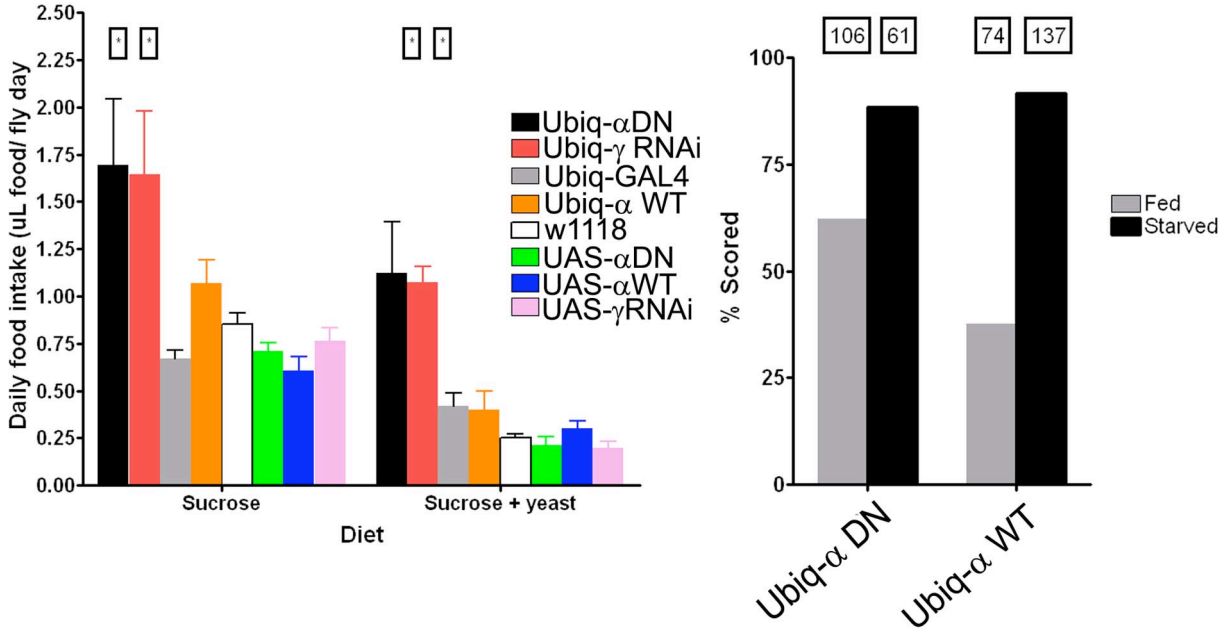

Figure 3-6. Altered feeding behaviors in animals with decreased AMPK function.

(A) Daily food intake in adult *Drosophila* with different AMPK functional levels with a sucrose diet (left) or a yeast-sucrose diet (right). Males and females showed no difference in food intake and data was pooled. Six trials consisting of three trials each were measured for one day. Asterisks denote statistical significance (ANOVA, $p < 0.05$) compared to all other genetic controls.

(B) Abdominal scoring in animals expressing either a wild-type or the dominant negative alpha subunit following either 12 hours of fed conditions (grey bars) or starvation (black bars). Numbers in boxes represent the total number of flies scored for a particular genotype. Animals were given two hours of exposure to the food with the dye and then anaesthetized and abdomens were scored for blue-dye.

Abnormal lipid accumulation phenotypes caused by altered AMPK. Animals with reduced AMPK function demonstrate hypersensitivity to starvation, despite also displaying hyperphagia, suggesting that these animals are persistently experiencing starvation. Because AMPK null mutants die as larvae and that these larvae are characteristically smaller than wild-type larvae of the same age (J.E. Brenman, personal observations), we speculated that persistent starvation may be the ultimate cause of reduced larval growth and lethality. We took advantage of previous observations that starvation induces an accumulation of lipid droplets in larval oenocytes (Gutierrez *et al.*, 2007), and consequently, we examined whether oenocytes from animals with reduced AMPK would show aberrant oenocyte lipid accumulation phenotypes. We introduced a lipid marker: LSD2-GFP, (Gronke *et al.*, 2003) in larvae with either wild-type AMPK function or lacking AMPK alpha gene function (Mirouse *et al.*, 2007). Under fed conditions, animals with wild-type AMPK function showed little to no lipid droplets present in oenocytes as expected and previously published (Gutierrez *et al.*, 2007), whereas in contrast, larvae lacking AMPK showed significantly more droplets and larger droplets per cell, resembling the starved phenotype of wild-type oenocytes (Figure 3-7). This phenotype was also evaluated using Oil-O-red staining which independently labels lipid droplets and circumvents over-expression of the LSD2-GFP marker, yet similar patterns were still observed (Figure 3-7A-G), specifically, that oenocytes with reduced AMPK function show significant lipid accumulation in the fed state.

Figure 3-7

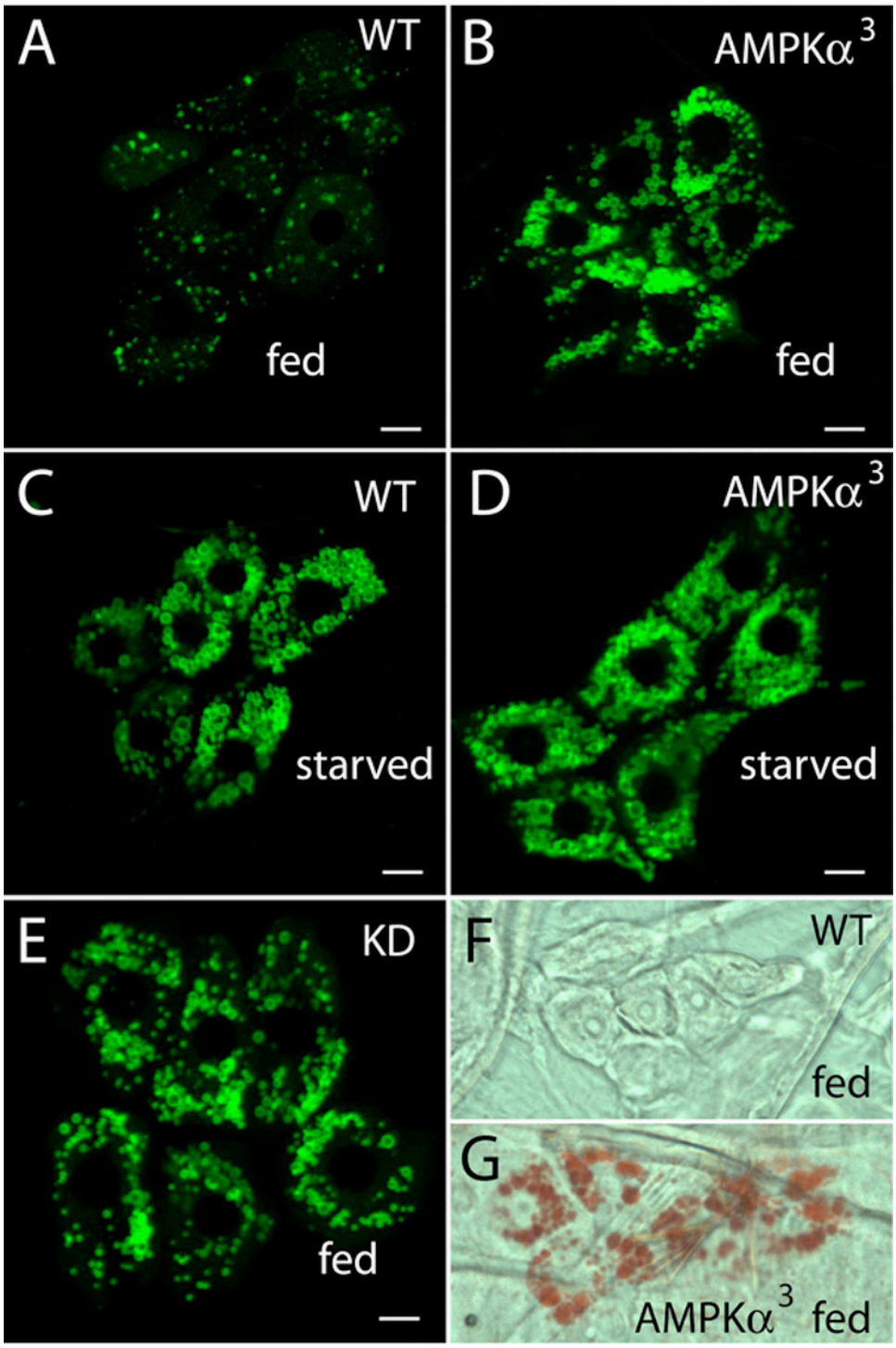
A
WT
fed
B
AMPKα3
fed
C
WT
starved
D
AMPKα3
starved
E
KD
fed
F
WT
fed
G
AMPKα3 fed

Figure 3-7. Abnormal lipid accumulation in oenocytes in animals with reduced AMPK. Oenocytes in the larva are a specialized cell type that functions similar to mammalian liver and accumulates lipids during starvation (Gutierrez et al., 2007). We introduced a LSD2-GFP fusion to oenocytes to observe the size and quantity of lipids in oenocytes in genetic backgrounds differing in AMPK function. In wild-type animals under fed conditions, the LSD2-GFP labeled lipid droplets were small and not prominent (**A**), whereas in the starved state, both the size and number of lipid droplets increase (**C**). In contrast, in AMPKα deficient larvae, there was no difference between the fed and starved states (**B** and **D**) and these closely resemble the starved state of wild-type animals. This phenotype was also exhibited in animals expressing the dominant negative AMPK subunit in oenocytes (**E**). Likewise, the amount of Oil-O red staining was notably absent in wild-type animals during fed conditions (**F**) but labeled significantly more droplets in animals lacking AMPK function during the fed state.

Altered metabolism in animals with reduced AMPK function. Given these observations of persistent starvation phenotypes, we then next tested to see if the size of stored energy reserves were impacted in animals with attenuated AMPK function. We quantified triglyceride levels in animals with either mutant or wild-type AMPK function during normal and starvation conditions. While there were no overt differences in weight, there was a significant impact of the $AMPK^{DN}$ on total triglyceride stores under fed conditions; with the average amount of triglyceride levels for the dominant negative being significantly lower than AMPK-wild type expressing flies (Figure 3-8A). Thus, the decreased amounts of triglyceride stores in flies with altered AMPK function, correlates with abbreviated lifespan during starvation.

While this observation might explain shortened survival under energetic stress, lower triglyceride levels resulting from a loss of AMPK function contradicts previous reports that activation of AMPK causes reduced triglyceride turnover (Zang *et al.*, 2004). However, this result is consistent with a previous observation in *C. elegans* demonstrating that AMPK mutants do not properly utilize lipid stores during energetic stress (Narbonne and Roy, 2009). In attempts to reconcile these observations, we hypothesized that reduced triglyceride levels concomitant with hyperphagia may be caused by increased metabolic demand present in animals with reduced AMPK function. If this were the case, then we reasoned that these animals might exhibit higher consumption of oxygen. Measurements of O_2 consumption in animals expressing the dominant negative alpha construct were consistently higher than in animals expressing a wild-type alpha subunit ($P< 0.0002$, $F = 47.07$, Repeated Measures ANOVA) (Figure 3-8B).

Figure 3-8

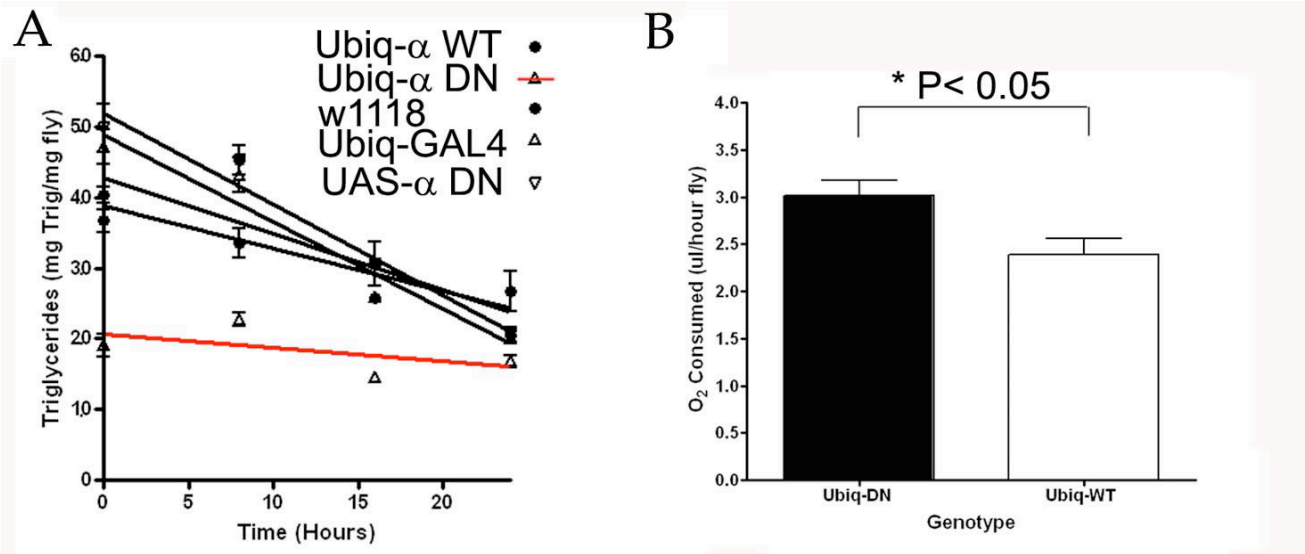

Figure 3-8. Altered metabolism in animals with reduced AMPK function. We quantified total triglyceride levels in adult females with different levels of AMPK function (**A**). Animals expressing the dominant negative construct under the control of the Ubiq-GAL4 driver show significantly lower triglyceride levels during fed conditions (Time 0). We also tested oxygen consumption in animals expressing either the dominant negative or a wild-type alpha subunit (**B**). Animals expressing the dominant negative have higher O_2 consumption than animals expressing a wild-type alpha subunit.

Rapamycin alleviates AMPK phenotypes. Given the observations that animals with reduced AMPK function have higher metabolic rates, we tested the hypothesis that this metabolic activity may stem from failures to reallocate energy during nutritional stress. AMPK signaling can intersect with the target of rapamycin (TOR); AMPK activation would lead to TOR inhibition, which would lead to decreased protein synthesis and decreased energy consumption. (Dowling *et al.*, 2010). We fed rapamycin, a TOR inhibitor, to larvae possessing the null allele of the α subunit and tested if this agent impacted the lethality associated with this mutation. We identified that the majority of AMPK deficient larvae died between the fifth and seventh day of the third instar, but that rapamycin increased the percentage of mutant larvae surviving to the seventh day (Figure 3-9A). We next tested whether rapamycin would likewise improve the starvation sensitivity in adult animals with reduced AMPK function. The median survival of these animals significantly improved during starvation conditions for females (P=0.003 T-Test) and males (P=0.0005 T-Test) (Figure 3-9B).

Figure 3-9

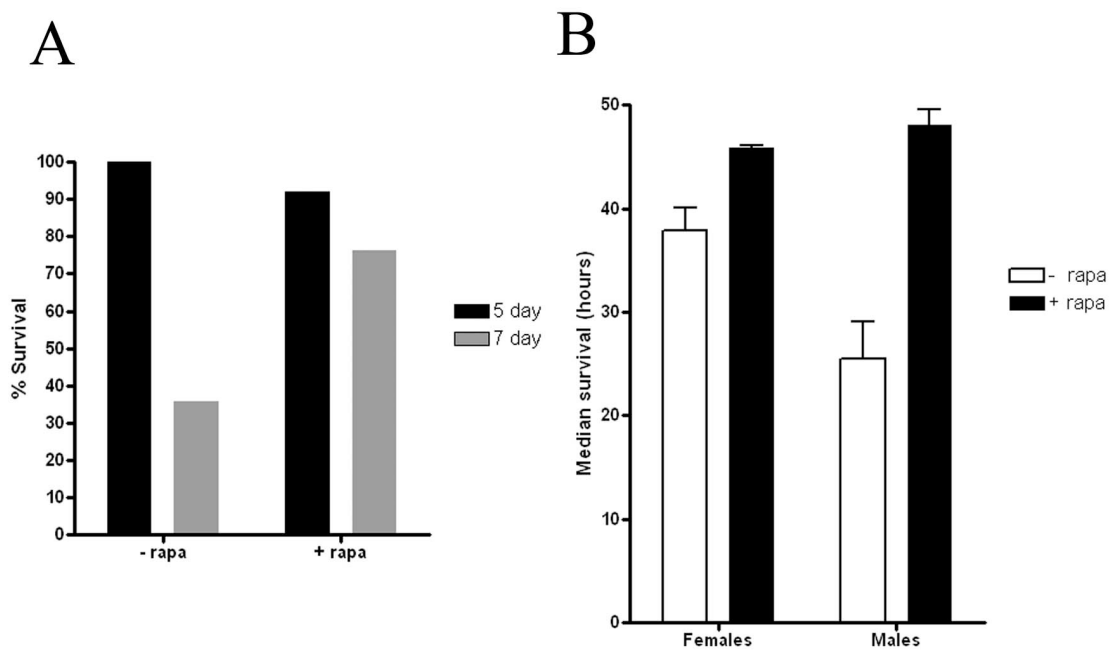

Figure 3-9. Rapamycin increases survival rates caused by reduced AMPK function. **(A)** We fed *AMPKα* mutant larvae rapamycin and evaluated survival. The majority of the lethal effects of the *AMPKα* mutation occur during the 5th and 7th day of the third instar. Incorporation of rapamycin reduced this lethality during this timeframe. **(B)** Likewise, rapamycin feeding for three days prior to starvation improved survival of adult animals expressing the dominant negative variant.

Discussion

We report that animals with reduced AMPK function are highly susceptible to the effects of starvation, and note significant changes in behavioral and physiological responses to starvation caused by altered AMPK function. Demonstrations of increased hyperactive locomotor responses, lower triglyceride levels, increased hunger-driven feeding behaviors, higher metabolic rates, and lipid accumulation phenotypes in liver-like oenocytes indicate that the loss of AMPK leads to a persistent starvation signal both at the organism and cellular levels.

These phenotypes suggest mechanisms underlying reduced survival during starvation in animals with altered AMPK activity. For example, starvation normally induces hyperactivity in flies with wild-type AMPK function (Isabel *et al.*, 2005; Lee *et al.*, 2005), and we observed larger hyperactive responses which occurred more rapidly in animals with reduced AMPK function. Hyperactive responses require more energy than rest but under normal conditions in the wild, might lead to increased foraging behavior and obtainment of new food sources. However, in cages without food in the laboratory, hyperactivity leads to diminished ability to withstand nutritional stress as increased energy expenditure is not offset by obtainment of new food sources. In addition, reduced triglyceride storage found in animals with reduced AMPK function further limits the ability to provide fuel for such energy-requiring responses. Similarly, oenocytes are thought to be involved in the processing of fats making them ready for utilization by the organism (Gutierrez *et al.*, 2007), suggesting that accumulation of lipids in oenocytes in AMPK mutants could be linked to the observed lower global triglyceride levels found elsewhere in the animal.

We suspect that many of these starvation phenotypes are consequences of a core metabolic defect. While we cannot rule out potential pleitropic effects, we suggest our results are consistent with an idea of compensatory changes in critical physiologies and behaviors stemming from defects in energy allocation, storage, or utilization. Our observations that rapamycin significantly extends lifespan, and that animals with reduced AMPK function consume more oxygen indicate inabilities to appropriately regulate cellular metabolic activities. Our observations are also consistent with multiple observations that AMPK is an important factor regulating cellular activities, and regulates expenditures of energy including protein synthesis (Deshmukh *et al.*, 2008). Furthermore, similar observations have been made regarding AMPK function in the nematode, *Caenorhabditis elegans*. Upon exposure to harsh environmental conditions, including nutrient limitation, *C. elegans* enters a dormant dauer state to become stress-resistant. *C. elegans* larvae lacking AMPK activity rapidly consume their energetic stores and die prematurely and are unable to utilize energy efficiently (Narbonne and Roy, 2009; Deshmukh *et al.*, 2008). In addition, altered locomotor activity, lower triglycerides, and higher oxygen consumption rates are also caused by a loss of AMPK function in this genetic model organism (Narbonne and Roy, 2009; Lee *et al.*, 2008); this parallels our findings in *Drosophila*. In mammals, a recent report of altered AMPK activity in the mouse also shows some similar phenotypes, specifically higher locomotor activity and reduced triglyceride levels (Dzamko *et al.*, 2010).

While such observations are consistent with the suspected roles of AMPK as a regulator of cellular activities, our results with an organismal reduction of AMPK function are not entirely expected based on all previous studies and predictions of AMPK function. For example, AMPK is involved in the release of orexigenic peptides from the mammalian

hypothalamus during starvation conditions, which acts to stimulate feeding during low energy (Claret *et al.*, 2007). Unexpectedly, our results with *Drosophila* with compromised AMPK function globally show a similar hyperphagic response, although the relationship to AMPK is opposite. Additionally, AMPK has been shown to inhibit lipolysis (Sullivan *et al.*, 1994), yet our results show that reduced AMPK function leads to lower triglyceride levels instead of higher levels. We maintain that our results do not specifically conflict with these reported roles for AMPK. We note that starvation induces a series of integrated cell-autonomous and hormonal mechanisms to coordinate organismal changes in behavior and physiology, with different cellular populations responding differently (Johnson and White, 2009). Therefore, AMPK is not the singular sensor for low energy levels, and indeed other mechanisms of restoring energy (i.e., autophagy), which can be independent of AMPK (Williams *et al.*, 2009), are presumably responsible for modified behavioral phenotypes in our whole animal reduction of AMPK function. Likewise, we suspect that in some tissues that the loss of AMPK may lead to resistance to nutrient deprivations, through the critical loss of behaviors and/or physiologies that would exhaust energy stores rapidly. We also note that the transgene-drivers employed here may not reduce AMPK levels in all cellular populations equally, although we note that two global drivers (Ubiq-GAL4 and hsp70-GAL4) do give similar phenotypes. Thus, our results may provide insight into the identification of other factor(s) that are likewise involved in mediating metabolic homeostasis.

We also note that these phenotypes likely stem from the acute loss of AMPK function as opposed to developmental defects caused by reduced AMPK signaling. While AMPK is clearly required for normal development, as null mutants are larval lethal (Mirouse *et al.*,

2007), we note that induced transgene expression solely in adults leads to reduced survivorship during starvation. We suspect that the metabolic phenotypes associated with attenuated AMPK signaling are also the cause of the lethality associated with the loss of AMPK function. These larvae are slow-growing, despite actively feeding. Our observations of abnormal lipid accumulation by larval oenocytes demonstrate that the loss of AMPK also leads to persistent starvation conditions (Gutierrez *et al.*, 2007) at least by some cell types.

Our results suggest a series of future experiments targeting AMPK function in various central populations that may mediate feeding behaviors, such as neurons that express neuropeptide F or the *hugin* – encoding peptides, both of which have been previously shown to modulate larval feeding (Wu *et al.*, 2003; Melcher and Pankratz, 2005). Likewise, other potential cell-specific roles of AMPK are now open to investigation in *Drosophila*, including muscle function, liver lipolysis, and locomotor behaviors. For example, the neuroendocrine cells that produce the Adipokinetic Hormons (AKH) are critical for the formation of starvation-induced hyperactive behaviors (Isabel *et al.*, 2005; Lee and Park, 2005). AMPK may participate in this specialized population of cells to regulate AKH signaling, as well as in other potential cells including insulin (Rulifson *et al.*, 2002), which is known to participate in the endocrine events that shape behavioral and physiological responses to starvation. During preparation of this manuscript, an independent report showed similar phenotypes stemming from reduced AMPK function (Tohyama and Yamaguchi, 2010). This report showed a similar sensitivity as we have in regards to *Drosophila* with reduced AMPK function through selective introduction of an RNAi element targeting the alpha subunit. Moreover, this report suggests that the selective loss of AMPK signaling in muscle tissue leads to heightened sensitivity to starvation. Again, we suspect multiple tissues to show

variable phenotypes; this suspicion is based on the multiple functional roles reported for the AMPK molecule, and we have begun assessing starvation phenotypes in selective tissues in *Drosophila*.

In addition, if AMPK functions noted here in *Drosophila* and previously in *C. elegans* (Gutierrez *et al.*, 2007; Greer *et al.*, 2007) are conserved in humans, some thought to global activation of AMPK as a therapeutic should be considered. Observations in *Drosophila* (here) and *C. elegans* suggest loss of AMPK activity leads to difficulty maintaining energy stores and inefficient utilization of them; in essence excessive use of energy stores and "slim" animals with limited triglycerides and higher metabolism. If the opposite were true during AMPK activation (for instance with an AMPK-activating drug in humans) one might predict highly-efficient and sparing utilization of energy stores (fat) in humans. Since Type 2 Diabetes is associated with obesity and a sedentary lifestyle, such an effect of AMPK activators would be very undesirable and potentially lead to increased energy stores (fat), complicating the use of AMPK-activators as drugs in humans.

References

Al-Anzi B, Tracey WD Jr, Benzer S. (2006). Response of *Drosophila* to wasabi is mediated by *painless*, the fly homolog of mammalian TRPA1/ANKTM1. *Curr. Biol*. 16:1034-40.

Birk JB, Wojtaszewski JF. (2006). Predominant alpha2/beta2/gamma3 AMPK activation during exercise in human skeletal muscle. *J Physiol.* 577:1021-32.

Broughton SJ, Piper MD, Ikeya T, Bass TM, Jacobson J, Driege Y, Martinez P, Hafen E, Withers DJ, Leevers SJ, Partridge L. (2005)**.** Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands. *Proc Natl Acad Sci USA*. 102:3105-10.

Claret M, Smith MA, Batterham RL, Selman C, Choudhury AI, Fryer LG, Clements M, Al-Qassab H, Heffron H, Xu AW, Speakman JR, Barsh GS, Viollet B, Vaulont S, Ashford ML, Carling D, Withers DJ. (2007). AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. *J. Clin. Invest*. 117:2325-36.

Deshmukh AS, Treebak JT, Long YC, Viollet B, Wojtaszewski JF, Zierath JR. (2008). Role of adenosine 5'-monophosphate-activated protein kinase subunits in skeletal muscle mammalian target of rapamycin signaling. *Mol Endocrinol*. 22:1105-12.

Dowling RJ, Topisirovic I, Fonseca BD, Sonenberg N. (2010). Dissecting the role of mTOR: Lessons from mTOR inhibitors. Biochim Biophys Acta. 1804:433-439.

Dzamko N, van Denderen BJ, Hevener AL, Jørgensen SB, Honeyman J, Galic S, Chen ZP, Watt MJ, Campbell DJ, Steinberg GR, Kemp BE. (2010). AMPK beta1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. J. Biol. Chem. 285:115-22.

Greer EL, Brunet A. (2009). Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*. *Aging Cell.* 8:113-27.

Greer EL, Dowlatshahi D, Banko MR, Villen J, Hoang K, Blanchard D, Gygi SP, Brunet A. (2007). An AMPK-FOXO pathway mediates longevity induced by a novel method of dietary restriction in *C. elegans*. *Curr. Biol*. 17:1646-56.

Grönke S, Beller M, Fellert S, Ramakrishnan H, Jäckle H, Kühnlein RP. (2003) Control of fat storage by a *Drosophila* PAT domain protein. *Curr Biol* 13:603-6.

Gutierrez E, Wiggins D, Fielding B, Gould AP. (2007). Specialized hepatocyte-like cells regulate *Drosophila* lipid metabolism. *Nature*. 445:275-80.

Hector C.E., C.A. Bretz, Y. Zhao, and E. C. Johnson. (2009) Functional differences between two CRF-Related Diuretic Hormone Receptors in *Drosophila*. J. Exp. Biol. 212: 3142-3147.

Isabel, G., Martin, J.R., Chidami, S., Veenstra, J.A., and Rosay, P. (2005). AKH-producing neuroendocrine cell ablation decreases trehalose and induces behavioral changes in *Drosophila*. *Am J Physiol Regul Integr Comp Physiol*., 288: R531-8.

Ja WW, Carvalho GB, Mak EM, de la Rosa NN, Fang AY, Liong JC, Brummel T, Benzer S. (2007). Prandiology of *Drosophila* and the CAFE assay. *Proc. Natl. Acad. Sci. USA*. 104:8253-6.

Johnson, E.C., and M. P. White (2009). Stressed-out Insects: Behavioral Modifications and Hormonal Actions. In: *Hormones, Brain and Behavior*. (2nd edition) Donald W. Pfaff, Arthur P. Arnold, Anne M. Etgen, Susan E. Fahrbach and Robert T. Rubin, editors. Vol 2. Academic Press; pp. 1069-1096.

Lee, G., and Park, J.H. (2005). Hemolymph sugar homeostasis and starvation-induced hyperactivity affected by genetic manipulations of the adipokinetic hormone-encoding gene in *Drosophila melanogaster. Genetics*, 167: 311-23.

Lee H, Cho JS, Lambacher N, Lee J, Lee SJ, Lee TH, Gartner A, Koo HS. (2008) The *Caenorhabditis elegans* AMP-activated protein kinase AAK-2 is phosphorylated by LKB1 and is required for resistance to oxidative stress and for normal motility and foraging behavior. *J. Biol. Chem.* 283:14988-93.

Long YC, Zierath JR.(2006). AMP-activated protein kinase signaling in metabolic regulation. *J. Clin. Invest*. 116:1776-83.

Melcher C, Pankratz MJ**.** (2005). Candidate gustatory interneurons modulating feeding behavior in the *Drosophila* brain. *PLoS Biol*. 3(9):e305.

Mirouse V, Swick LL, Kazgan N, St Johnston D, Brenman JE. (2007). LKB1 and AMPK maintain epithelial cell polarity under energetic stress. *J. Cell. Biol*. 177:387-92.

Nagata D, Kiyosue A, Takahashi M, Satonaka H, Tanaka K, Sata M, Nagano T, Nagai R, Hirata Y. (2009). A new constitutively active mutant of AMP-activated protein kinase inhibits anoxia-induced apoptosis of vascular endothelial cell. *Hypertens Res*.32:133-9.

Narbonne P, Roy R. (2009) *Caenorhabditis elegans* dauers need LKB1/AMPK to ration lipid reserves and ensure long-term survival. *Nature*. 457:210-4.

Narkar VA, Downes M, Yu RT, Embler E, Wang YX, Banayo E, Mihaylova MM, Nelson MC, Zou Y, Juguilon H, Kang H, Shaw RJ, Evans RM. (2008). AMPK and PPARdelta agonists are exercise mimetics. *Cell*. 134:405-15.

Pan DA, Hardie DG**.** (2002). A homologue of AMP-activated protein kinase in *Drosophila melanogaster* is sensitive to AMP and is activated by ATP depletion. *Biochem. J.* 367:179-86.

Riek U, Scholz R, Konarev P, Rufer A, Suter M, Nazabal A, Ringler P, Chami M, Müller SA, Neumann D, Forstner M, Hennig M, Zenobi R, Engel A, Svergun D, Schlattner U, Wallimann T. (2008). Structural properties of AMP-activated protein kinase: dimerization, molecular shape, and changes upon ligand binding. *J Biol Chem*. 283:18331-43.

Rulifson EJ, Kim SK, Nusse R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. Science. 296:1118-20.

Schulz C, Perezgasga L, Fuller MT. (2001). Genetic analysis of dPsa, the *Drosophila* orthologue of puromycin-sensitive aminopeptidase, suggests redundancy of aminopeptidases. *Dev Genes Evol*. 211:581-8.

Sullivan JE, Brocklehurst KJ, Marley AE, Carey F, Carling D, Beri RK. (1994). Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase. FEBS Lett. 353:33-6.

Tohyama D, Yamaguchi A. (2010). A critical role of SNF1A/dAMPKalpha (Drosophila AMP-activated protein kinase alpha) in muscle on longevity and stress resistance in Drosophila melanogaster. Biochem Biophys Res Commun. 394:112-8.

Viollet B, Lantier L, Devin-Leclerc J, Hebrard S, Amouyal C, Mounier R, Foretz M, Andreelli F. (2009). Targeting the AMPK pathway for the treatment of Type 2 diabetes. *Front Biosci.* 14:3380-400.

Williams T, Forsberg LJ, Viollet B, Brenman JE. (2009). Basal autophagy induction without AMP-activated protein kinase under low glucose conditions. Autophagy. 5:1155-65.

Winder W, Thomson D (2007) Cellular energy sensing and signaling by AMP-activated proten kinase. *Cell Biochem Biophys* 47**,** 332-47.

Woods A, Azzout-Marniche D, Foretz M, Stein SC, Lemarchand P, Ferré P, Foufelle F, Carling D. (2000). Characterization of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. *Mol. Cell. Biol.* 20:6704-11.

Wu, Q., Wen, T., Lee, G., Park, J.H., Cai, H.N., and Shen, P. **(**2003). Developmental control of foraging and social behavior by the *Drosophila* neuropeptide Y-like system. *Neuron*. 39: 147-61.

Zang M, Zuccollo A, Hou X, Nagata D, Walsh K, Herscovitz H, Brecher P, Ruderman NB, Cohen RA. (2004). AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. J. Biol. Chem. 279(46):47898-905.

Zhao, Y., S. A. Hawksworth, C.A. Bretz, J. Hirsh, and E.C. Johnson. (2010) Corazonin neurons participate in sexually dimorphic circuitry that shape behavioral responses to stress in *Drosophila*. PLoS ONE 5: e9141.

CHAPTER IV

Identification of a nuclear export signal in the catalytic subunit of AMP-activated protein kinase

Abstract

The metabolic regulator AMP-activated protein kinase (AMPK) helps maintain cellular homeostasis through activation or inhibition of proteins involved in energy-producing and energy-consuming pathways. While AMPK phosphorylation targets include both cytoplasmic and nuclear proteins, the precise mechanisms that regulate the localization of AMPK, and thus its access to these substrates, are unclear. We identify highly conserved carboxy-terminal hydrophobic amino acids that function as a Leptomycin-B sensitive, CRM1-dependent nuclear export sequence (NES) in the AMPK catalytic subunit (AMPKα). When this sequence is modified AMPKα shows increased nuclear localization via a Ran-dependent import pathway. Cytoplasmic localization can be restored by substituting well-defined snurportin-1 or protein kinase A inhibitor (PKIA) CRM1-binding NESs into AMPKα. We further demonstrate a functional requirement *in vivo* for the AMPKα carboxy-terminal NES, as transgenic *Drosophila* expressing AMPKα lacking this C-terminal tail fail to rescue lethality of AMPKα null mutant flies. Sequestered to the nucleus, this truncated protein shows highly reduced phosphorylation at the key Thr172 activation residue, suggesting that the activation of AMPK predominantly occurs in the cytoplasm under unstressed conditions. Thus, modulation of CRM1-mediated export of AMPKα via its C-

terminal NES provides an additional mechanism for cells to use in the regulation of AMPK activity and localization.

Introduction

AMP-activated protein kinase (AMPK) consists of a trimer containing a catalytic serine-threonine kinase subunit (α) and two regulatory subunits (β and γ) (Davies *et al.*, 1994; Stapleton *et al.*, 1994) (Gao *et al.*, 1996). Many cellular stressors activate AMPK including oxidative stress, heat shock and low energy levels (low ATP/AMP ratios). In response, AMPK restores energetic balance by inhibiting anabolic processes that consume energy, while activating catabolic pathways to conserve energy within the cell (for review (Hardie, 2007)). As an example, AMPK phosphorylates and inhibits cytoplasmic acetyl coenzyme A carboxylase (ACC), a rate-limiting enzyme in energy-consuming fatty acid synthesis (Carling *et al.*, 1987) (Davies *et al.*, 1989), with concomitant activation of energy-producing fatty acid oxidation, likely due to activation of carnitine-palmitoyl transferase I (CPT1) by increased malonyl-CoA (Hardie *et al.*, 1998).

For most kinases, distinguishing *in vivo* from *in vitro* targets remains a difficult endeavor, as homogenized cells and tissues frequently used for *in vitro* kinase assays allow proteins that are normally either spatially or temporally separated to come into contact with each other. One means for regulating kinase access to targets *in vivo* is through such spatio-temporal control of each component. Although AMPK can be found both in the nucleus and the cytoplasm (Salt *et al.*, 1998), the exact mechanisms regulating its subcellular localization have not been fully elucidated. In yeast, only alkaline pH has been shown to induce movement of SNF1 - the AMPKα orthologue in yeast - from the cytoplasm to the nucleus (Hong and Carlson, 2007). In mammalian cells, leptin (Suzuki *et al.*, 2007) and heat shock - possibly through MEK signaling (Kodiha *et al.*, 2007), can also cause translocation of AMPKα subunits to the nucleus. In addition, isoform-specific AMPK subunits have also

been shown to accumulate in the nucleus in a circadian fashion (Lamia *et al.*, 2009), and nuclear translocation can also be induced *in vivo* in muscle cells after exercise stress (McGee *et al.*, 2003).

AMPK subcellular localization could have many important functional consequences. The most apparent expected effects of increasing nuclear localization would include an increase in phosphorylation of nuclear substrates of AMPK, such as the peroxisomes proliferator-activated receptor gamma coactivator-1α (PGC-1α), while cytoplasmic targets such as ACC would show decreased phosphorylation. To add further complexity to AMPK regulation, one of the upstream AMPK activators, the LKB1 kinase, also shuttles in and out of the nucleus (Dorfman and Macara, 2008), but is largely activated in the cytoplasm (Boudeau *et al.*, 2003a).

Primary amino acid sequence analysis can sometimes predict structure/function relationships for domains of a protein. For single subunit proteins, nuclear localization sequences and nuclear export sequences within the protein itself help identify mechanisms for its localization. However, for multi-subunit kinases like AMPK and the LKB1/STRAD/MO25 complex, such regulation is more complicated.

Although the AMPKα amino-terminus is highly conserved, containing the serine-threonine kinase domain, the AMPKα carboxy terminus does not contain any known functional motifs outside of the β/γ binding sites. We previously noted that the final carboxy-terminal 20 amino acids of AMPKα are highly conserved across diverse species (Brenman and Temple, 2007). In this study, we utilized genetic and cell biological approaches to evaluate potential functions for the AMPKα c-terminus. We identify a critical

new function for the carboxy-terminal amino acids of AMPKα *in vivo*, which affects AMPKα subcellular localization, phosphorylation and ultimately organismal viability.

Materials and Methods

Generating Transgenic Flies. Truncated *dAMPKαΔC* was cloned into a pUAST vector as a BglII-EcoR1 fragment of a *dAMPKα-RA* transcript (www.flybase.org) by inserting a stop codon after Proline 561 of wild type *dAMPKα* using PCR-based mutagenesis. The GFP::dAMPKα and mCherry::dAMPKαΔC fusion proteins were made using GFP or mCherry at the fused in-frame to the N-terminus of *dAMPKα* in the pUAST vector. Transgenes were introduced into a w^{1118} stock via P-element mediated-transformation by the Duke Animal Models Core facility.

Fly Stocks and Crosses. *UAS-dAMPKα* wt and *UAS-dAMPKαΔC* alleles expressed in sensory neurons using a *l(2)109-80-GAL4* driver (Gao *et al.*, 1999) and recombined with *UAS-actin::GFP* to visualize sensory neurons, as described previously (Medina *et al.*, 2006). For rescue experiments, both constructs were expressed using the *Ubiquitin-Gal4* driver and crossed into *dampk*α3 null mutants. Adult males were scored for rescue by non-bar eye phenotype due to the lack of FM7 balancer chromosome. All flies were maintained at 25ºC in yeast-cornmeal vials. Heat shock experiments were performed by crossing mCherry and GFP constructs of wild-type *dAMPKα* to *Ubiquitin-Gal4*. Third instar larvae were subjected to heat shock for 1 hr at 37ºC and allowed to recover for 15 minutes at 25ºC. Live larval imaging was performed as described (Mirouse *et al.*, 2007) using the 488-nm argon line for GFP and 543-nm Helium neon line for mCherry on a Zeiss LSM510 confocal microscope. *UAS-GFP::APC2* flies were a gift from Dave Roberts and Mark Peifer (UNC-Chapel Hill).

Plasmid Construction. For cell culture studies, both wild-type and truncated versions of AMPKα2 were amplified by PCR using Pfu DNA Polymerase (Stratagene) from rat AMPKα2 (AddGene:plasmid 15991) and inserted into pEGFP-C1 (Clontech

Laboratories, Inc). Mouse AMPKα1 wild-type and truncated versions were generated using PCR amplification from mouse cDNA and inserted into pEGFP-C1. The SV40-NLS (PKKKRKVG), AMPKα2 and AMPKα1 C-terminal tail tags were cloned into the C-terminus of the pEGFP-C1:: GFP construct. For expressing the SV40NLS and the AMPKα2 C-terminal tag together, the SV40NLS coding sequence was inserted into the N-terminal forward primer for amplification of the GFP coding sequence. The SV40NLS::GFP amplicon was then inserted into a pEGFP-C2 plasmid containing the AMPKα2 C-terminal tail coding sequence at the C-terminus of that plasmid.

L546A and L550A substitutions in the AMPKα2 C-terminal tail sequence was inserted into the reverse primer sequence and cloned using site directed mutagenesis. HA-tagged human AMPKγ1 and rat AMPKβ1 constructs were gifts from Reuben Shaw (UCSD), mCherry::AMPKγ1 was produced by inserting human AMPKγ1 into a modified pEGFP-N1 vector with GFP replaced by Flag::mCherry (gift from Tom Maynard, UNC-Chapel Hill). Myc-tagged clones of wild-type AMPKα2, AMPKα2ΔC, and AMPKα2$^{L,L\text{-}A,A}$ constructs were all generated using standard site directed mutagenesis and inserted into the pCMV-myc vector (Clontech, 631604). The RanQ69L clone was a gift from Andrew Wilde (University of Toronto), which was then inserted into a pmCherry-C1 plasmid for live cell visualization.

To generate AMPK-CRM1-NES fusions, sequences encoding residues Met1 to Val14 (human Snurportin-1) or Ser35 to Ile47 (human cAMP-dependent protein kinase inhibitor alpha (PKIA)) were added after Asp538 of rat AMPKα2, replacing the C-terminal tail. Truncated AMPKα2 missing only the last 14 amino acids (AMPKα2ΔC^{538}) was produced by introducing a UGA stop codon after Asp538. For GFP-tagged constructs, products were amplified using PCR, ligated into the pEGFP-C1 vector and sequenced to verify fidelity. For

myc-tagged constructs, the coding regions were amplified from the above GFP-AMPKα2 plasmids and inserted into the pCMV-myc vector.

Immunohistochemistry. AMPKα localization in fly tissue was determined using standard dissection and immunostaining procedures (Medina *et al.*, 2006). The primary antibody was anti-AMPKα (mouse) (Abcam: ab51025) and the secondary antibody was Cy-3 conjugated anti-mouse (1:200, Jackson ImmunoResearch Laboratories, Inc.). During the wash steps, ToPro-3 (Invitrogen) was added to the wash solution and incubated for 30 minutes to stain nuclei. Larval fillets were mounted on slides in 70% glycerol in 1xPBS. Images were collected by confocal microscopy with a 40x oil immersion lens (LSM 510; Carl Zeiss Microimaging Inc.) using suitable GFP, Cy3 and ToPro-3 excitation wavelengths.

AMPKα Immunoprecipitation. *Drosophila* protein lysates for immunoprecipitation were prepared by collecting equal numbers of male and female flies (50 total) of each genotype in a 1.5 ml tube. One ml lysis buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate 1 mM b-glycerolphosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 1:500 dilution of Sigma mammalian protease inhibitor cocktail) was added to each sample. Flies were then ground to homogeneity with a pestle, sonicated, and centrifuged at 16,000xg for 10 minutes at 4°C to remove insoluble material and debris. Supernatants were collected, and the protein concentration was determined using the Bio-Rad Dc Protein Assay. Immune complexes were formed by incubation of 100 µl of anti-dAMPKα mouse monoclonal antibody (Abcam ab51025) with 1 mL of 1 mg/mL fly lysate overnight at 4°C. Antibodies were precipitated by incubation with 20µl protein A/G agarose (Pierce Protein Research Products) for 3 hours, followed by centrifugation and washing with lysis buffer. Proteins were eluted by 10 minute

70°C incubation in LDS-loading buffer (Invitrogen Corporation) and separated on 4-12% Bis-Tris NuPAGE gels along with 50 μg of crude lysates. Electrophoresis was followed by Western blotting and probing for phospho-AMPKα (Cell Signaling Technology, #2535) and total dAMPKα, with anti-Lamin C (LC28.26, Developmental Studies Hybridoma Bank) used as a loading control.

AMPKα/β/γ Co-Immunoprecipitation. HEK293 cells were transiently transfected with myc-tagged AMPKα2, HA-tagged AMPKβ1, and FLAG-tagged Cherry-AMPKγ1 in 10 cm dishes. Cells were harvested and lysed in 0.5 ml lysis buffer (see above), sonicated, and centrifuged at 16,000x*g* for 10 minutes at 4°C. Protein concentrations of supernatants were determined followed by overnight incubation of 1 ml of 1 mg/ml of each lysate with 100 μL of anti-myc antibody (9E 10, Developmental Studies Hybridoma Bank). Antibody precipitation and blotting were performed as described above. Primary antibody dilutions were anti-Myc (1:100), anti-HA (1:250, Santa Cruz, F-7), and anti-FLAG (1:1000, Sigma, M2). All blots were scanned and quantified using the Odyssey Infrared System (LI-COR Biosciences) using fluorescently-labeled secondary antibodies (LI-COR, IRDye 680 anti-rabbit IgG and IRDye 800 anti-mouse IgG).

Cell Culture and Treatments. HEK293, HeLa and CHO cells were cultured in DMEM medium supplemented with 10% Fetal Bovine Serum and penicillin-streptomycin. Cells were plated 24 hr prior to transfection. DNA transfection of cells (1 μg of DNA to 2ml of medium) was performed using PolyJet DNA Transfection Reagent (SignaGen Laboratories), as directed by the supplier. Leptomycin B (10 ng/ml, Sigma) and cycloheximide (1 μg/ml, Roche) were pre-mixed in culture medium and added as indicated.

Cells were treated with cycloheximide for 1 hr before addition of Leptomycin B mix for indicated times.

Nuclear Localization Assay, Live Cell Imaging and Tiling. HEK293 cells were transfected with the constructs indicated and scored 24 hours later using fluorescence microscopy to observe the sub-cellular localization of GFP. 2xGFP alone was the negative control for localization and for treatment with Leptomycin B. For each GFP fusion, 200 cells were counted and scored as: predominantly nuclear, nuclear and cytoplasmic, or predominantly cytoplasmic localization. For imaging, cells were fixed and stained with DRAQ-5 (Biostatus Limited) to visualize nuclei. For live cell imaging, 24 hours post-transfection the cells were treated with cycloheximide (1μg/ml) for 1 hour followed by treatment with Leptomycin B (10ng/ml). Pictures were taken every 10 minutes for GFP localization using an Olympus FV100 microscope. Tiling images were also taken using the Olympus FV100 Multi-Area Time Lapse Imaging program followed by counting and deciphering of the subcellular localization of GFP as previously described (Henderson, 2000). To control for biased counting, 50 cells from each of the three classes (N, NC, and C) were selected and the fluorescence intensities were quantified as previously described (Henderson, 2000). For cells identified as predominantly nuclear, the ratio of nuclear to cytoplasmic GFP level in a cell was greater than 2, for nuclear and cytoplasmic localization the same ratio was between 0.7 and 2, and for predominantly cytoplasmic localization the ratio was below 0.7.

Results

The AMPKα Carboxy-Terminus is Required for Function. Despite containing a highly conserved carboxy terminus ((Brenman and Temple, 2007) and (Figure, 4-1A and B)), the AMPKα C-terminal 23 amino acids do not contain predicted functional motifs. Co-crystal structures of the carboxy terminus of AMPKα with β/γ subunits indicate that the carboxy-tail participates in intermolecular interactions with the β subunit and intramolecular interactions with the rest of the C-terminal domain (Amodeo *et al.*, 2007; Xiao *et al.*, 2007), although previous studies also suggest that the AMPKα carboxy-tail is not required for association with the β/γ subunits (Iseli *et al.*, 2005).

To test the functional significance of the AMPKα carboxy terminus, we generated transgenic *Drosophila* expressing full-length AMPKα lacking the final 22 amino acids (AMPKαΔC) for reintroduction into *AMPKα* mutant or wild type backgrounds. While vertebrates contain two largely genetically redundant *AMPKα* genes, *Drosophila* encodes only a single *AMPKα* gene thus greatly simplifying *in vivo* genetic analyses. Expression of full-length (amino terminally-tagged or untagged) versions of AMPKα in a null background rescues the previously identified neuronal phenotype ((Mirouse *et al.*, 2007) and Figure 4-1C), while AMPKαΔC versions do not (Figure 4-1C). Further, although both N-terminally GFP-tagged or mCherry-tagged AMPKα rescue lethal null mutations to full viability at expected Mendelian ratios (data not shown), neither untagged nor tagged AMPKαΔC rescue any mutant alleles to viability (scoring over one thousand potential rescue events). These experiments indicate a crucial function for the carboxy-terminal 22 amino acids of *Drosophila AMPKα* in neuronal maintenance and viability.

Figure 4-1

A
Kinase Domain
AID
Subunits
11
278
286
336
398
472
535-552
ΔC
T172
B
523
552
Human STLSSVSPRL GSHTMDFFEM CASLITTLAR
Rattus STLSSASPRL GSHTMDFFEM CASLITALAR
Drosophila VSGVMPLQPT GHHTMEFFEM CAALIIQLAR
C.elegans TPPPSPGGAK LSQTMQFFEI CAALIGTLAR
S.cerevisiae VSNISEDEMS TFSAYPFLHL TTKLIMELAV NSQSN
C
WT
AMPKα3
80>UAS-AMPKα
AMPKα3
80>UAS-AMPKαΔC
AMPKα3

Figure 4-1. AMPKα contains a highly conserved carboxy-terminal tail required for function *in vivo*. (A) Schematic of AMPKα homology with blocks highlighting regions with >50% conservation among bilateria (rat AMPKα2 as template; calculated by ProPhylER). Amino acid numbers denote the ranges. (AID: autoinihibitory domain; 398-472: previously mapped site for β and γ subunit binding) **(B)** The carboxy-terminal tail sequence alignment of AMPKα orthologues with conserved residues highlighted in blue and invariant residues in red (AMPKα2 for species with multiple AMPKα subunits). **(C)** Peripheral neurons visualized by *Gal4 109(2)80*-driven expression of *UAS-actin::GFP* in wild type (WT) *Drosophila* 2nd instar larvae, *AMPKα* loss of function ($AMPK\alpha^3$) larvae, $AMPK\alpha^3$ expressing a wild type *AMPKα* transgene (80>*UAS-AMPKα*), or $AMPK\alpha^3$ expressing a C-terminally truncated *AMPKα* transgene (80>*UAS-AMPKα* ΔC). Dendritic swellings are highlighted with arrows. Bar, 10 μm.

The Carboxy-Terminal 22 Amino Acids of AMPKα are Required for Normal Localization in vivo. In order to explore why a 22 amino acid carboxy terminal deletion of AMPKα fails to rescue *AMPKα* null mutants, we examined the localization of the truncated protein both by antibody immunohistochemistry and visualization of fluorescently tagged fusion protein. Expression of untagged AMPKα in transgenic animals and subsequent detection of AMPKα by immunohistochemistry revealed very clear subcellular localization differences between full-length and truncated protein (AMPKαΔC) (Figure 4-2A). AMPKαΔC localizes predominantly in the nucleus whereas full-length protein is both cytoplasmic and nuclear, using either immunohistochemistry of untagged protein (Figure 4-2A) or live animal images with fluorescently tagged proteins (Figure 4-2B). The observation that AMPKαΔC is highly enriched in the nucleus was observed in diverse tissues including neurons, muscle, fat bodies and salivary glands (Figure 4-2 and data not shown). Differential subcellular localization of AMPKα with and without the carboxy terminus is most clearly demonstrated using a live transgenic animal simultaneously expressing both full length and AMPKαΔC in the same cells (Figure 4-2B). This observation was not restricted to *Drosophila in vivo*, as a conceptually similar result was observed in transiently transfected mammalian cells (HEK293) *in vitro* (Figure 4-2C). Counting of transfected mammalian cells for subcellular localization demonstrated a clear difference between full-length AMPKα and AMPKαΔC (Figure 4-2D).

Figure 4-2

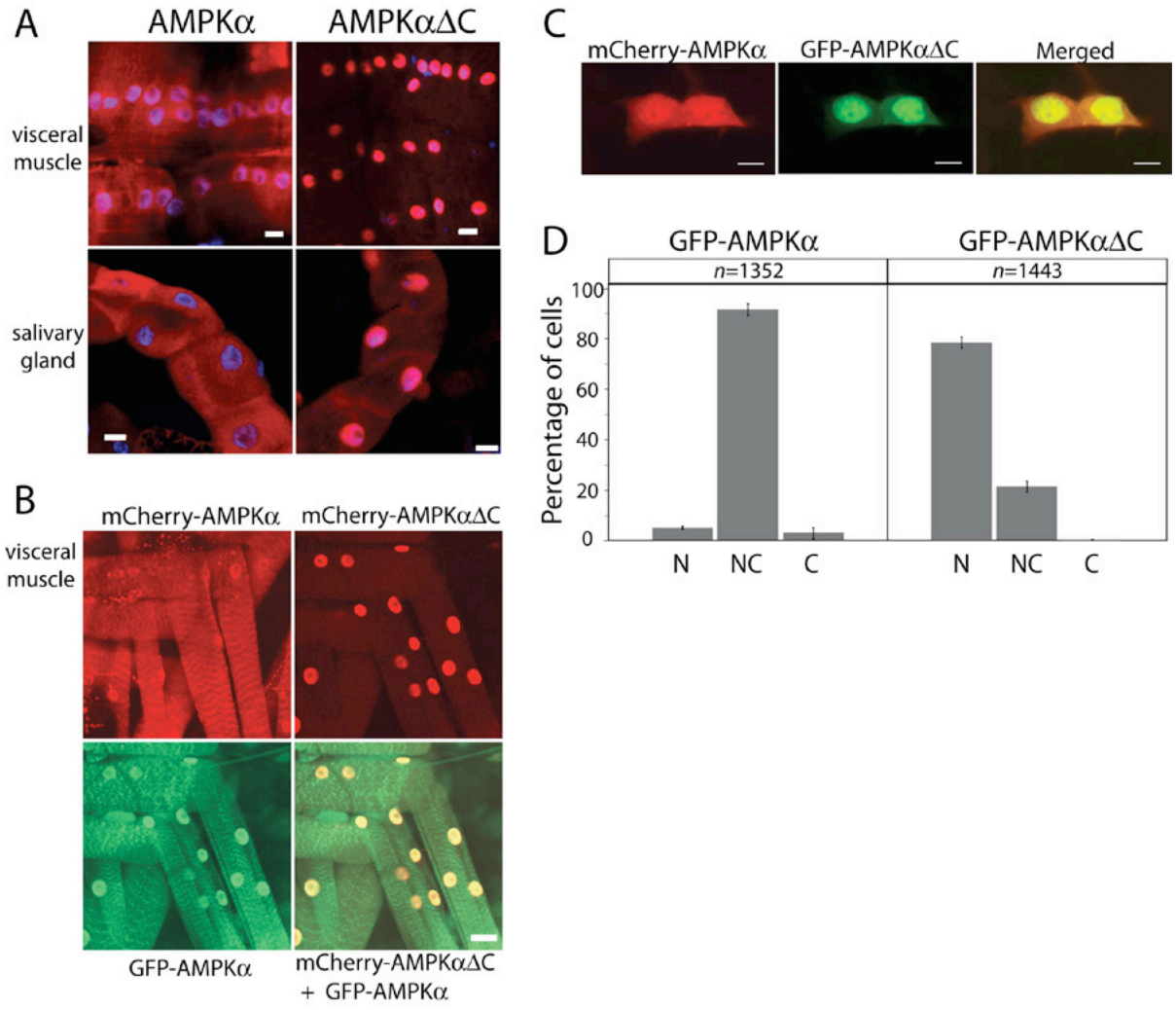
A
AMPKα
AMPKαΔC
visceral muscle
salivary gland
B
mCherry-AMPKα
mCherry-AMPKαΔC
visceral muscle
GFP-AMPKα
mCherry-AMPKαΔC + GFP-AMPKα
C
mCherry-AMPKα
GFP-AMPKαΔC
Merged
D
GFP-AMPKα
GFP-AMPKαΔC
n=1352
n=1443
Percentage of cells
100
80
60
40
20
0
N
NC
C
N
NC
C

Figure 4-2. AMPKα lacking the carboxy terminus localizes predominantly to nuclei *in vivo* and *in vitro*. **(A)** Indirect immunofluorescence of AMPKα (red) staining with anti-dAMPKα antibody on transgenic *Drosophila* third-instar larvae expressing either full-length wild type AMPKα or the carboxy-truncated AMPKα (AMPKαΔC). All transgenic proteins are expressed using the *Gal4-UAS* system, driven by *Ubiquitin-Gal4* (A and B). Nuclei were stained with ToPro-3 (blue). **(B)** Live animal images of larvae expressing amino-terminal mCherry-tagged wild type (mCherry-AMPKα) or truncated (mCherry-AMPKαΔC) AMPKα, GFP-tagged wild type α (GFP-AMPKα) alone or in combination with mCherry-tagged truncated α (mCherry-AMPKαΔC+GFP-AMPKα). Bar, 10μm. **(C)** Co-expression of amino-terminal fluorescently-tagged full-length AMPKα2 (mCherry-AMPKα, red) and truncated (GFP-AMPKαΔC, green) in the same co-transfected mammalian cells (HEK293). **(D)** Scoring of transfected cells for subcellular localization of GFP-tagged full-length (GFP-AMPKα) or truncated (GFP-AMPKαΔC) AMPKα as primarily nuclear (N), both nuclear and cytoplasmic (NC) or primarily cytoplasmic (C). Proper scoring for each group was confirmed by quantification of nuclear and cytoplasmic fluorescence for >50 cells in each group (N: C fluorescence ratios were >2.0, 0.7-2.0, and <0.7, for N, NC, and C, respectively).

The AMPKα Carboxy-Tail Functions as a Leptomycin B-Sensitive Nuclear Export Sequence (NES). To elucidate whether the carboxy-terminus might act as a nuclear export sequence in AMPKα localization, we used a previously described mammalian cell assay to test for sequences that alter the subcellular localization of proteins (Frederick *et al.*, 2008). We fused the AMPKα carboxy-tail in frame at the C-terminus of two tandem GFP molecules (Figure 4-3A). Two consecutive fused GFPs (2xGFP, ~54kD) localize diffusely within the nucleus and the cytoplasm (as previously reported (Frederick *et al.*, 2008) and Figure 4-3B). Adding the 23 amino acid carboxy tail of rat AMPKα2 (2xGFP-α2 tail) leads to localization predominantly in the cytoplasm (Figure 4-3B).

As a previous study demonstrated that endogenous (untagged) AMPKα protein in HeLa cells enriches in the nucleus upon treatment with Leptomycin B (LMB) (Kodiha *et al.*, 2007), a specific inhibitor of CRM1-mediated nuclear export (Kutay and Guttinger, 2005), we tested whether LMB specifically inhibits this AMPKα tail-dependent nuclear export. Indeed, LMB treatment does result in altered localization of the 2xGFP-α2 tail from predominantly cytoplasmic (Figure 4-3B) to both nucleus and cytoplasm (Figure 4-3D). This effect of LMB can also be illustrated using time-lapse experiments, showing accumulation of proteins containing the carboxy tail of AMPKα2 in the nucleus within 10 minutes of LMB addition (Figure 4-4).

Since CRM1 NESs are often leucine-rich (Kutay and Guttinger, 2005), we specifically mutated two conserved leucines in the carboxy tail (2xGFP-α2$^{L,L\rightarrow A,A}$) (Figure 4-3D), which altered localization to both nuclei and cytoplasm similar to treatment of 2xGFP-α2 tail with LMB. Identical results were obtained using the carboxy tail of AMPKα1 (data not shown), which also contains the same conserved bulky hydrophobic residues (including leucines) at

the same positions and suggests that any difference between AMPKα1 and AMPKα2 localization is not due to their tail sequences. Using a larger GFP-tagged construct containing full length AMPKα with the dual leucine mutations (GFP-AMPKα$^{L,L\rightarrow A,A}$) resulted in even more pronounced accumulation in the nucleus (Figure 4-3, E and F), perhaps due to diminished non-directional diffusion through the nuclear pore or the presence of nuclear import signals elsewhere on AMPK.

Addition of the carboxy terminal tail of AMPKα2 to 2xGFP was even sufficient to overcome nuclear targeting via the SV40 NLS (SV40-NLS α2 tail, Figure 4-3B). This effect was not due to inactivation of the SV40 NLS, as treatment with LMB induced accumulation of the NLS-containing protein in the nucleus (Figure 4-4). As the tail appears to act as a CRM1-dependent NES, we compared the AMPKα tail sequence to known CRM1-dependent nuclear export sequences (NESs) (Figure 4-3C). Although the precise positioning of key residues for CRM1-dependent NESs vary, they are generally highly enriched for bulky hydrophobic amino acids (ϕ = leucine, isoleucine, phenylalanine, valine, methionine) at specified spacings (ϕ-x-2/3-ϕ-x-2/3-ϕ-x-ϕ) (Kutay and Guttinger, 2005), generally consistent with an α helix. AMPKα carboxy tails are also enriched for bulky hydrophobic amino acids (Figure 4-1B), only one residue away from being a canonical NES (Figure 4-3C). (Yeast SNF1 does indeed match the consensus NES.) However, other proteins with defined NESs, including the nuclear-cytoplasmic shuttling heat stress protein, HsfA2, also vary from the canonical consensus containing either four or five bulky hydrophobic residues at more flexible spacings (Heerklotz *et al.*, 2001). According to these more flexible criteria, AMPKα proteins in animals may also match the CRM1 consensus sequence.

AMPKα carboxy termini contain other conserved residues - including a conserved cysteine and threonine (Figure 4-1B), suggesting that these residues may be modified *in vivo* to alter either AMPK activity or localization. In *Drosophila*, mutating the carboxy tail Cys573 to serine rescued both the neuronal phenotype and lethality of AMPKα null mutants (data not shown), indicating that this cysteine is not essential. Further, in mammalian cells, mutating Thr536 to either a phosphomimetic (aspartate) residue or to an alanine failed to affect the localization of the 2xGFP-α2 tail protein compared to the wild type tail (data not shown). Therefore we focused our further investigation on the conserved bulky hydrophobic amino acids in the carboxy terminus as functionally important for the AMPK NES.

Figure 4-3

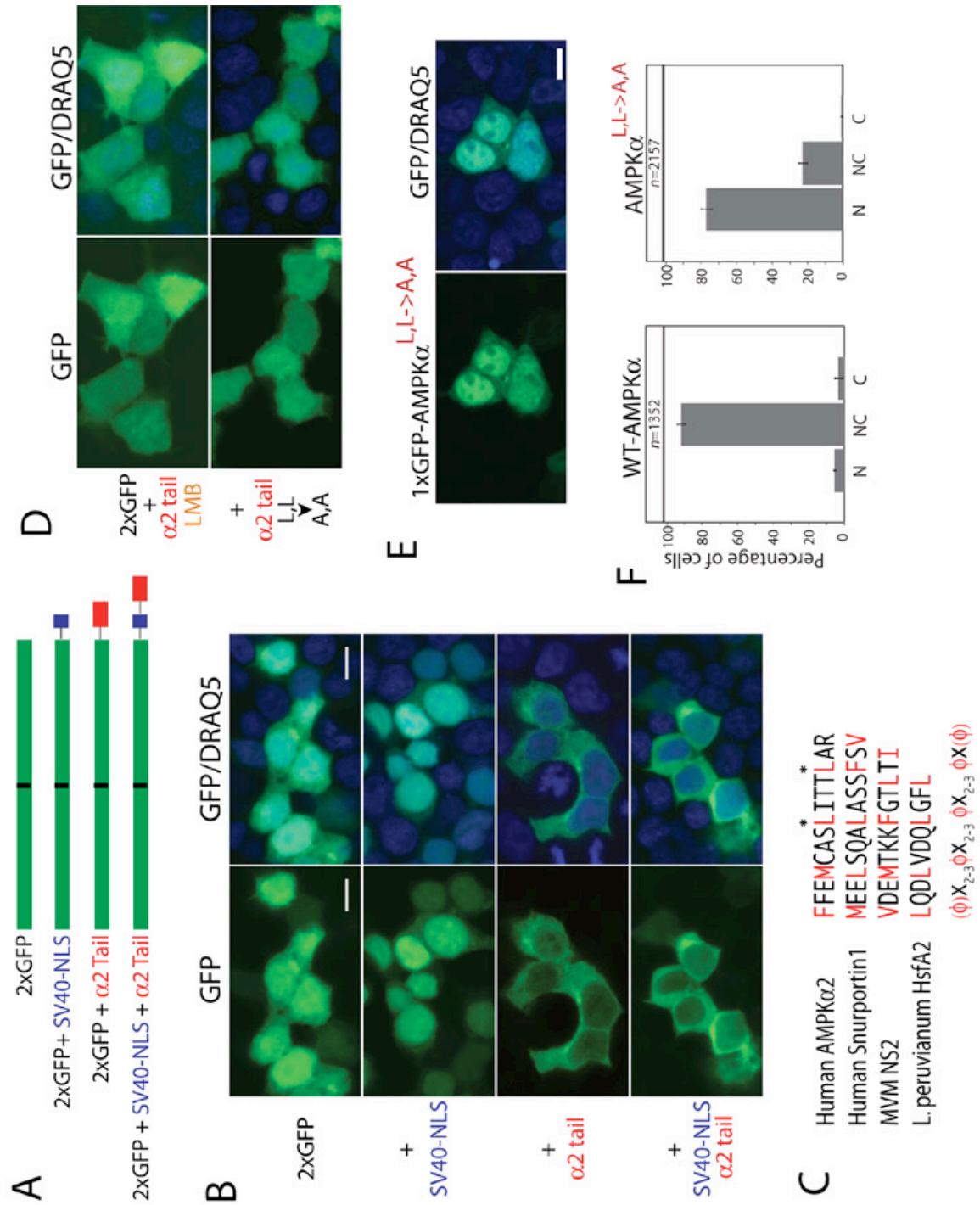
A
2xGFP
2xGFP+ SV40-NLS
2xGFP + α2 Tail
2xGFP + SV40-NLS + α2 Tail
B
GFP
GFP/DRAQ5
2xGFP
+ SV40-NLS
+ α2 tail
+ SV40-NLS α2 tail
C
Human AMPKα2 FFEMCASLITTLAR
Human Snurportin1 MEELSQALASSFSV
MVM NS2 VDEMTKKFGTLTI
L.peruvianum HsfA2 LQDLVDQLGFL
(ϕ)X2-3ϕX2-3ϕX2-3ϕX(ϕ)
D
GFP
GFP/DRAQ5
2xGFP + α2 tail LMB
+ α2 tail L,L→A,A
E
1xGFP-AMPKα L,L->A,A
GFP/DRAQ5
F
WT-AMPKα
n=1352
AMPKα L,L->A,A
n=2157
Percentage of cells
0
20
40
60
80
100
N
NC
C

Figure 4-3. The carboxy-terminal tail of AMPKα functions as a nuclear export sequence (NES). (A) Schematic representation of the four constructs containing two tandem GFP molecules (2xGFP) with or without the carboxy-terminal 23 amino acids of AMPKα2 (red) and/or the SV40 NLS (blue) used in (B). **(B)** HEK293 cells transfected with constructs containing 2xGFP alone, 2xGFP with the SV40-NLS, 2xGFP with the AMPKα2 tail, or 2xGFP with both the SV40 NLS and the α2 tail. Nuclei are stained with DRAQ5 (blue). **(C)** Alignment of the AMPKα2 tail with confirmed CRM1-dependent nuclear export sequences. The CRM1-NES consensus sequence is shown, consisting of either four or five bulky hydrophobic amino acids (ϕ, red) with variable spacing between them. Key leucine residues (often enriched in CRM1 NES sequences), L546 and L550, that were mutated for (D) are highlighted with asterisks (*). **(D)** Cells expressing either 2xGFP fused to the wild type α2 tail and treated with the CRM1-specific inhibitor Leptomycin B (LMB), or 2xGFP with L546A and L550A (L,L→A,A) mutations in the α2 tail, to block CRM1-mediated nuclear export (compare to α2 tail in (B). **(E)** Cells transfected with a GFP-tagged full-length AMPKα2$^{L,L\rightarrow A,A}$ mutant. **(F)** Scoring of cells for subcellular localization of GFP-AMPKα with the L,L→A,A mutations to nucleus (N), nucleus and cytoplasm (NC) or cytoplasm (C)(as in Figure 4-2D). Bar, 10μm.

Figure 4-4

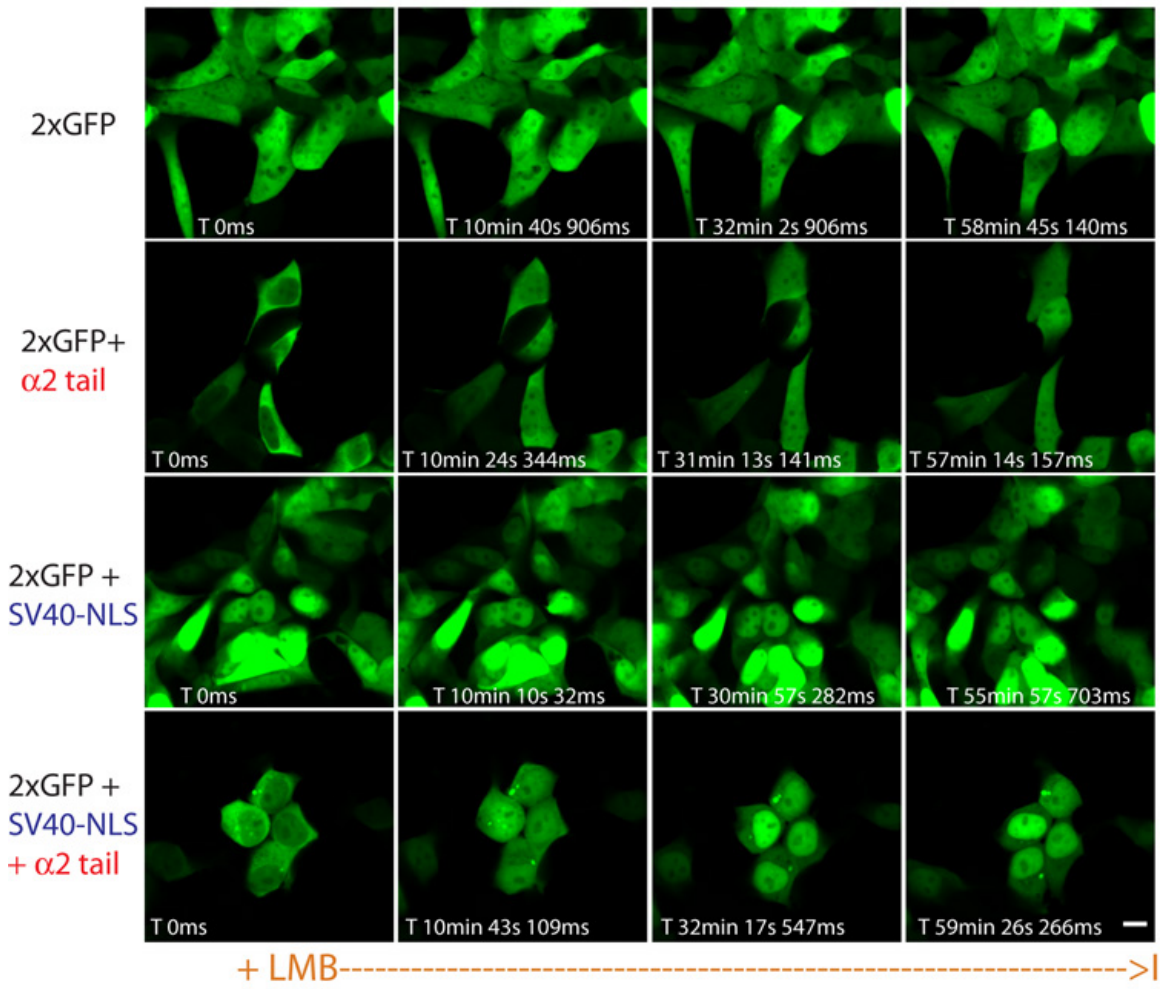

Figure 4-4. Leptomycin B (LMB) treatment, a specific inhibitor of CRM1-mediated nuclear export, induces accumulation of AMPKα carboxy tail-containing proteins in the nucleus in a time-dependent manner. HEK 293 cells transiently transfected with either 2xGFP or 2xGFP fusions containing the AMPKα2 C-terminal tail, the SV40 NLS, or both tail and NLS, imaged before and at indicated time points after treatment with Leptomycin-B (LMB). Bar,10μm.

AMPKα Cytoplasmic Localization can be Restored Using Other Defined Nuclear Export Sequences. Having identified sequences required for NES function at the carboxy-terminus of AMPKα, we wondered whether other well-documented NESs would function *in lieu* of the AMPKα carboxy-tail sequence. First, we further refined the putative AMPKα NES to within the carboxy-terminal 14 amino acids based on sequence alignment with other previously characterized CRM1-dependent NESs (Figure 4-3C) and confirmed the functional consequences of truncating only the final 14 amino acids (AMPKαΔC^{538}), as this shorter truncation also localizes to the nucleus (Figure 4-5A). We then chose two well-characterized NESs to replace the putative AMPKα NES; there is a crystal structure of snurportin-1 (SNUPN) bound to CRM1 (Dong *et al.*, 2009), while protein kinase A inhibitor (PKIA) contains a distinct but also well-characterized NES (Fornerod *et al.*, 1997). Both NESs served to restore the AMPKαΔC^{538} fusion protein to the cytoplasm (Figure 4-5A), The transplanted NESs did not act by disrupting nuclear import, as both constructs showed increased nuclear localization in the presence of LMB (data not shown). Additionally, the SNUPN-NES and PKIA-NES AMPKα chimeric constructs retain significant affinity for β and γ by co-immunoprecipitation (Figure 4-5B), despite having no sequence identity with the AMPKα tail, indicating that these localization changes are not due to disruption of the AMPK heterotrimer. As further confirmation that AMPK α/β/γ binding is not responsible for these α-tail-mediated changes in localization, we found that the construct which most strongly abolishes AMPKα cytoplasmic localization (AMPKα$^{L,L\rightarrow A,A}$), and contains the fewest amino acid changes, retains essentially wild-type binding to the β and γ subunits (Figure 4-5B).

Figure 4-5

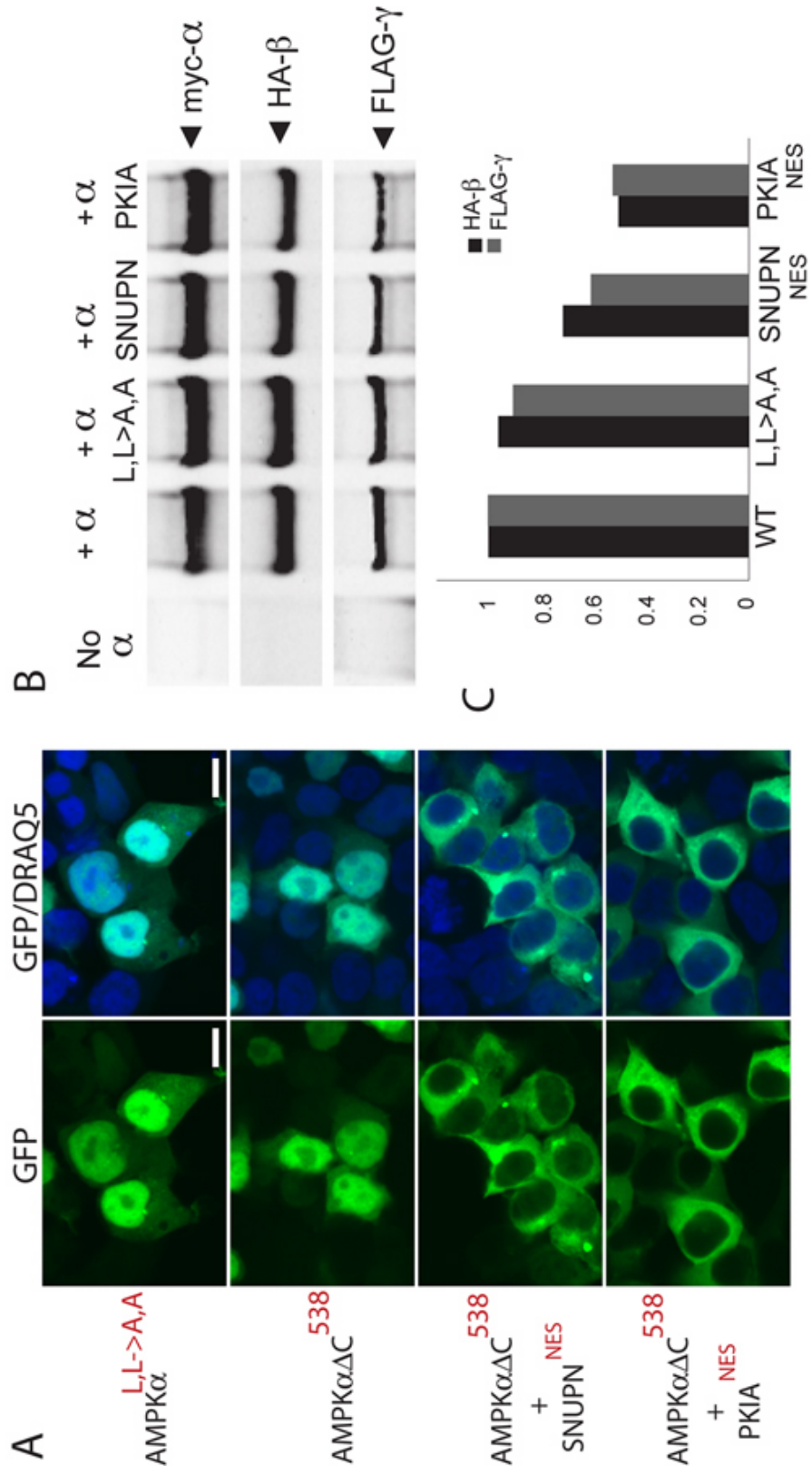
A
GFP
GFP/DRAQ5
AMPKα L,L->A,A
AMPKαΔC 538
AMPKαΔC 538 + SNUPN NES
AMPKαΔC 538 + PKIA NES
B
No α
+ α
+ α L,L>A,A
+ α SNUPN
+ α PKIA
◀ myc-α
◀ HA-β
◀ FLAG-γ
C
HA-β
FLAG-γ
1
0.8
0.6
0.4
0.2
0
WT
L,L>A,A
SNUPN NES
PKIA NES

Figure 4-5. Previously identified Nuclear Export Sequences (NESs) can substitute for the carboxy-terminus of AMPKα to restore cytoplasmic localization. (A) HEK 293 cells transiently transfected with GFP-tagged AMPKα2 constructs containing the L546A and L550A mutations (AMPKα$^{L,L\rightarrow A,A}$), a 14 amino acid C-terminal deletion (AMPKαΔC^{538}), or with the 14 C-terminal residues of AMPKα2 replaced with the CRM1-dependent nuclear export sequences from human snurportin-1 (SNUPN, MEELSQALASSFSV, 14 amino acids) or cyclic-AMP-dependent protein kinase inhibitor α (PKIA, SNELALKLAGLDI, 13 amino acids). Bar, 10 μm. **(B)** AMPKα, β, and γ subunits were co-immunoprecipitated with anti-myc antibody from HEK cell lysates either expressing HA-tagged AMPKβ1 and FLAG-tagged AMPKγ1 alone (first lane), or coexpressed with myc-tagged wild type AMPKα, L546A/L550A (L,L→A,A) AMPKα, or SNUPN and PKIA AMPKα-NES chimeras. **(C)** The relative association of β and γ subunits with myc-tagged wild type, L,L→A,A mutant, or NES chimera AMPKα variants normalized to AMPKα wild type levels, calculated from the protein band intensities in (B).

The Carboxy-Terminal AMPKα Tail is not Required for Binding β/γ Subunits. Although the carboxy tail of AMPKα appears to act as a nuclear export sequence, previous immunoprecipitation experiments with transfected cells mapped the β binding site to amino acids 313-473 of AMPKα (Iseli *et al.*, 2005), indicating that the carboxy tail of AMPKα is not required for association with the β/γ subunits. The AMPK heterotrimer crystal structures also indicate that the γ subunit has minimal contact with the carboxy tail of AMPKα/SNF1 (Amodeo *et al.*, 2007; Xiao *et al.*, 2007). Using co-immunoprecipitation experiments, we also confirmed that the carboxy tail is not required for AMPKα/β/γ association in transfected cells, as β and γ both still associate with AMPKαΔC (Figure 4-6). However, the tail may increase complex association, affinity and/or stability ((Iseli *et al.*, 2005) and Figure 4-6).

Figure 4-6

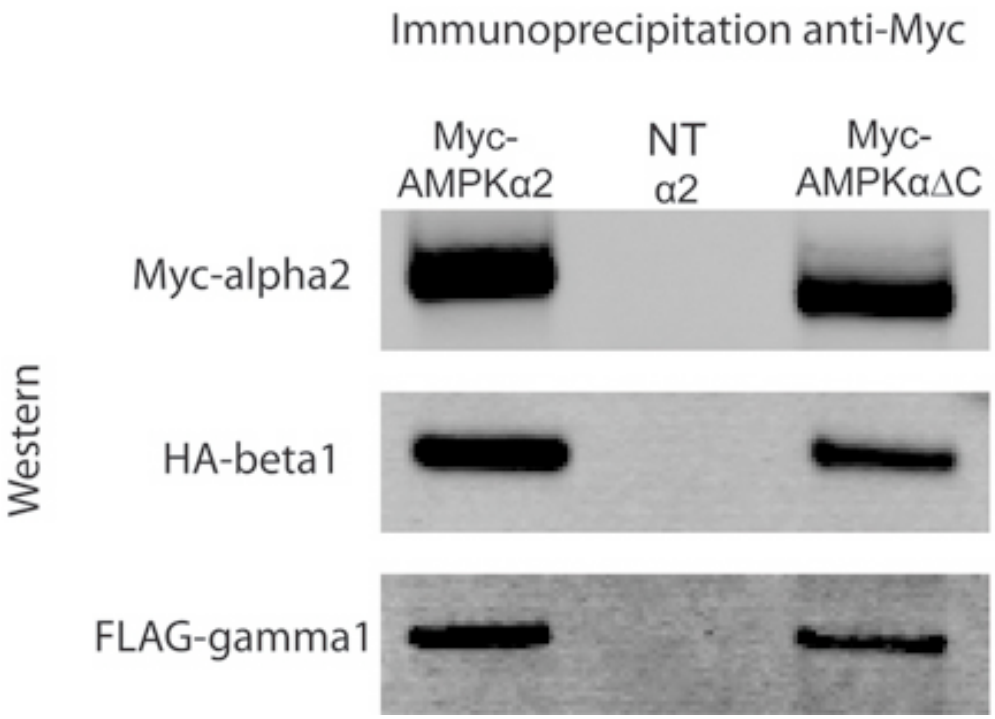

Figure 4-6. Truncated AMPKα lacking the carboxy-terminal 23 amino acids (AMPKαΔC) associates with β/γ subunits in transfected cells by anti-myc immunoprecipitation. Western blot of anti-myc immunoprecipitations from lysates of HEK 293 cells co-transfected with HA-tagged AMPKβ, FLAG-tagged AMPKγ (all three lanes), and either myc-tagged full-length AMPKα2 (lane 1) or C-terminally truncated AMPKαΔC (lane 3). Cells with no myc-AMPKα were used as a negative control (lane 2).

AMPKα Nuclear Entry is Dependent on Ran. As the AMPK trimer complex is too large to passively diffuse into the nucleus, we sought to clarify the mechanism of its active transport. While many proteins are imported into the nucleus through Ran-dependent binding to members of the Importin (Imp) family (Strom and Weis, 2001) (for review (Weis, 2003)), proteins may also be translocated through Ran-independent pathways, such as through direct binding to the nuclear pore complex (Matsubayashi *et al.*, 2001).

Several signaling proteins, including ERK2 and MEK1, have a conserved sequence motif (TPT or SPS), in which phosphorylation of these residues will induce translocation into the nucleus in a Ran-independent manner (Chuderland *et al.*, 2008). While the AMPKα isoforms contain similar sequences (TPS in α1 and TPT in α2), mutation of these residues to alanine or phosphomimetic glutamic acid residues failed to affect the localization of the truncated form of AMPKα (data not shown).

To further distinguish between the possible import pathways responsible for AMPK translocation, we examined the effect of a Ran mutant (RanQ69L) that halts Ran-dependent nuclear import and export by blocking its GTP hydrolysis (Bischoff *et al.*, 1994). Transiently transfecting cells with this Ran mutant along with GFP-tagged AMPKα, we see that AMPKα is restricted to the cytoplasm (2nd row, Figure 4-7), as is its upstream activator LKB1 ((Dorfman and Macara, 2008) and 4th row, Figure 4-7). Even the carboxy-terminal AMPKα truncation (AMPKαΔC), that is normally strongly nuclear, localizes similarly to full-length AMPKα in the presence of RanQ69L (3rd row, Figure 4-7), indicating that AMPKα is normally basally imported via a Ran-GDP-dependent pathway.

Conventional nuclear localization signals (NLSs) are typically enriched for a single cluster of basic amino acids (K/R) (e.g. SV40 NLS), or separated in a bipartite fashion by a

linker region of 10 to 12 residues (Leung *et al.*, 2003). AMPKα2 but not AMPKα1 has been proposed to contain a K-K/R-x-K/R NLS within the kinase domain that is activated by leptin in C2C12 cells (Suzuki *et al.*, 2007), allowing differential localization between AMPKα1 and AMPKα2. Although it is not known whether HEK cells respond to leptin, we did not observe any difference in localization between AMPKα1 and AMPKα2 with or without mutations in this putative kinase domain NLS (data not shown), indicating that this leptin-stimulated nuclear translocation does not function in HEK cells. Along with our findings using RanQ69L and the nuclear localization of the truncated AMPKα in several cell types in *Drosophila*, these results ultimately suggest that AMPK contains another Ran-dependent NLS, either elsewhere in α, or in the β or γ subunits that is basally active.

We also found that the phosphorylation state of the truncated AMPKα does not affect its nuclear translocation, as HEK cells transfected with C-terminally truncated AMPKα constructs containing T172D and T172A mutations in the activation loop localized similarly to truncated AMPKα (data not shown),

Figure 4-7

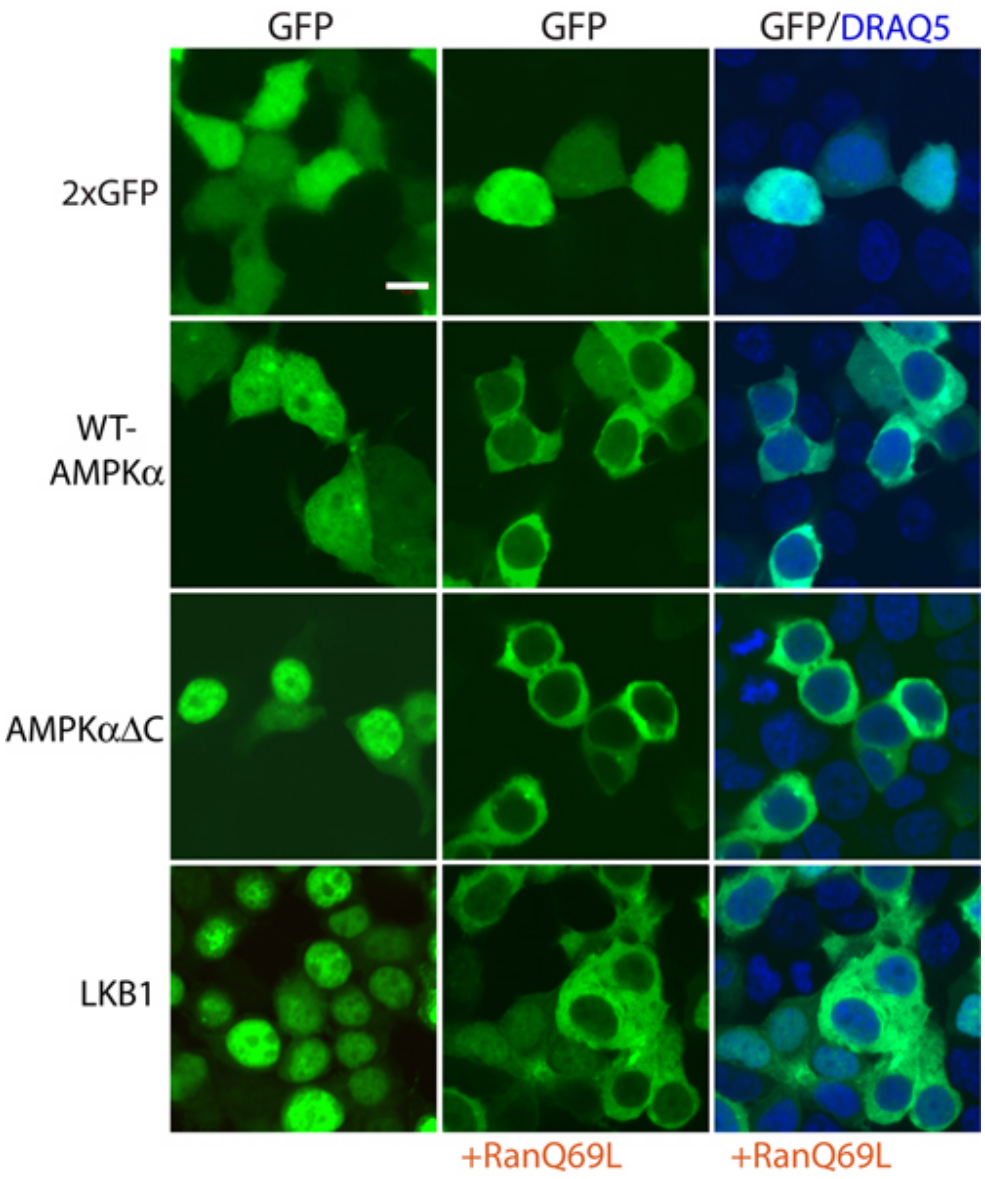

Figure 4-7. AMPKα, like LKB1, requires Ran-GTP hydrolysis for nuclear import. HEK 293 cells transiently transfected with constructs expressing 2 fused GFP molecules (2xGFP), GFP-tagged full-length AMPKα2, truncated AMPKα2 (AMPKαΔC) or LKB1, with or without the GTP-hydrolysis defective Ran mutant RanQ69L. Nuclei are stained with DRAQ5 (blue). Bar, 10μm.

Heat Shock Increases Nuclear AMPKα *in vivo*. Numerous *in vitro* studies have suggested changing cellular environments/conditions may change AMPKα localization, including alkaline pH, heat shock and oxidative stress, and leptin stimulation (Hong and Carlson, 2007; Kodiha *et al.*, 2007; Suzuki *et al.*, 2007). We wondered whether these stressors that affect AMPKα localization *in vitro*, might also affect AMPKα localization *in vivo*. Indeed, using live transgenic animal (larvae) imaging, we found that heat shock induced nuclear enrichment of AMPKα *in vivo* (Figure 4-8). While both GFP- and mCherry-tagged AMPKα increased nuclear enrichment upon heat shock (Figure 4-8, D and E), other GFP-tagged proteins, including APC2, which contains both NES (Rosin-Arbesfeld *et al.*, 2000) and NLS (Zhang *et al.*, 2000) sequences, did not localize to the nucleus under heat shock (Figure 4-8F). Other stressors, including inducing oxidative stress by feeding larvae paraquat and food starvation, did not alter AMPKα localization as they had *in vitro* (data not shown).

Figure 4-8

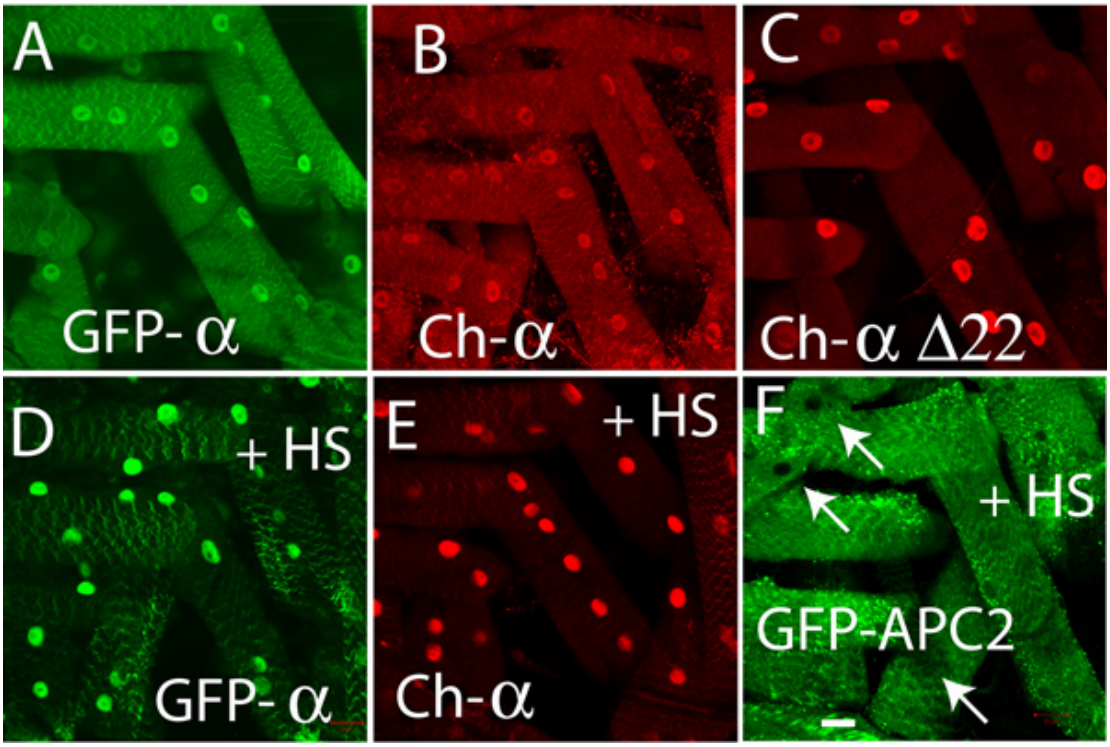

Figure 4-8. Heat shocked transgenic larvae demonstrate increased nuclear localization of AMPKα *in vivo*. Live animal images of 3rd instar *Drosophila* larvae expressing GFP-tagged (A, D) or mCherry-tagged wild type AMPKα (B, E), mCherry-tagged truncated AMPKαΔC (C), or GFP-tagged APC2 (containing both NLS and NES sequences). Animals in D-F were subjected to heat shock at 37°C for 1 hour, followed by a 15 minute recovery at 25°C. All transgenic proteins are expressed using the *Gal4-UAS* system, driven by *Ubiquitin-Gal4*. Bar, 20 μm.

The Truncated Nuclear AMPKα Isoform Shows Reduced Phosphorylation *in vivo.* Although there are clear differences in AMPKα localization dependent on the carboxy-terminal putative NES, we wondered what downstream consequences might be elicited. For example, LKB1, an upstream activator of AMPKα, requires cytoplasmic localization for activation (Baas *et al.*, 2003; Boudeau *et al.*, 2003a). To determine whether or not the differentially localized AMPKα, with or without the putative NES, might also affect the phosphorylation of the invariant Thr172 (Thr184 in *Drosophila*) that is required for AMPK activity (Lizcano *et al.*, 2004), we measured the phosphorylation levels of Thr184 in transgenic *Drosophila* animals expressing either wild type or truncated AMPKαΔC by western blot. Quantification of phospho-AMPKα in either total lysates or immunoprecipitated AMPKα, normalized to total AMPKα levels, indicates that only ~20% of the truncated nuclear-enriched protein (Figure 4-2) is phosphorylated relative to wild type full length protein (Figure 4-9).

Figure 4-9

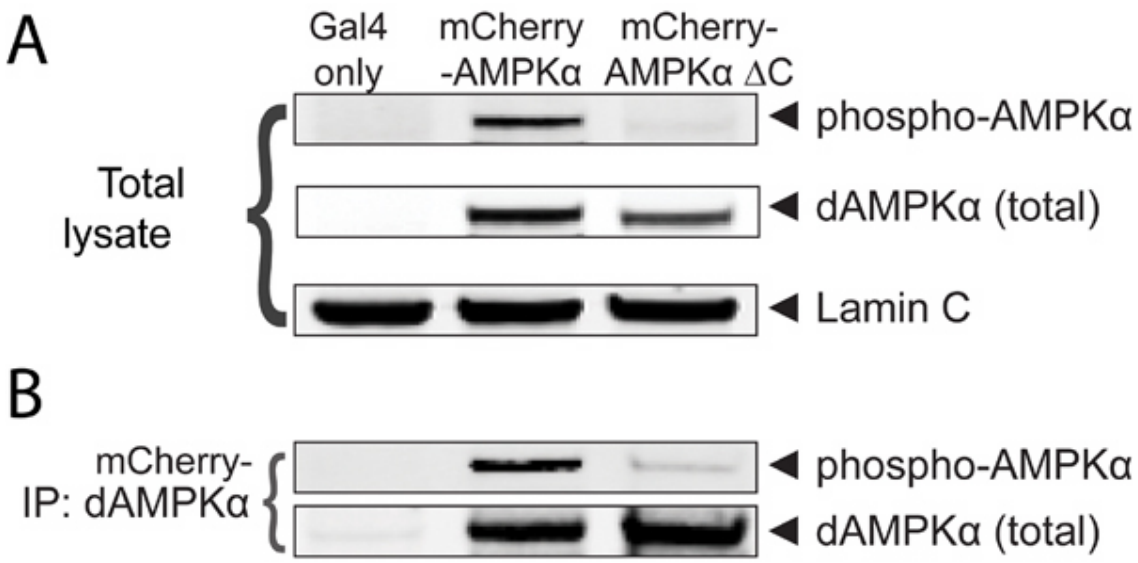

Figure 4-9. Phosphorylation of the activation loop threonine (T184) in *Drosophila* AMPKα is reduced in a nuclear enriched form *in vivo*. (A) Western blot of total lysates from transgenic *Drosophila* flies expressing Gal4 alone, mCherry-tagged full length AMPKα, or mCherry-tagged truncated AMPKα (AMPKαΔC) probed for phospho-AMPKα, total AMPKα, and Lamin C (loading control). **(B)** Western blot of proteins immunoprecipitated with anti-AMPKα antibody from fly lysates from the above fly lines, probed for phospho- and total AMPKα. Both AMPKα protein constructs are overexpressed using the *Gal4-UAS* system, driven by *Ubiquitin-Gal4*.

Discussion

For enzymes in particular, subcellular protein localization plays a key role in the proper functioning of cells by enabling interaction with required substrates and preventing unwanted side-reactions. Regulation of subcellular localization, studied for numerous nucleocytoplasmic-shuttling signal transducing proteins (Xu and Massague, 2004), adds an additional layer of complexity, enabling changes in access to substrates as well as upstream activators and inhibitors depending on the needs of the cell.

As an example, the upstream AMPK activator LKB1, under different stimuli, can localize to either the nucleus or cytoplasm, greatly affecting its own activity. Some LKB1 mutations that cause human Peutz-Jeghers syndrome constitutively sequester LKB1 to the nucleus, and despite being outside the kinase domain, are phenotypically indistinguishable from mutations that abolish enzymatic activity, (Nezu *et al.*, 1999; Boudeau *et al.*, 2003b; Xie *et al.*, 2009). Although the mechanism of LKB1 localization is complex, it involves other proteins, including the STRADs (STE related adapter) that form a complex with LKB1, promoting nuclear export and inhibiting nuclear import (Boudeau *et al.*, 2003a; Dorfman and Macara, 2008). Although LKB1 is an upstream activator of AMPKα (Lizcano *et al.*, 2004) and both proteins are kinases, LKB1 can function without other subunits bound, while AMPK is generally thought of as an obligate trimer (Hardie, 2007).

Previous studies have elucidated both nuclear and cytoplasmic targets of AMPK. In the cytoplasm, AMPK most notably phosphorylates and inhibits acetyl Co-A carboxylase (ACC), a rate-limiting enzyme required for fatty acid synthesis (Carling *et al.*, 1989). Conversely, there are several known nuclear targets of AMPK (Leff, 2003; Bronner *et al.*, 2004; Jager *et al.*, 2007; Narkar *et al.*, 2008) including PGC1α and PPARα/γ/δ that regulate

transcription in the nucleus. Furthermore, AMPK accumulation in or dispersion from the nucleus can be regulated by exercise, cellular stress and circadian rhythms. In one study, exercise increased induced nuclear translocation of AMPKα2 in skeletal muscle, (McGee *et al.*, 2003), where AMPK is known to activate PGC1α and subsequent gene transcription (Jager *et al.*, 2007). Another more recent study has demonstrated that AMPKα1 in the nucleus fluctuates in a circadian manner, regulating the circadian clock by inducing degradation of cryptochrome 1 (Lamia *et al.*, 2009). Clearly, mechanisms that regulate AMPK subcellular localization are widely utilized to modulate its access to downstream substrates.

The nuclear pore complex (NPC) plays a key role as a molecular sieve to help compartmentalize proteins between nucleus and cytoplasm. Indeed, many nucleocytoplasmic shuttling proteins contain signals to direct them in and/or out through the NPC (Yasuhara *et al.*, 2009). An AMPK trimer would far exceed the generally accepted nuclear pore diffusional cut-off size of 40 kDa (Gorlich and Kutay, 1999), and would thus also need such NPC shuttling signals. Despite distinct AMPK targets in both the nucleus and cytoplasm, the mechanisms for regulating its localization remain unclear, particularly in organisms that have only single α/β/γ subunits (*e.g. Drosophila*), where localization models based on different genetically-encoded isoforms are not applicable.

One previous model for nuclear AMPK localization proposes that AMPKα2, but not AMPKα1, contains an NLS in the kinase domain that becomes functional only upon addition of leptin (Suzuki *et al.*, 2007). As not all organisms encode leptin, and we did not observe any localization differences for AMPKα2 when mutating key residues in the proposed leptin-stimulated NLS in transfected HEK293 cells (data not shown), regulation of AMPK

localization is likely cell type-dependent. This can also be seen in the differential localization between AMPKα1 and AMPKα2 isoforms in insulinoma cells (Salt *et al.*, 1998), in contrast to HEK cells, where α1 and α2 localize similarly (data not shown). These effects are also seen with the β subunit, as only AMPKβ1 enriches in the nucleus upon mutation of two phosphorylation sites in HEK cells (Warden *et al.*, 2001), while AMPK complexes containing β2 preferentially localize in the nucleus in C2C12 cells under leptin treatment (Suzuki *et al.*, 2007). Distinct SNF1 β subunits are also thought to promote differential subcellular localization in yeast (Vincent *et al.*, 2001). Altogether, these results suggest that cells differentially regulate AMPK localization, and thus activity, through multiple pathways, depending on their unique metabolic requirements and hormonal responses.

The findings herein identify amino acids at the carboxy terminus of AMPKα that modulate its nuclear export (Figures 1 and 3) that are nearly universal, with these sequences found across phyla (Figures 1 and 3), closely matching the consensus sequence for the leucine-rich CRM1-dependent nuclear export sequences (la Cour *et al.*, 2004). As C-terminally truncated AMPKα localizes to the nucleus *in vitro* in HEK cells, and *in vivo* in *Drosophila* under unstressed conditions, this suggests that AMPKα is basally imported to the nucleus, and that regulation of AMPK localization in response to stress would predominantly be affected through modulation of the export pathway. Adding further complexity, localization of the AMPK complex and partitioning of specific subunits may also be both cell type and context-dependent. For instance, in multicellular organisms certain tissues (e.g. fat) provide energy to other tissues/organs (e.g. muscle) at their own expense. In these cases, AMPK activation likely leads to different physiological outcomes between cell types, such as increased lipid mobilization in fat cells *versus* increased lipid uptake in muscle cells. In these situations,

differential localization of AMPK in distinct cell types could be used to generate these different cellular responses.

A further avenue of inquiry in regulation of AMPK localization is in the possible effects of post-translational modification of AMPK subunits on the accessibility of the carboxy-tail of AMPKα. As the AMPKα carboxy-tail folds into a pocket formed by the α and β subunits after a long flexible loop (Amodeo *et al.*, 2007; Xiao *et al.*, 2007), altering the strength of these interactions could change its accessibility to CRM1, thus activating or inhibiting nuclear export. Although there are conserved residues that could be post-translationally modified in the AMPKα carboxy-tail adjacent to the putative NES, we have so far been unable to identify residues flanking the NES that change the subcellular localization of AMPKα *in vitro* or are required for genetic rescue *in vivo*, as described earlier. One tantalizing possibility is that the potential phosphoserine mutations in β1 increase nuclear localization of AMPK by enhancing β interactions with the AMPKα tail, thus blocking nuclear export.

Whatever mechanisms determine AMPK localization, they must take into account two general observations: 1) AMPKα1 and AMPKα2 are largely genetically and functionally redundant in the mouse and 2) many organisms encode only a single isoform for individual AMPK subunits. In many mouse strains AMPKα1 and AMPKα2 are genetically redundant as single α1 knockouts or α2 knockouts are viable, yet double knockouts are lethal (personal communication B. Viollet), suggesting that different AMPKα isoforms are functionally redundant for activities required for life *in vivo*. Therefore the elucidation of mechanisms that regulate AMPKα subcellular localization beyond isoform distinctions, such

as the ones identified in this study, is vitally important to the understanding AMPK regulation *in vivo*.

References

Amodeo, G.A., Rudolph, M.J., and Tong, L. (2007). Crystal structure of the heterotrimer cerevisiae AMPK homologue SNF1. Nature *449*, 492-495.

Baas, A.F., Boudeau, J., Sapkota, G.P., Smit, L., Medema, R., Morrice, N.A., Alessi, D.R., and Clevers, H.C. (2003). Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD. Embo J *22*, 3062-3072.

Bischoff, F.R., Klebe, C., Kretschmer, J., Wittinghofer, A., and Ponstingl, H. (1994). RanGAP1 induces GTPase activity of nuclear Ras-related Ran. Proc Natl Acad Sci U S A *91*, 2587-2591.

Boudeau, J., Baas, A.F., Deak, M., Morrice, N.A., Kieloch, A., Schutkowski, M., Prescott, A.R., Clevers, H.C., and Alessi, D.R. (2003a). MO25alpha/beta interact with STRADalpha/beta enhancing their ability to bind, activate and localize LKB1 in the cytoplasm. Embo J *22*, 5102-5114.

Boudeau, J., Sapkota, G., and Alessi, D.R. (2003b). LKB1, a protein kinase regulating cell proliferation and polarity. FEBS Lett *546*, 159-165.

Brenman, J.E., and Temple, B.R. (2007). Opinion: alternative views of AMP-activated protein kinase. Cell Biochem Biophys *47*, 321-331.

Bronner, M., Hertz, R., and Bar-Tana, J. (2004). Kinase-independent transcriptional co-activation of peroxisome proliferator-activated receptor alpha by AMP-activated protein kinase. Biochem J *384*, 295-305.

Carling, D., Clarke, P.R., Zammit, V.A., and Hardie, D.G. (1989). Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. Eur J Biochem *186*, 129-136.

Carling, D., Zammit, V.A., and Hardie, D.G. (1987). A common bicyclic protein kinase cascade inactivates the regulatory enzymes of fatty acid and cholesterol biosynthesis. FEBS Lett *223*, 217-222.

Chuderland, D., Konson, A., and Seger, R. (2008). Identification and characterization of a general nuclear translocation signal in signaling proteins. Mol Cell *31*, 850-861.

Davies, S.P., Carling, D., and Hardie, D.G. (1989). Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. Eur J Biochem *186*, 123-128.

Davies, S.P., Hawley, S.A., Woods, A., Carling, D., Haystead, T.A., and Hardie, D.G. (1994). Purification of the AMP-activated protein kinase on ATP-gamma-sepharose and analysis of its subunit structure. Eur J Biochem *223*, 351-357.

Dong, X., Biswas, A., Suel, K.E., Jackson, L.K., Martinez, R., Gu, H., and Chook, Y.M. (2009). Structural basis for leucine-rich nuclear export signal recognition by CRM1. Nature *458*, 1136-1141.

Dorfman, J., and Macara, I.G. (2008). STRAD{alpha} Regulates LKB1 Localization by Blocking Access to Importin-{alpha}, and by Association with Crm1 and Exportin-7. Mol Biol Cell *19*, 1614-1626.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I.W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051-1060.

Frederick, E.D., Ramos, S.B., and Blackshear, P.J. (2008). A unique C-terminal repeat domain maintains the cytosolic localization of the placenta-specific tristetraprolin family member ZFP36L3. J Biol Chem *283*, 14792-14800.

Gao, F.B., Brenman, J.E., Jan, L.Y., and Jan, Y.N. (1999). Genes regulating dendritic outgrowth, branching, and routing in Drosophila. Genes Dev *13*, 2549-2561.

Gao, G., Fernandez, C.S., Stapleton, D., Auster, A.S., Widmer, J., Dyck, J.R., Kemp, B.E., and Witters, L.A. (1996). Non-catalytic beta- and gamma-subunit isoforms of the 5'-AMP-activated protein kinase. J Biol Chem *271*, 8675-8681.

Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol *15*, 607-660.

Hardie, D.G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol *8*, 774-785.

Hardie, D.G., Carling, D., and Carlson, M. (1998). The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu Rev Biochem *67*, 821-855.

Heerklotz, D., Doring, P., Bonzelius, F., Winkelhaus, S., and Nover, L. (2001). The balance of nuclear import and export determines the intracellular distribution and function of tomato heat stress transcription factor HsfA2. Mol Cell Biol *21*, 1759-1768.

Henderson, B.R. (2000). Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. Nat Cell Biol *2*, 653-660.

Hong, S.P., and Carlson, M. (2007). Regulation of snf1 protein kinase in response to environmental stress. J Biol Chem *282*, 16838-16845.

Iseli, T.J., Walter, M., van Denderen, B.J., Katsis, F., Witters, L.A., Kemp, B.E., Michell, B.J., and Stapleton, D. (2005). AMP-activated protein kinase beta subunit tethers alpha and gamma subunits via its C-terminal sequence (186-270). J Biol Chem *280*, 13395-13400.

Jager, S., Handschin, C., St-Pierre, J., and Spiegelman, B.M. (2007). AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U S A *104*, 12017-12022.

Kodiha, M., Rassi, J.G., Brown, C.M., and Stochaj, U. (2007). Localization of AMP kinase is regulated by stress, cell density, and signaling through the MEK-->ERK1/2 pathway. Am J Physiol Cell Physiol *293*, C1427-1436.

Kutay, U., and Guttinger, S. (2005). Leucine-rich nuclear-export signals: born to be weak. Trends Cell Biol *15*, 121-124.

La Cour, T., Kiemer, L., Molgaard, A., Gupta, R., Skriver, K., and Brunak, S. (2004). Analysis and prediction of leucine-rich nuclear export signals. Protein Eng Des Sel *17*, 527-536.

Lamia, K.A., Sachdeva, U.M., DiTacchio, L., Williams, E.C., Alvarez, J.G., Egan, D.F., Vasquez, D.S., Juguilon, H., Panda, S., Shaw, R.J., Thompson, C.B., and Evans, R.M. (2009). AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. Science *326*, 437-440.

Leff, T. (2003). AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins. Biochem Soc Trans *31*, 224-227.

Leung, S.W., Harreman, M.T., Hodel, M.R., Hodel, A.E., and Corbett, A.H. (2003). Dissection of the karyopherin alpha nuclear localization signal (NLS)-binding groove: functional requirements for NLS binding. J Biol Chem *278*, 41947-41953.

Lizcano, J.M., Goransson, O., Toth, R., Deak, M., Morrice, N.A., Boudeau, J., Hawley, S.A., Udd, L., Makela, T.P., Hardie, D.G., and Alessi, D.R. (2004). LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. Embo J *23*, 833-843.

Matsubayashi, Y., Fukuda, M., and Nishida, E. (2001). Evidence for existence of a nuclear pore complex-mediated, cytosol-independent pathway of nuclear translocation of ERK MAP kinase in permeabilized cells. J Biol Chem *276*, 41755-41760.

McGee, S.L., Howlett, K.F., Starkie, R.L., Cameron-Smith, D., Kemp, B.E., and Hargreaves, M. (2003). Exercise increases nuclear AMPK alpha2 in human skeletal muscle. Diabetes *52*, 926-928.

Medina, P.M., Swick, L.L., Andersen, R., Blalock, Z., and Brenman, J.E. (2006). A novel forward genetic screen for identifying mutations affecting larval neuronal dendrite development in Drosophila melanogaster. Genetics *172*, 2325-2335.

Mirouse, V., Swick, L.L., Kazgan, N., St Johnston, D., and Brenman, J.E. (2007). LKB1 and AMPK maintain epithelial cell polarity under energetic stress. J Cell Biol *177*, 387-392.

Narkar, V.A., Downes, M., Yu, R.T., Embler, E., Wang, Y.X., Banayo, E., Mihaylova, M.M., Nelson, M.C., Zou, Y., Juguilon, H., Kang, H., Shaw, R.J., and Evans, R.M. (2008). AMPK and PPARdelta agonists are exercise mimetics. Cell *134*, 405-415.

Nezu, J., Oku, A., and Shimane, M. (1999). Loss of cytoplasmic retention ability of mutant LKB1 found in Peutz-Jeghers syndrome patients. Biochem Biophys Res Commun *261*, 750-755.

Rosin-Arbesfeld, R., Townsley, F., and Bienz, M. (2000). The APC tumour suppressor has a nuclear export function. Nature *406*, 1009-1012.

Salt, I., Celler, J.W., Hawley, S.A., Prescott, A., Woods, A., Carling, D., and Hardie, D.G. (1998). AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. Biochem J *334 (Pt 1)*, 177-187.

Stapleton, D., Gao, G., Michell, B.J., Widmer, J., Mitchelhill, K., Teh, T., House, C.M., Witters, L.A., and Kemp, B.E. (1994). Mammalian 5'-AMP-activated protein kinase non-catalytic subunits are homologs of proteins that interact with yeast Snf1 protein kinase. J Biol Chem *269*, 29343-29346.

Strom, A.C., and Weis, K. (2001). Importin-beta-like nuclear transport receptors. Genome Biol *2*, REVIEWS3008.

Suzuki, A., Okamoto, S., Lee, S., Saito, K., Shiuchi, T., and Minokoshi, Y. (2007). Leptin stimulates fatty acid oxidation and peroxisome proliferator-activated receptor alpha gene expression in mouse C2C12 myoblasts by changing the subcellular localization of the alpha2 form of AMP-activated protein kinase. Mol Cell Biol *27*, 4317-4327.

Vincent, O., Townley, R., Kuchin, S., and Carlson, M. (2001). Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. Genes Dev *15*, 1104-1114.

Warden, S.M., Richardson, C., O'Donnell, J., Jr., Stapleton, D., Kemp, B.E., and Witters, L.A. (2001). Post-translational modifications of the beta-1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization. Biochem J *354*, 275-283.

Weis, K. (2003). Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle. Cell *112*, 441-451.

Xiao, B., Heath, R., Saiu, P., Leiper, F.C., Leone, P., Jing, C., Walker, P.A., Haire, L., Eccleston, J.F., Davis, C.T., Martin, S.R., Carling, D., and Gamblin, S.J. (2007). Structural basis for AMP binding to mammalian AMP-activated protein kinase. Nature *449*, 496-500.

Xie, Z., Dong, Y., Zhang, J., Scholz, R., Neumann, D., and Zou, M.H. (2009). Identification of the serine 307 of LKB1 as a novel phosphorylation site essential for its nucleocytoplasmic transport and endothelial cell angiogenesis. Mol Cell Biol *29*, 3582-3596.

Xu, L., and Massague, J. (2004). Nucleocytoplasmic shuttling of signal transducers. Nat Rev Mol Cell Biol *5*, 209-219.

Yasuhara, N., Oka, M., and Yoneda, Y. (2009). The role of the nuclear transport system in cell differentiation. Semin Cell Dev Biol *20*, 590-599.

Zhang, F., White, R.L., and Neufeld, K.L. (2000). Phosphorylation near nuclear localization signal regulates nuclear import of adenomatous polyposis coli protein. Proc Natl Acad Sci U S A *97*, 12577-12582.

CHAPTER V

Summary and Future Directions

AMPK and cell polarity. Although AMPK is a cellular energy sensor, its primary sequence is highly similar to well-known polarity genes, such as SAD kinase and *par-1*. Recent cell culture studies suggest AMPK has a role in polarization of MDCK cells (Zhang et al., 2006). This raises the question as to whether AMPK has other roles besides sensing the cellular energy level.

The other reason to suggest AMPK has a role in cell polarity is due to LKB1, an AMPK activator, itself being an essential polarity gene. That being said, LKB1 regulates par-1 polarity gene and there are claims LKB1 mediates its cell polarity function through par-1 and cellular energy level control through AMPK. With our data, we have shown that AMPK has a role in epithelial cell polarity. But we do not know the signaling mechanism of this effect. A second article after our initial publication on AMPK's role in cell polarity claimed AMPK mediates cell polarity through the phosphorylation of non-muscle myosin regulatory light chain (MRLC) protein (Lee et al., 2007). The authors demonstrated that a constitutively active copy of MRLC rescues the loss of polarity phenotype due to loss of AMPK activity both in fruit flies and in mammalian cell culture studies. Preliminary data from our lab suggests that loss of MRLC in neurons shows a similar phenotype to loss of AMPK (data not shown).

In addition, since it is already known that LKB1 has a cell polarity effect through *par-1*, it is essential to determine whether a loss of *par-1* has a similar effect on epithelial-like tissues in post-developmental stages. It is also essential to over-express an active copy of *par-1* in an *lkb1* mutant background and determine if the *lkb1* null cell phenotype is rescued.

AMPK, metabolism and behavior. Since we were the first lab to identify AMPKα mutations in fruit flies and since mammals have multiple α subunits, i.e., redundancy, it is wise to assess the effect of loss of AMPK on the whole animal. However, it is essential to keep in mind that AMPK has diverse actions in different tissues and organs. That is why it is important to express an AMPK dominant-negative in different tissues and determine its effects on different organs.

One tissue we have determined the effect of loss of AMPK either by an AMPKα null mutant or a dominant-negative is the liver-like oenocytes. Oenocytes are 4-6 cells that exist in every segment of the larvae and adults. They are responsible for the translocation of fatty acids from fat bodies to them and so they can metabolize those lipids and transport to the neighboring cells (Gutierrez et al., 2007). As we show in Chapter III, Figure 3-7, oenocytes accumulate neutral lipids when the larvae are starved. In AMPKα null mutant larvae, they accumulate neutral lipids without starvation. This means under non-starvation conditions, our AMPKα null mutants display a starvation phenotype. At the same time, we do not know whether they are aberrantly processing the lipids in the oenocytes or if the lipids are excessively accumulated in the oenocytes in our AMPKα null mutants. The total triglyceride levels of the AMPK mutants are also low even though they are hyperphagic. So, how can they be both hyperphagic and have less stored lipid? The most likely hypothesis is they are incapable of using their stored triglycerides in the absence of AMPK, i.e., their capability of

storing, processing and breaking down fatty acids is reduced without AMPK. Another hypothesis is a loss of AMPK in the gut cells leads to reduced nutrient uptake due to impaired peristaltic activity and this explains the larvae being hyperphagic and low stored lipids at the same time (Bland et al., 2010).

AMPK subcellular localization. As mentioned in Chapter IV, AMPK sub-cellular localization, nucleus *vs.* cytoplasm, is essential for AMPK's access to its downstream and upstream targets. That being said, we have not seen any effects on direct nuclear targets of AMPK in the literature up to this date. In our hands, p-AMPK localizes mostly in the cytoplasm in fruit flies cells (Mirouse et al., 2007). There are other groups that have shown AMPK is localized to the nucleus under stress or under adipose-derived hormone, leptin, treatment (Kodiha et al., 2007) (Suzuki et al., 2007). In our hands, we were unable repeat any of these experiments. Neither heat shocking nor Leptomycin B treatment of HEK293 or HeLa cells followed by fractionation of endogenous proteins resulted in any increased nuclear localization of either AMPKα or AMPKβ. But this could very well be due a difference in fractionation efficiency or technique between us and those authors. Leptin treatment in HEK293 cells did not change the localization of AMPKα, but this might be due to HEK293 cell's insensitivity to leptin treatment.

Trying to look for a change in the phosphorylation status of downstream targets of AMPK in HEK293 cells, we transfected with our constructs (AMPKα wild-type, AMPKαΔC, AMPKα$^{L,L\rightarrow A,A}$, or AMPKα Dominant-negative) but did not find any significant results either. We were checking the p-ACC level as an indicator of the activity of AMPK. Since AMPK has to be a complex to be functional, we went ahead and transfected cells not only with AMPKα alone but concomitantly with β and γ. We then again checked the

p-ACC level and did not see any significant difference between non-transfected and transfected cells. We then thought maybe we should stress these cells after transfection by fully starving them and determine whether the transfected cells showed any difference in p-ACC level. In addition, we treated some of the transfected cells with the AMPK activator AICAR with or without starvation and measured the difference in p-ACC levels. None of the above conditions showed any change in p-ACC level. Then we realized both in flies and in cell culture, AMPK activity is regulated when we over-express a wild-type or mutant version of AMPK. For instance, when we over-express a wild-type copy of AMPKα in cells, the endogenous AMPKα phospho-level will drop only slightly since the exogenous construct can also be phosphorylated (data not shown). On the other hand, when we overexpress a Dominant Negative copy of AMPKα both in flies and cell culture, the endogenous AMPKα phosphorylation level increases. So we conclude there is an internal regulation of total functional AMPK and its activity level in the cells *in vitro* and *in vivo*. Unless we have AMPKα mutant cells, we will not be able to express different constructs and observe a change in p-ACC levels or other downstream targets. Currently, we have AMPKα1 and α2 subunit null mouse embryonic fibroblast (MEF) cells in our lab. These cells have a very low p-ACC level as we have published before (Williams et al., 2009). Our efforts to transfect MEF-KO cells were unsuccessful since MEF-KO cells proved to have a very low rate of transfection. We might consider trying to make stable cell lines expressing constructs of AMPKα.

During this time, we have also decided to use shRNA to downregulate the endogenous AMPKα in HEK293 cells and to then over-express the AMPK wild-type or mutant constructs to observe their effects on p-ACC levels. Normally the shRNA would

knock down the over-expression constructs since the constructs are also translating AMPKα. However, we will use a shRNA that is specific to only human AMPKα and then over-express the rat AMPKα wild-type and mutant constructs to observe the effect on p-ACC levels. Through this method, we hope to show a biological tie to change of localization of AMPK using *in vitro* studies. We cannot repeat some of these experiments in fruit flies since we do not have an antibody that recognizes *Drosophila* p-ACC levels.

New nuclear target of AMPK. During our studies, a new study emerged claiming a new nuclear target of AMPK. The authors claim AMPK regulates the Circadian Clock by translocating to the nucleus during the day and phosphorylating, and thereby leading to the degradation of, CRY1. This will then cause the expression of Clock-controlled genes (Lamia et al., 2009). They have done fractionation studies from mouse liver nucleus. We have decided to do the same experiments (mentioned above in our shRNA endogenous AMPKα and over-expression of wild-type or mutant copy of AMPKα experiments) and check the total level of CRY1 in addition to looking at p-ACC levels. We will overexpress a Flag-tagged version of CRY1 and check the total levels of CRY1 when expressing different constructs of AMPKα. We will then use cycloheximide, an inhibitor of protein biosynthesis, and observe the total level of CRY1 by over-expression of AMPKα constructs.

Fractionation of AMPK complex. During our studies, one of the questions we asked was whether AMPKα goes into nucleus as a heterotrimeric complex attached to the β and γ subunits, or if it enters alone? We have immunoprecipitation experiments (Chapter IV; Figures 4- 8) of the over-expression of the α, β and γ subunits in HEK293 cells where α is either wild-type or an LL->AA mutation. In that experiment, even the nuclear AMPKα is still bound to the β and γ subunits 80-90% of the time indicating AMPK enters the nucleus as a

heterotrimer. We also have immunostaining experiments that show when AMPKα is in the nucleus, the regulatory subunits tend to localize with it. But we still want to over-express these constructs and then fractionate nuclear proteins and determine if AMPKα, β and γ are together in the nucleus. Despite our efforts to fractionate the over-expression proteins, we were unable to successfully complete these experiments. This was not due to any intrinsic property of AMPK itself since our control fractionation of over-expressed GFP-SV40NLS, a construct which normally localizes to the nucleus, was also not successful. We noticed as soon as the cells were broken, the nuclear GFP began to leak out of the nucleus within minutes.

Restoring the cytoplasmic localization of AMPKα in Drosophila. As we have shown in Chapter IV, cytoplasmic localization of truncated AMPKαΔC^{538} can be rescued by attaching a known NES sequence at the C-terminus end. We will generate transgenic flies expressing the same construct and check (i) localization and phosphorylation level of that construct. If that construct localizes to the cytoplasm and can be phosphorylated, then we will know the phosphorylation of AMPK takes place in the cytosol. (ii)We will also cross that line to an AMPKα mutant background and see whether it will rescue the lethality of AMPKα null flies.

Determining the mechanism of the loss of AMPKα phenotype in sensory neurons. As we have shown in Chapter II, the AMPKα mutant phenotype causes a morphological defect in the fruit fly sensory neurons. The dendritic enlargement phenotype only happens in dendrites whereas the axons and the filopodia seem normal. We observe a similar phenotype with over-expression of the dominant-negative, as shown in Chapter III. We wanted to check whether any mutants of the regulatory subunits of AMPK, the β and γ

subunits, exist in the Flybase. There was no *Drosophila* β subunit mutation at hand until recently and so we were unable to check the sensory neuron phenotype in those flies. On the other hand, we have found multiple P-element translocation lines in Flybase. P-elements are transposable elements that are randomly inserted into the fly genome and produced to randomly disrupt genes (Spradling et al., 1995). Their localization in the genome is confirmed by sequencing from each end of the transposable element. We have chosen around nine of these P-elements that are mapped to *Drosophila* AMPKγ subunit, the Snf4 gene. We thought some of these P-elements might cause a null genotype of the Snf4 gene. We chose the ones that are partly or wholly located in the exons of Snf4. Five of these P-elements had a dendritic enlargement phenotype identical to the AMPKα loss of function, but less severe (Fig. 5-1). Later on, we did rescue the Snf4 mutant phenotype and its lethality by overexpressing a wild-type copy of Snf4 in these neurons (data not shown).

This above data shows that the phenotype that we see in these neurons is due to loss of AMPK activity. There is another group (Tschape et al., 2002) claiming loss of Snf4 in *Drosophila* causes progressive neurodegeneration and neuronal cell death. Sections from the Snf4 mutant fly brain showed accumulation of vacuoles. We suspected the phenotype that we see in fly sensory neurons is due to some vacuolar defect or some endocytotic pathway defect. We ordered mutations in genes that are in the endocytotic or autophagy pathway. We checked whether any of them with or without the AMPKα mutant would show a similar phenotype to the loss of AMPK phenotype. None of the mutants or the over-expression of those genes either rescued or made the phenotype worse.

Figure 5-1.

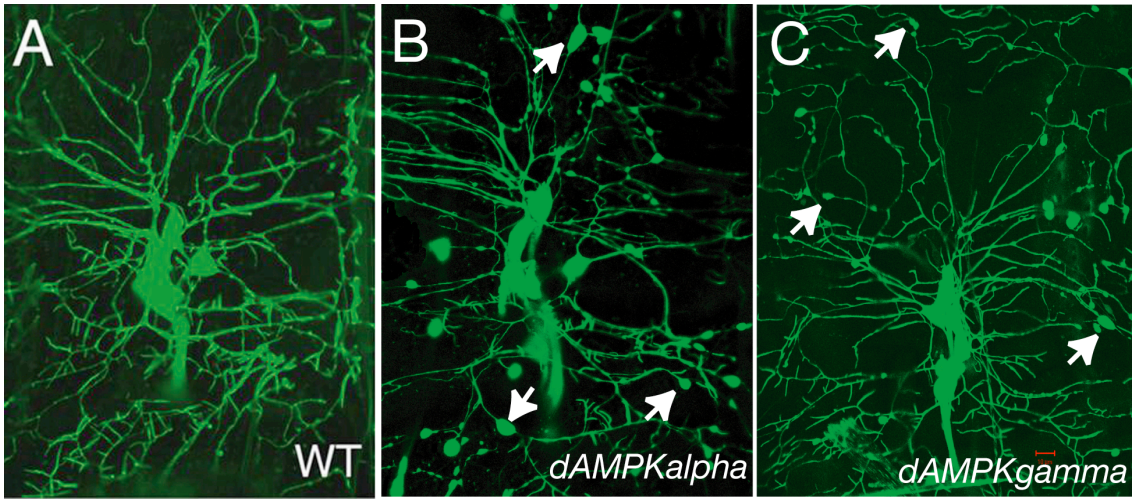

Figure 5-1. Both *dAMPK α* and *dAMPK γ* mutants lead to enlarged dendrite membrane swellings/domains. *Drosophila* dendritic arborization (da) neurons **(A)**, and mutant *dAMPK α* **(B)** and a P element allele of *dAMPK γ* **(C)**. Both mutants show similar dendritic phenotypes, stronger with *dAMPKα* mutant. Arrows point at striking membrane enlargements are seen in dendrites. Second instar larvae expressing Actin::GFP in da neurons.

When we over-express a UAS- Dominant Negative copy of AMPKα under an ubiquitous Gal4 line and incubate them in higher temperature (29°C), they will not survive till adulthood. When we overexpress AMPKα wild-type copy, it will rescue the lethality phenotype whereas over-expression of UAS-GFP does not rescue the lethality. Then, we ordered UAS lines of candidate genes that are either involved in the AMPK signaling pathway or have a role in endocytotic machinery. None of the overexpression of candidate genes gave us any rescued flies at higher temperature.

That is why we have decided to design a screen to identify and characterize novel downstream and upstream regulators of AMPK. The lab is currently working on an EMS mutagenesis screen using either shRNA of AMPKα or Dominant negative AMPKα as their starting flies where the EMS mutation will suppress the lethality of these fly lines. It is hoped new modifier screens will help elucidate novel components of AMPK signaling to help explain diverse AMPK mutant phenotypes.

References

Bland, M.L., Lee, R.J., Magallanes, J.M., Foskett, J.K., and Birnbaum, M.J. (2010). AMPK Supports Growth in Drosophila by Regulating Muscle Activity and Nutrient Uptake in the Gut. Dev Biol.

Gutierrez, E., Wiggins, D., Fielding, B., and Gould, A.P. (2007). Specialized hepatocyte-like cells regulate Drosophila lipid metabolism. Nature *445*, 275-280.

Kodiha, M., Rassi, J.G., Brown, C.M., and Stochaj, U. (2007). Localization of AMP kinase is regulated by stress, cell density, and signaling through the MEK-->ERK1/2 pathway. Am J Physiol Cell Physiol *293*, C1427-1436.

Lamia, K.A., Sachdeva, U.M., DiTacchio, L., Williams, E.C., Alvarez, J.G., Egan, D.F., Vasquez, D.S., Juguilon, H., Panda, S., Shaw, R.J.*, et al.* (2009). AMPK regulates the circadian clock by cryptochrome phosphorylation and degradation. Science *326*, 437-440.

Lee, J.H., Koh, H., Kim, M., Kim, Y., Lee, S.Y., Karess, R.E., Lee, S.H., Shong, M., Kim, J.M., Kim, J.*, et al.* (2007). Energy-dependent regulation of cell structure by AMP-activated protein kinase. Nature *447*, 1017-1020.

Mirouse, V., Swick, L.L., Kazgan, N., St Johnston, D., and Brenman, J.E. (2007). LKB1 and AMPK maintain epithelial cell polarity under energetic stress. J Cell Biol *177*, 387-392.

Spradling, A.C., Stern, D.M., Kiss, I., Roote, J., Laverty, T., and Rubin, G.M. (1995). Gene disruptions using P transposable elements: an integral component of the Drosophila genome project. Proc Natl Acad Sci U S A *92*, 10824-10830.

Suzuki, A., Okamoto, S., Lee, S., Saito, K., Shiuchi, T., and Minokoshi, Y. (2007). Leptin stimulates fatty acid oxidation and peroxisome proliferator-activated receptor alpha gene expression in mouse C2C12 myoblasts by changing the subcellular localization of the alpha2 form of AMP-activated protein kinase. Mol Cell Biol *27*, 4317-4327.

Tschape, J.A., Hammerschmied, C., Muhlig-Versen, M., Athenstaedt, K., Daum, G., and Kretzschmar, D. (2002). The neurodegeneration mutant lochrig interferes with cholesterol homeostasis and Appl processing. EMBO J *21*, 6367-6376.

Williams, T., Forsberg, L.J., Viollet, B., and Brenman, J.E. (2009). Basal autophagy induction without AMP-activated protein kinase under low glucose conditions. Autophagy *5*, 1155-1165.

Zhang, L., Li, J., Young, L.H., and Caplan, M.J. (2006). AMP-activated protein kinase regulates the assembly of epithelial tight junctions. Proc Natl Acad Sci U S A *103*, 17272-17277.