THE REGULATION OF THE CELL SURFACE PROTEOME BY AMP-ACTIVATED PROTEIN KINASE

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Abstract

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Eden Ross

Master of Science, Molecular Science, Ryerson University, 2014

The cell-surface proteome controls numerous cellular functions and is dynamically controlled by endocytosis and recycling under different cellular conditions. Energy stress is a state in which a cell must engage adaptive responses to ensure survival, including remodelling of the cell-surface proteome. AMP-activated protein kinase (AMPK) is an important metabolic regulator in the cell. Recent studies suggest AMPK activation may alter the endocytosis of a few specific proteins. How increased AMPK activity globally regulates the cell surface proteome is not known. I have developed a method to isolate the cell surface proteome from cultured cells. Coupling this method to quantitative mass spectrometry has allowed systematic identification of changes in the cell-surface proteome upon metabolic regulation. I found that activation of AMPK results in robust changes in the cell surface proteome, including cell adhesion and migration proteins. I confirmed that AMPK activation elicits a decrease in the cell surface abundance of the adhesion and migration protein β1-integrin, and that this is correlated with altered function of the endocytosis protein Dab2. Thus, my research furthers our understanding of how AMPK regulates the cell surface proteome and the specific mechanism by which AMPK regulates cellular adhesion and migration.

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List of abbreviations

Abbot – A-769662

ACC – Acetyl-CoA carboxylase

ADP – Adenosine diphosphate

AICAR – 5-Aminoimidazole-4-carboxamide ribonucleotide; AICA ribonucleotide; Acadesine

AMP – Adenosine monophosphate

AMPK – AMP-activated protein kinase

AP-2 – Adaptor complex 2

ARRDC – Arrestin-domain-containing protein

ATP – Adenosine triphosphate

bFGF – Basic fibroblast growth factor

BM – Basement membrane

BSA – Bovine Serum Albumin

CaMKK - Ca^{2+}/calmodulin-dependent kinase kinase

CCP – Clathrin coated pit

CCV – Clathrin coated vesicle

CD – Cluster of differentiation

CHC – Clathrin heavy chain

CLASP – Clathrin-associated sorting protein

CLC – Clathrin light chain

CME – Clathrin mediated endocytosis

Dab2 – Disabled-2

DTT – dithiothreitol

EAP – Endocytic accessory protein

ECM – Extracellular matrix

EE – Early endosome

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

EH – Eps-homology

ESI – electrospray ionization

f-actin – Filamentous actin

FAK – Focal adhesion kinase

FBS – Fetal bovine serum

GDP – Guanosine diphosphate

GFP – Green fluorescent protein

GLUT-1 – Glucose transporter 1

GLUT-4 – Glucose transporter 4

GO – Gene ontology

GTP – Guanosine triphosphate

HAX1 – HCLS1-associated protein

LC-ESI-MSMS – Liquid chromatography electrospray ionization tandem mass spectrometry

LDL – Low density lipoprotein

LDL-R – Low density lipoprotein receptor

LKB-1 – Liver kinase B1

LSB – Laemmli Sample Buffer

MALDI – matrix-assisted laser desorption ionization

MHC – Major Histocompatibility Complex

MMP – Matrix metalloproteinase

MS – Mass spectrometry

MudPIT – Multidimensional protein identification technology

NA – Numerical Aperture

NHS – *N*-hydroxysuccinimide

O-GlcNAc – *O*-linked N-acetylglucosamine

OGT – *O*-linked N-acetylglucosamine transferase

p- – Phospho-

p130CAS – P130 CRK-associated substrate

p38 MAPK – p38 mitogen-activated protein kinase

PBS – Phosphate buffered saline

PFA – Paraformaldehyde

PIP_2 – Phosphatidylinositol-(4,5)-bisphosphate

PIP5K – Phosphatidylinositol-4-phosphate-5-kinase

PKC – Protein kinase C

PM – Plasma membrane

PY – Phosphotyrosine

RE – Recycling endosome

RPE – Retinal pigment epithelial cells

SDS – Sodium dodecyl sulfate

SDS PAGE – SDS Polyacrylamide gel electrophoresis

SFK – Src family kinase

SILAC – Stable isotope labeling by amino acids in cell culture

SS – Disulphide

Tfn – Transferrin

TfR – Transferrin receptor

TIRFM – Total internal reflection fluorescence microscopy

TXNIP – Thioredoxin-interacting protein

VALAP – **Va**seline, **La**nolin, **P**arafin wax

ZMP – 5-Aminoimidazole-4-carboxamide ribonucleoside

Introduction

Proteins on the surface of cells control numerous aspects of cellular function including signaling, nutrient uptake, and adhesion. The profile of all the proteins on the cell surface is termed the "cell surface proteome". These proteins exact their function by necessarily gaining access to the extracellular milieu – extracellular matrix proteins, hormones, nutrients, and so on. Nutrient transporters and carriers are crucial for obtaining essential nutrients and regulating their metabolic balance within the cell. For example, Glucose Transporter type 1 (GLUT-1) is an important sugar transporter responsible for the uptake of glucose into the cell. GLUT-1 deficiency is implicated as a cause of severe learning difficulties and developmental delay (Gordon & Newton, 2003). Adhesion molecules are responsible for forming contact sites with the extracellular matrix (ECM) or with other cells. Integrins are adhesion proteins that facilitate coupling of the ECM to the cytoskeleton and regulate cellular adhesion and migration.

The surface of a cell is not a static compartment as the cell surface proteome is dynamically regulated by multiple cellular mechanisms in response to changing cellular conditions. Remodelling of the cell surface proteome plays an important role in cell growth and differentiation. For example, such remodelling is crucial for cell type determination in T-cell development: $CD4^-8^-$ progenitor cells transition through a double-positive ($CD4^+8^+$) stage where both MHC coreceptors are present on the cell surface before maturing into single-positive thymocytes with only one coreceptor in the PM ($CD4^+8^-$ or $CD4^-8^+$). Plasticity of the cell surface proteome is a key feature that allows cells to respond to changing environments and metabolic states. Changes in the cell surface proteome may be long-term/chronic and result from changes

in expression of different cell surface proteins, or these changes may be short-term/acute and result from changes in membrane traffic.

Cell-surface protein membrane traffic

Perturbing the abundance of cell surface proteins may have vast implications on general cell health and maintenance. Proteins are regulated at the cell surface by their biosynthesis, degradation, endocytosis, recycling, and exocytosis (**Figure 1**). The cell surface proteome can be dramatically different between different cell types and between healthy and diseased cells of the same type (Billah et al., 2011; Hynes, 1976; Shin et al., 2003; Sun, 2012).

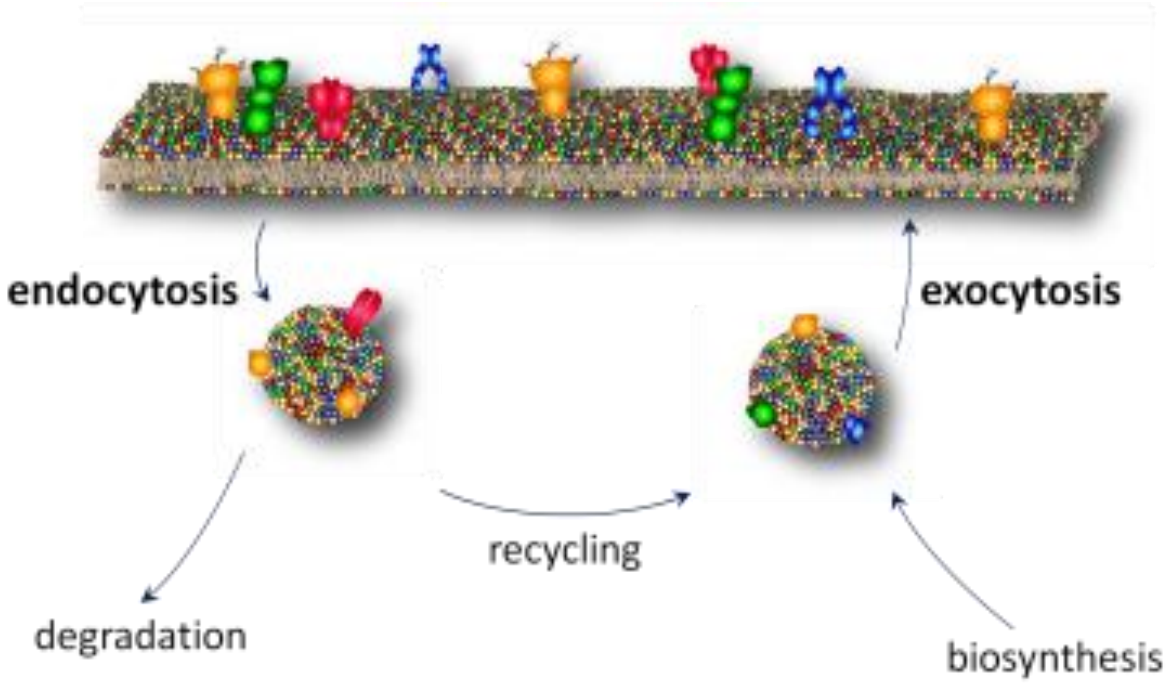


Figure 1 **Cell surface proteins are involved in many important functional pathways. Cell surface proteins are regulated by multiple cellular mechanisms including endocytosis, degradation, recycling, biosynthesis, and exocytosis.**

Regulation of endocytosis is one way to impact cell surface content. If endocytosis of a protein is interrupted, the interruption will result in an accumulation of that protein on the cell surface. Increasing the rate of endocytosis of a protein, on the other hand, would result in a decrease in the cell surface content of that protein.

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is the principal route of internalization for membrane proteins and initiates by recruitment of clathrin and other proteins to cargo (protein)-enriched areas of the plasma membrane (PM) known as clathrin-coated pits (CCPs). Clathrin and other proteins assemble on the inner leaflet of the plasma membrane, a process during which the plasma membrane becomes invaginated at the sites of CCPs. At any time, hundreds of CCPs are found in a cell (Traub, 2009). After a maturation stage, CCPs may eventually undergo scission from the PM to yield intracellular clathrin-coated vesicles (CCVs). The net result of formation of a CCV is the transfer of specific proteins from the cell surface into intracellular vesicles and subsequently endosomes (**Figure 2**).

There are many proteins and lipids that are required for the formation of a CCP leading to a CCV, underlying the complexity of this process. Despite the complexity of the formation of CCPs, some key molecules emerge: 1) clathrin, which self-assembles to form a scaffold, 2) AP-2, which binds to clathrin, cargo proteins, and the membrane, 3) Phosphatidylinositol-(4, 5)-bisphosphate (PIP_2), which is the main lipid binding partner of the PM for CME proteins including AP-2, and 4) cargo proteins, which are also bound and recruited by AP-2.

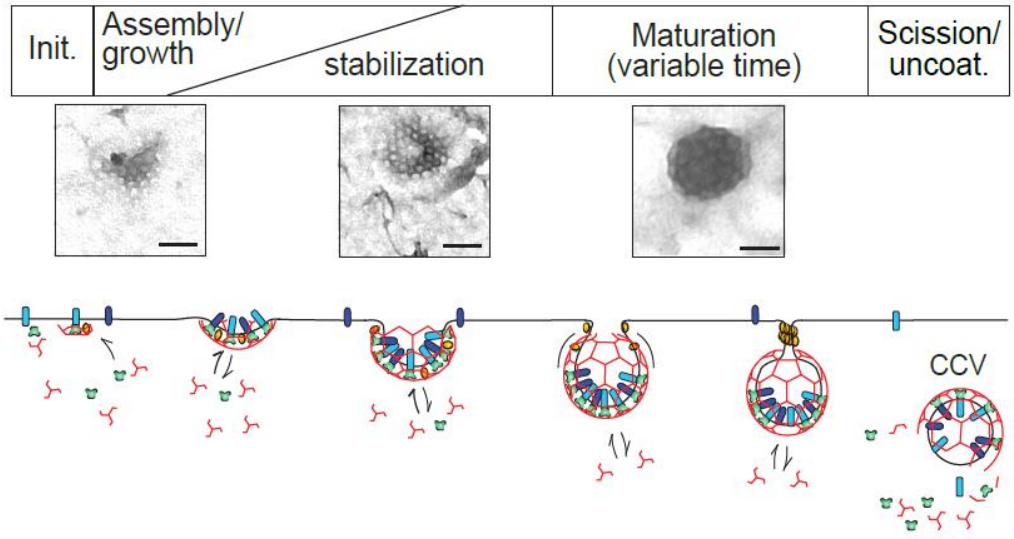


Figure 2 **Formation of clathrin-coated vesicles occurs as a result of regulated initiation, assembly, maturation and scission of clathrin-coated pits. CME initiates by recruitment of clathrin, AP-2, and other proteins to cargo-enriched areas of the PM known to assemble CCPs. After maturation, CCPs undergo dynamin-dependent scission from the PM to yield intracellular CCVs. Upon internalization, the clathrin coat disassembles**

Clathrin, a protein first isolated in 1975 by Barbara Pearse (Pearse, 1976), is the scaffold molecule in CME. It forms a triskelion shape with three clathrin light chains and three clathrin heavy chains. Clathrin self-assembles into a polyhedral basket-like structure that stabilizes early endocytic structure and facilitates CME (Brodsky, Chen, Knuehl, Towler, & Wakeham, 2001). The 190 kDa clathrin heavy chain C-terminus binds many accessory factors that regulate the progression of endocytosis (Schmid & McMahon, 2007; ter Haar, Harrison, & Kirchhausen, 2000). This feature of clathrin is why it is considered a major interaction hub of endocytosis.

The adaptor protein complex AP-2 is considered another interaction hub in endocytosis. AP-2 is a heterotetrameric protein complex with a trunk domain that binds to cargo and lipids, and two appendage domains, connected to the trunk with flexible linkers, that bind to many accessory proteins when concentrated in forming CCPs (Brett & Traub, 2006; Owen et al., 1999; Schmid & McMahon, 2007). AP-2 binds to clathrin, endocytic cargo, and accessory proteins

and so mediates cargo recruitment, maturation, and scission of the CCP. Some accessory proteins that AP-2 binds include the Eps-homology (EH) domain proteins Eps15 and intersectin.

PIP_2 is the main lipid binding partner of the PM for CME proteins including AP-2 and is another interaction hub in endocytosis. It is crucial for the recruitment of proteins to CCPs for CME and regulates CCP initiation, stabilization and size (Antonescu, Aguet, Danuser, & Schmid, 2011). PIP_2 synthesis is largely a result of phosphatidylinositol-4-phosphate-5-kinase (PIP5K) phosphorylation of phosphatidylinositol-4-phosphate in mammalian cells (Doughman, Firestone, & Anderson, 2003).

CME cargo proteins are cell surface receptors and their bound ligands. Internalization of cargo proteins regulates their abundance at the cell surface but cargo can regulate CME as well (Loerke et al., 2009; Mettlen, Loerke, Yarar, Danuser, & Schmid, 2010; Wilde et al., 1999). For example, EGFR is a transmembrane receptor protein responsible for the internalization and signaling of EGF. Ligand-bound EGFR stimulates Src phosphorylation of clathrin which influences clathrin redistribution and EGF uptake (Wilde et al., 1999).

There are approximately 30-50 other proteins that are recruited to CCPs in addition to the four "key" molecules described above. Some are required for membrane bending such as BAR domain or ENTH-domain containing proteins and others, such as clathrin-associated sorting proteins (CLASPs) are required for the incorporation of specific cargoes to CCPs and for their internalization. CLASPs are involved in the endocytosis of some cargoes (Traub, 2009). For example, Dab2 is a CLASP that is required for the endocytosis of specific cargo, such as LDL-R and β1-integrin. Dab2 contains a phosphotyrosine (PY)-binding domain that recognizes PIP_2 and the FxNPxY internalization motif found in LDL-R and β1-integrin (Morris & Cooper, 2001; Chao & Kunz, 2009; Ezratty, Bertaux, Marcantonio, & Gundersen, 2009; Teckchandani et al.,

2009). Like AP-2, Dab2 also binds to EH-domain containing proteins Eps15 and intersectin (Teckchandani, Mulkearns, Randolph, Toida, & Cooper, 2012).

There are hundreds to thousands of cell surface proteins; however three proteins, EGFR, transferrin (Tfn) receptor (TfR) and Low-Density Lipoprotein (LDL) receptor (LDL-R) are studied and used as general representatives of all cell surface proteins. Studies have shown that their endocytosis mechanisms and regulation are different (Antonescu et al., 2010; Loerke et al., 2009; Mettlen et al., 2010). Specifically, TfR requires TTP for its internalization, EGFR requires Epsin or Grb2, and LDLR requires ARH/Dab2. Recent developments in CME research have shown that CCPs are heterogeneous (Antonescu, Mcgraw, & Klip, 2014; Loerke et al., 2009; Mettlen et al., 2010) and that CME as well is a heterogeneous process that exhibits distinct mechanisms and regulation for the endocytosis of distinct receptors.

Most vesicular carriers originating from the PM are targeted to the early endosome, an organelle with specific proteins such as EEA1 and Rab5. From here, proteins are either sorted to the degradative pathway (in lysosomes, through traffic to late endosomes and then multi-vesicular bodies) or recycled back to the PM either directly or through a separate Rab11-positive membrane compartment.

While there is much to be learned about the molecular mechanisms that underlie endocytosis, it is now clear that different receptors (i.e. TfR and LDLR) can be distinctly regulated by CME, in part due to their differential use of CLASPs like Dab2. Not only does CME need to differentially regulate the cell surface levels of different proteins, but it also has to adjust this regulation to meet the changing needs of a cell. One of the most critical parameters of the cell that needs to be regulated in order to ensure homeostasis and survival is energy.

Energetic stress and AMPK

ATP is the powerhouse molecule that allows cells to undergo many energetically demanding processes. Phosphodiester bonds within ribonucleoside phosphates are the energy currency of the cell, and the end product of glycolysis and oxidative phosphorylation is ATP production. Fuel sources for ATP production primarily include glucose and fatty acids in most cell types. Adenylate kinase catalyzes the reaction 2ADP ↔ATP+AMP which is maintained close to equilibrium in most eukaryotic cells (G. D. Hardie, 2014). In unstressed cells, catabolism maintains the ATP:ADP ratio at around 10:1, which drives the adenylate kinase reaction towards ADP so that low levels of AMP are maintained.

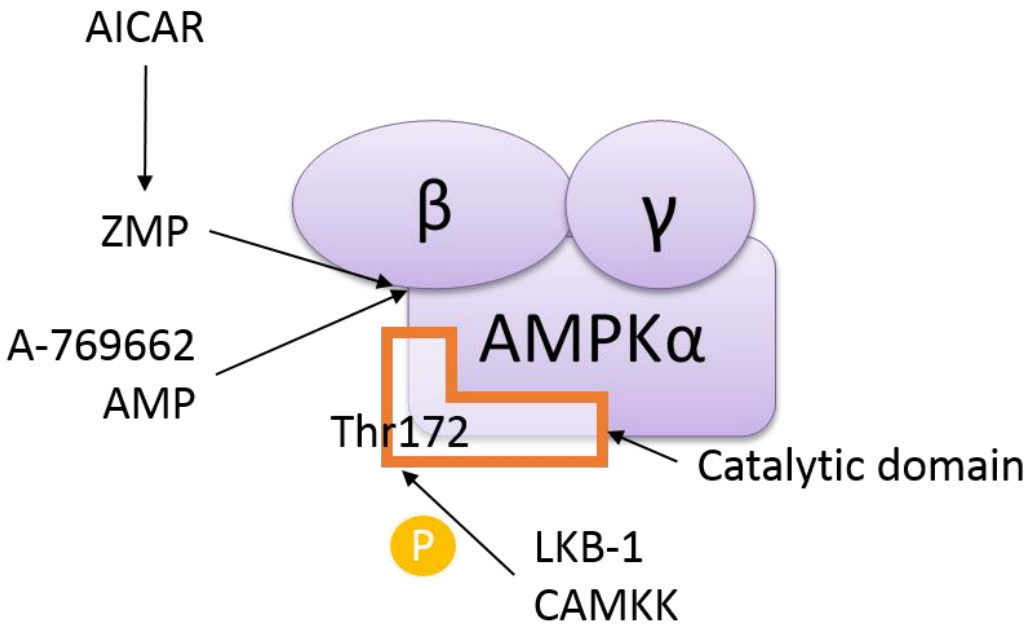


Figure 3 **AMPK is a heterotrimeric complex comprised of a catalytic alpha subunit and beta and gamma regulatory subunits (Mitchelhill et al., 1994). AMPK is phosphorylated at threonine 172 within the catalytic domain of the alpha subunit by LKB-1 or CAMKK. AICAR is converted to ZMP upon internalization. ZMP, A-769662, and AMP bind to the regulatory beta subunit near the alpha catalytic domain (G. D. Hardie, 2014)**

Certain physiological conditions (e.g. muscle contraction, lung cell hypercapnia) can result in dramatic changes in ATP levels (Antonescu et al., 2014). When cells experience this metabolic stress, the ATP:ADP ratio falls and the adenylate kinase reaction is displaced towards AMP (Gowans, Hawley, Ross, & Hardie, 2013). AMP concentration starts at a very low level so the changes in stressed cells are always much larger than the changes in ATP or ADP levels (Gowans et al., 2013). Therefore cellular AMP concentration is a sensitive indicator of energy stress. AMP-activated protein kinase (AMPK) is a metabolic stress sensor of the cell that is responsible for detecting and correcting energetic stress. AMPK becomes activated after recognizing a decrease in the AMP:ATP ratio. Activated AMPK directly and indirectly enacts many cellular responses that allow the cell to return to a metabolically favourable state.

AMPK is a heterotrimer that is structurally and functionally homologous with the yeast protein Snf1 (Mitchelhill et al., 1994). AMPK has a catalytic alpha (α1 or α2) subunit and beta (β1 or β2) and gamma (γ1, γ2, or γ3) non-catalytic subunits (**Figure 3**) (Mitchelhill et al., 1994). At the ATP concentrations typically found in an unstressed state, AMPK favours binding ATP over AMP, given the different relative affinity of the kinase for these two molecules (Gowans et al., 2013). In metabolic stress conditions, as the concentration of ATP drops, the ratio of AMP:ATP increases and AMPK favours binding to AMP. AMP binding to AMPK results in a conformational change exposing threonine 172 on the alpha subunit of AMPK that can be phosphorylated by the tumour suppressor liver kinase B1 (LKB-1) or by Ca^{2+}/calmodulin-dependent kinase kinase (CaMKK) (Corton, Gillespie, Hawley, & Hardie, 1995). Phosphorylated AMPK is active and can rapidly alter cellular metabolism.

AMPK can be regulated by post-translational modifications such as *O*-GlcNAcylation and phosphorylation. AMPK α- and γ- subunits are *O*-GlcNAcylated by *O*-GlcNAc transferase

(OGT) and this modification on the γ-subunit of AMPK increases AMPK activity (Bullen et al., 2014). AMPK shuttles between the nucleus and the cytoplasm under normal growth conditions (Kodiha, Rassi, Brown, & Stochaj, 2007). This localization can be controlled by phosphorylation by other kinases such as Erk which are in turn regulated by a variety of stimuli and can be perturbed in stress-conditions (Kodiha et al., 2007). Hence, the physiological action of AMPK may be controlled not only by its intrinsic affinity of specific substrate proteins, but also by its O-GlcNAcylation and by its cellular compartmentalization.

AMPK's effects can acutely phosphorylate its own downstream targets and also exhibits certain long-term effects by influencing gene transcription (**Figure 4**). In order to conserve cellular energy, AMPK inhibits anabolic pathways and activates catabolic pathways in response to metabolic stress (Chen et al., 1999).

Activated AMPK phosphorylates (and thereby inactivates) acetyl-coA carboxylase (ACC) to down-regulate fatty acid synthesis and promote fatty acid oxidation. ACC is an enzyme that makes malonyl-CoA. Malonyl-Coa is the building block for fatty acid production and is also an inhibitor of the carnitine acyltransferase, which is the first step in fatty acid breakdown to make ATP. So by phosphorylating and inhibiting ACC, the cell shifts to burning more fatty acids to make more ATP.

AMPK as a regulator of membrane traffic

In addition to controlling the enzymatic activity of metabolic enzymes and to regulating gene transcription, AMPK is also emerging as a key regulator of membrane traffic (Antonescu et al., 2014). For example, AMPK phosphorylates and promotes the degradation of thioredoxin-interacting protein (TXNIP), an arrestin-domain-containing protein (ARRDC) that promotes GLUT1 internalization through CCPs (Antonescu et al., 2014; Wu et al., 2013). Therefore AMPK induced degradation of TXNIP will result in increased plasma membrane GLUT1 levels, allowing for increased glucose uptake and ATP generation. Furthermore, AMPK activation in muscles increases GLUT4 (another glucose transporter) presence at the cell surface (Antonescu, Díaz, Femia, Planas, & Klip, 2008; Xi, Han, & Zhang, 2001). The cell surface pump protein Na^+/K^+-ATPase consumes up to 30% of resting ATP in some cells (Antonescu et al., 2014). AMPK activation results in the internalization and consequential inactivation of Na^+/K^+-ATPase which may preserve cellular ATP levels.

Since AMPK plays a central role in the control of glucose homeostasis and lipid metabolism, it is an important therapeutic target in Type 2 Diabetes and obesity. Low-level activation of AMPK is likely to contribute to the global rise in obesity and diabetes (D. G. Hardie, 2011). The role of AMPK in regulating systemic metabolism may be primarily due to AMPK in muscle and liver (G. D. Hardie, 2014).

AMPK in development and migration

AMPK also plays other roles in many other cell types, as can be seen by the fact that a double AMPK α1/α2 knockout mouse is embryonic lethal (Viollet et al., 2009). The role of

AMPK in other cell types is poorly understood however there is some evidence of a link between AMPK and cell polarity and cell migration (D. G. Hardie, 2011; Kanellis et al., 2006). Activation of AMPK reduces cell adhesion in endothelial progenitor cells (Kaiser, Friedrich, Chavakis, Böhm, & Friedrich, 2012), and reduces cell migration in vascular smooth muscle cells (Liang et al., 2008), U937 monocytes (Kanellis et al., 2006), and glioblastoma cells (Ferla, Haspinger, & Surmacz, 2012). The molecular mechanism by which AMPK may control cell adhesion and migration is unknown.

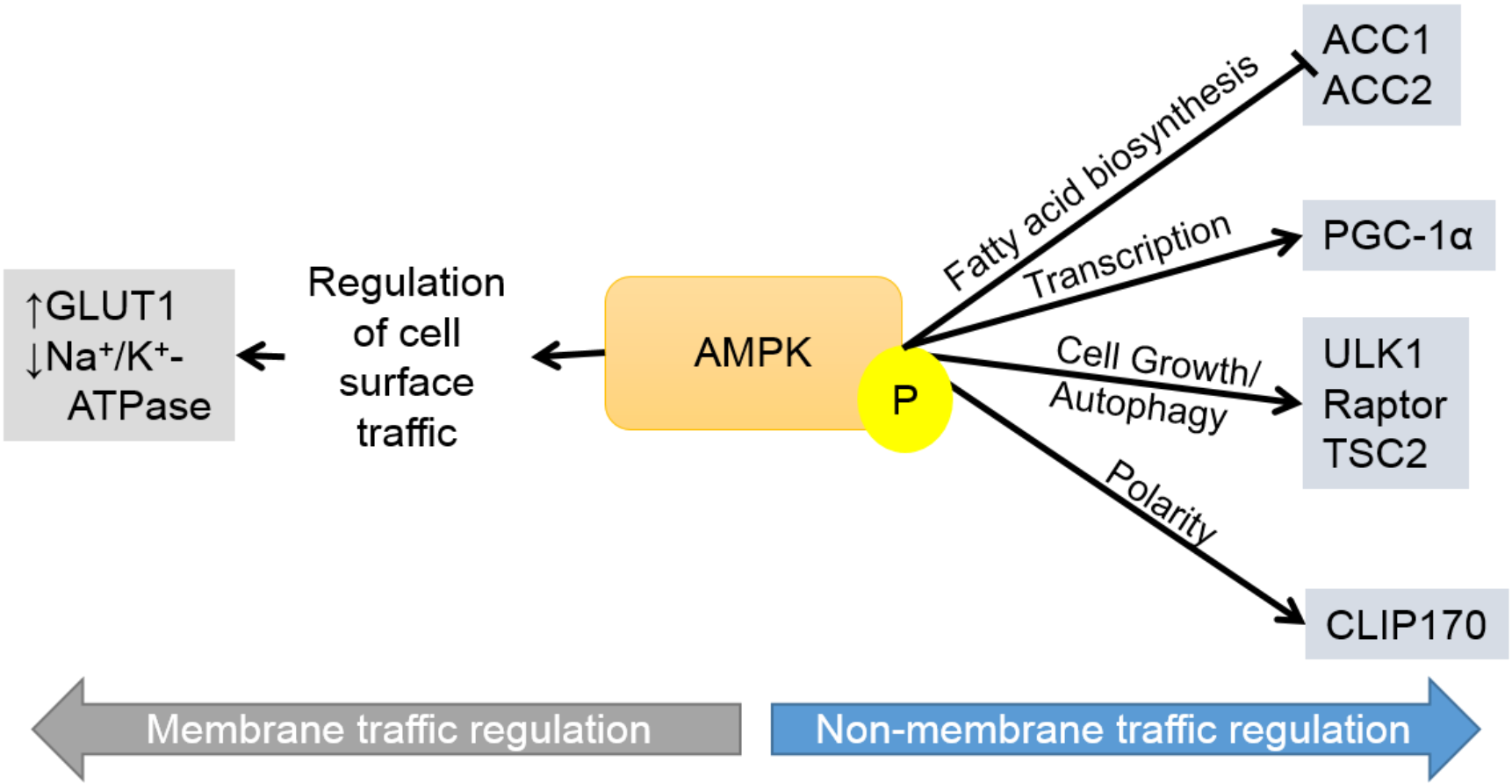


Figure 4 **AMPK phosphorylation is implicated in many cellular responses to metabolic stress. Fatty acid biosynthesis is inhibited after activated AMPK phosphorylates ACC1 and 2. PGC-1α, ULK1, Raptor, TSC2 and CLIP170 are other proteins involved in important cellular responses known to be phosphorylated by AMPK activation. Increased GLUT1 at the cell surface and decreased Na+/K+-ATPase at the cell surface have been shown, however the mechanism has not been elucidated.**

Cellular migration and adhesion

Cell migration is the process by which cells move throughout their environment. This process requires cells to polarize distribution of certain key PM proteins and extend protrusions (large, broad lamellipodia; long, thin pseudopodia; or spike-like filopodia) according to the direction of travel. Lamellipodia and filopodia formation are driven by actin polymerization and are stabilized by adherence to the ECM or adjacent cells. These adhesions form traction sites for migration as the cell moves forward over them and are disassembled at the cell rear allowing for detachment (**Figure 5**) (Ridley et al., 2003).

Maintenance of a polarised state in migration requires active focal adhesion disassembly and coordinated adhesion molecule membrane traffic so that new contacts can form near the front of the cell (M. C. Jones, Caswell, & Norman, 2006). Cell migration involves cell deformation and deformations of the surrounding tissue as the migrating cell moves along (Lange & Fabry, 2013). These cellular deformations are coupled to a build-up of mechanical stresses that must be overcome by generation of mechanical forces by cytoskeletal proteins (Lange & Fabry, 2013).

Adhesion molecule membrane traffic

Integrins are a family of adhesion molecules involved in interactions between the cell and the extracellular matrix (ECM) that surrounds the cell. Past descriptions of integrin membrane traffic led to proposals that endocytosis and recycling drive cell migration by moving adhesion receptors from the back to the front of moving cells (Bretscher, 1996). Although there is not yet evidence that this long-range transport operates during cell migration, recent discoveries show

that Rab21-positive endosomes mediate the redistribution of integrins from one end of the cell to the other during cytokinesis (Pellinen et al., 2006).

On the other hand, the influence of integrin trafficking on cell migration is likely mediated by modulating Rho GTPase signalling (White, Caswell, & Norman, 2007) and by controlling the spatial restriction of integrins (P. T. Caswell et al., 2007). Rab proteins are members of the Ras superfamily of GTPases that function in endomembrane traffic by acting as molecular switches that oscillate between GTP- and GDP-bound conformations. The GTP-bound form is active and the regulatory function of Rab proteins is restricted to the compartment where they are localized. While the function of some Rab proteins like Rab5 are mostly ubiquitous, affecting the early endosome membrane traffic of a large number of proteins, the function of some Rab proteins appears to be more selective for some cargo.

The Rab11 subfamily, comprised of Rab11a, Rab11b, and Rab25 (also known as Rab11c), controls the recycling of internalized membrane proteins to the cell surface (Galvez, Gilleron, Zerial, & O'Sullivan, 2012; Zerial & McBride, 2001). Rab25 is expressed exclusively in epithelial tissues and promotes a mode of migration on three-dimensional matrices that is characterized by the extension of long pseudopodia (P. T. Caswell et al., 2007). Rab25 directly interacts with the β1 integrin cytoplasmic tail and promotes the localization of vesicles that deliver integrins to the PM at the pseudopodial tips at the leading edge of a migrating cell (P. T. Caswell et al., 2007).

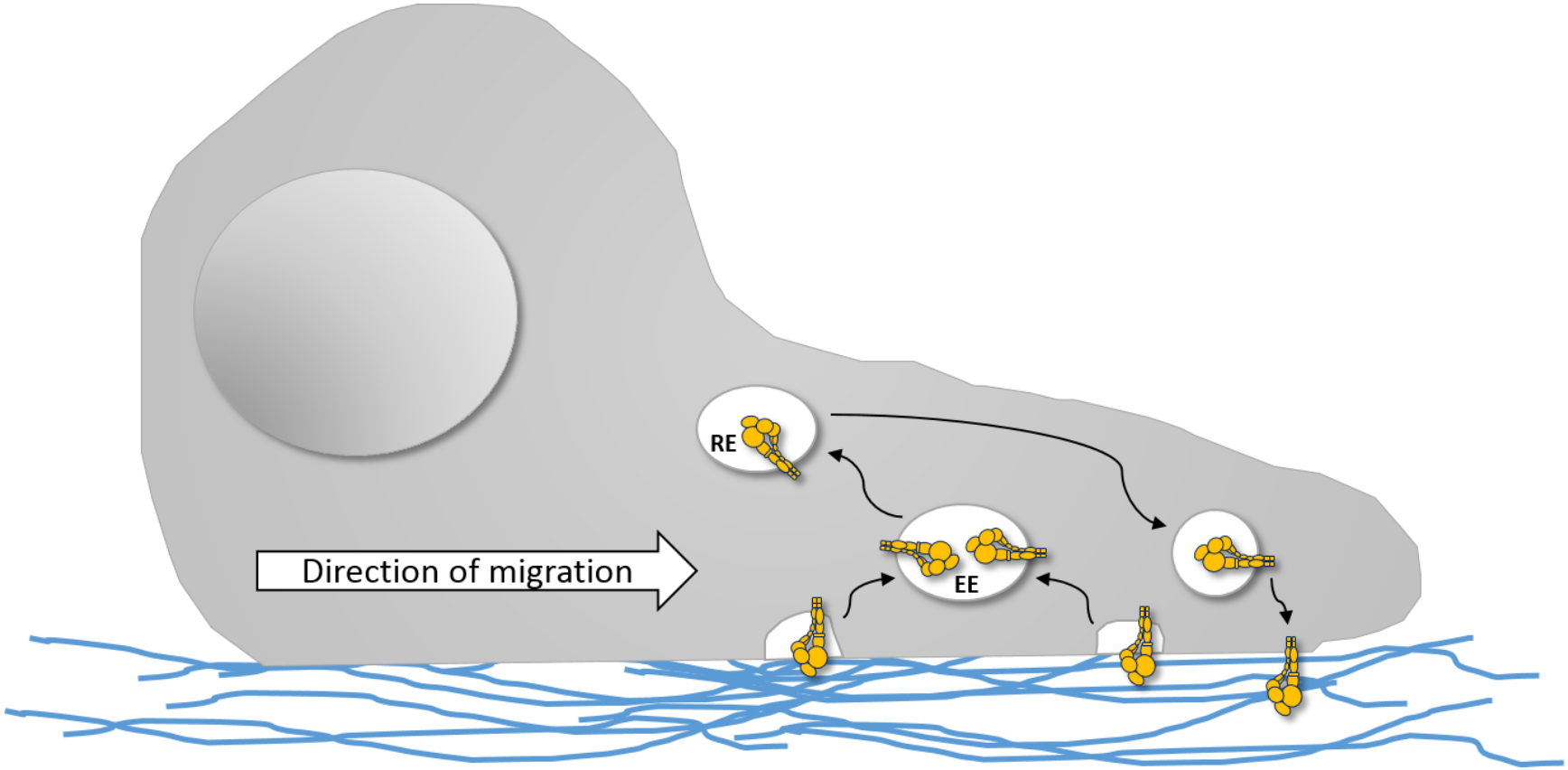


Figure 5 **Cellular migration requires coordinated regulation of adhesion molecules at the cell surface. Adhesion molecules forming focal contacts are internalized and recycled to be deposited at the leading edge of the cell. New focal contacts form in the protruding lamellipodia of migrating cells which the cell uses to generate locomotive force, and finally as the cell moves past the sites of focal adhesion, these are disassembled and the parts recycled.**

Integrins

Integrins are cell adhesion receptors on the cell surface that facilitate coupling of the extracellular matrix (ECM) to the cytoskeleton. Integrins are heterodimeric, transmembrane glycoproteins (Felding-Habermann, 2003). An integrin heterodimer (**Figure 6**) has one α-subunit non-covalently linked to one β-subunit (Felding-Habermann, 2003). Each subunit contains a single membrane spanning helix and a short unstructured cytoplasmic tail (Campbell & Humphries, 2011). The α- and β- subunits are constructed from several domains with flexible linkers between them (Campbell & Humphries, 2011). α-subunits have approximately 1000 amino acids and β-subunits have approximately 750 amino acids (Desgrosellier & Cheresh, 2010). There are 18 α-subunits and 8 β-subunits in mammals that make at least 24 αβ heterodimers (Desgrosellier & Cheresh, 2010).

The profile of integrin heterodimers on a certain cell`s surface dictates the extent to which it will adhere to and migrate on different surfaces (**Table 1**) (Desgrosellier & Cheresh, 2010). Integrins recognize specific adhesive sequences and bind directly to ECM components such as fibronectin, fibrinogen, osteopontin, vitronectin, collagen, and laminin (Desgrosellier & Cheresh, 2010). For example, αv integrins and α5β1 recognize the RGD sequence on their ligands (Desgrosellier & Cheresh, 2010). Other adhesive sequences are EILDV and REDV sequences (Desgrosellier & Cheresh, 2010). Binding to the ECM and cytoskeletal components such as actin, talin, and kindlin provides necessary traction for cell motility and invasion (Desgrosellier & Cheresh, 2010).

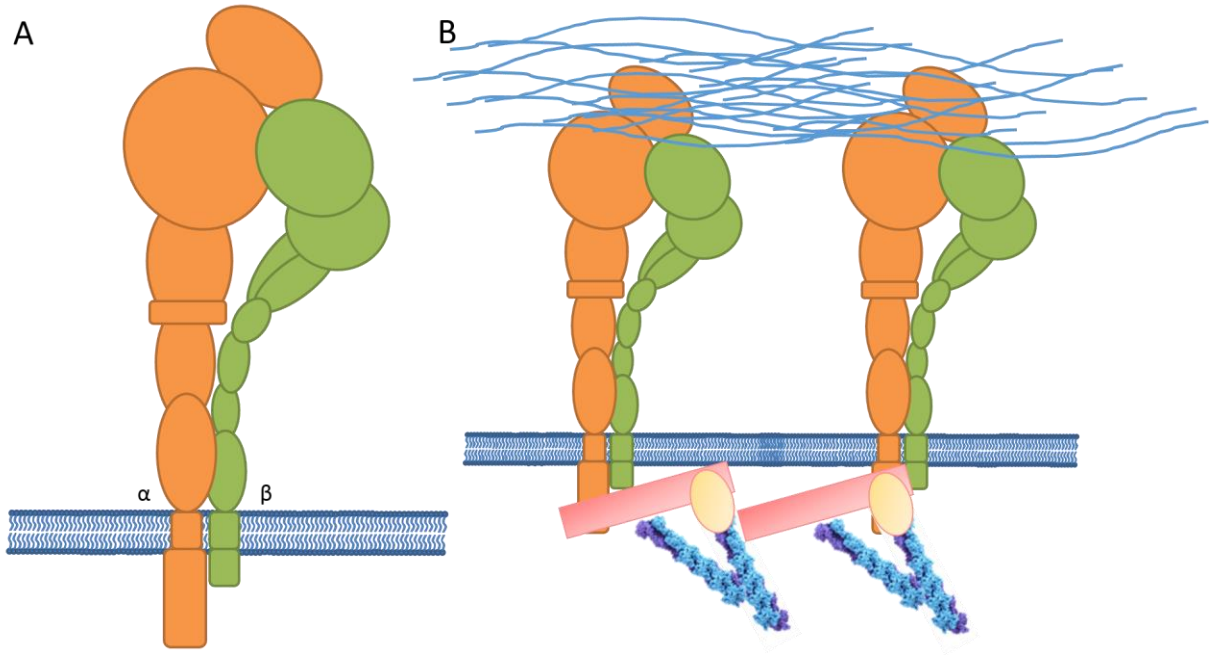


Figure 6 **A) An integrin heterodimer has one α-subunit and one β-subunit non-covalently linked together. Each subunit contains a single membrane spanning helix and a short unstructured cytoplasmic tail. The α- and β- subunits are constructed from several domains with flexible linkers between them. B) Integrin heterodimers bind extracellular matrix proteins with their extracellular domains and bind cytoskeletal proteins such as talin, kindlin, and actin (pictured in red, light orange and blue, respectively) with their intracellular domains to form focal contacts.**

Table 1 **Examples of integrin heterodimers and their ligands**

Name	Distribution	Ligands
$\alpha_1\beta_1$	Many	Collagens, laminins
$\alpha_7\beta_1$	Muscle, glioma	laminins
$\alpha_v\beta_1$	Ocular melanoma; neurological tumours	Vitronectin; fibrinogen
$\alpha_v\beta_3$	Activated endothelial cells, melanoma, glioblastoma	Vitronectin, fibronectin, fibrinogen, osteopontin
$\alpha_v\beta_5$	Fibroblasts, epithelial cells, & more	Vitronectin and adenovirus
$\alpha_6\beta_4$	Epithelial cells	laminin

β1-integrin is the principle binding partner of many α-integrins (**Table 1**) and as such is a key adhesion molecule (Desgrosellier & Cheresh, 2010). Although many integrin complexes contain β1-integrin, there exist differences in ECM binding between them. For example, integrin α1β1 binds collagen and laminin, α4β1 binds fibronectin and VCAM1, α5β1 binds fibronectin and L1-CAM, α6β1 binds laminin, merosin, and kalinin (Avraamides, Garmy-Susini, & Varner, 2008) an αvβ1 binds vitronectin and fibrinogen (J. F. Marshall et al., 1995). Differential expression of β1-containing integrin complexes in various cell types or tissues (**Table 1**) may specify particular adhesion and migration properties but distinct β1-integrin heterodimers may share some aspects of regulation that depend on β1-integrin.

Integrin ligation to the ECM results in a clustering of integrins in the plane of the plasma membrane (PM) which recruits various signalling and adaptor proteins to form focal adhesions (Desgrosellier & Cheresh, 2010). Focal adhesions are dynamic macromolecular complexes that link the ECM to the actin cytoskeleton through integrins (Desgrosellier & Cheresh, 2010). Although integrins lack kinase activity, their clustering recruits and activates kinases, such as focal adhesion kinases (FAKs) and Src family kinases (SFKs), and scaffolding molecules such as P130 CRK-associated substrate (p130CAS). Integrin clustering in focal adhesions involves cytoskeletal reorganization and therefore can lead to changes in cell shape (Felding-Habermann, 2003).

Endocytosis and recycling of integrins

Integrins are internalized by clathrin-dependent and clathrin-independent endocytic mechanisms (P. T. Caswell, Vadrevu, & Norman, 2009). A given integrin heterodimer can

follow more than one internalization route (P. T. Caswell et al., 2009). Clathrin adaptors for clathrin-mediated endocytosis that are involved in the clathrin-mediated internalization of integrins include HCLS1-associated protein X1 (HAX1), disabled (Dab), and Numb (P. T. Caswell et al., 2009). Most recently, the clathrin adaptor protein Dab2 was shown to recruit certain β1-integrins to Dab2-rich clathrin coated pits (CCPs) across the cell surface and that their endocytosis is Dab2 dependent (Teckchandani et al., 2009). Upon internalization, integrin heterodimers localize to early endosomes from which they can be either returned directly to the PM in a Rab4 dependent manner or further trafficked to the perinuclear recycling compartment (PNRC) before recycling through Rab11- and/or Arf6-dependent mechanisms (P. Caswell & Norman, 2008; M. C. Jones et al., 2006; Mai et al., 2011; Pellinen et al., 2006). The journey of internalized β1-integrins through the endomembrane system is shown in **Figure 7**. β1-integrins localize to Rab21-positive early endosomes where they are retained by their interaction with Rab21 (Mai et al., 2011; Pellinen et al., 2006). P120RasGAP displaces the Rab21-integrin interaction allowing for β1-integrin exit from Rab21-endosomes (Mai et al., 2011). β1-integrins are returned to the PM by Rab11-positive recycling endosomes (Mai et al., 2011; Pellinen et al., 2006).

Integrins in proliferation and angiogenesis

Integrins have been shown to exhibit cellular functions other than those involved in cellular adhesion. For example, integrins can regulate proliferation (Assoian & Klein, 2008) and, when ligated, can enhance cell survival through several mechanisms including by activation of the PI3K-Akt pathway (Aoudjit & Vuori, 2001). The β1 class of integrins have been shown to be required for angiogenesis – the formation of new blood vessels (Bloch et al., 1997).

Angiogenesis is highly important throughout processes such as embryonic development, tissue repair, and fertility, but also a key step in chronic inflammation, tumour growth and tumour metastasis (Avraamides et al., 2008). New blood vessels in tumours can grow by sprouting from pre-existing vessels to provide blood flow to support a growing tumour or to a location where the metastasized tumour cells colonize. β1-integrin-null embryos die by embryonic day 9.5-10.5 *in utero* due to defects in implantation (Fassler & Meyer, 1995; Stephens et al., 1995) and animals with endothelial cell-specific deletion of β1-integrin die by embryonic day 10.5 with severe vascular defects (Tanjore, Zeisberg, Gerami-Naini, & Kalluri, 2008). Furthermore, endothelial cell proliferation and vessel branching is absent in β1-integrin-null embryoid bodies (Bloch et al., 1997). The β1-integrin family has a clear, crucial role in angiogenesis.

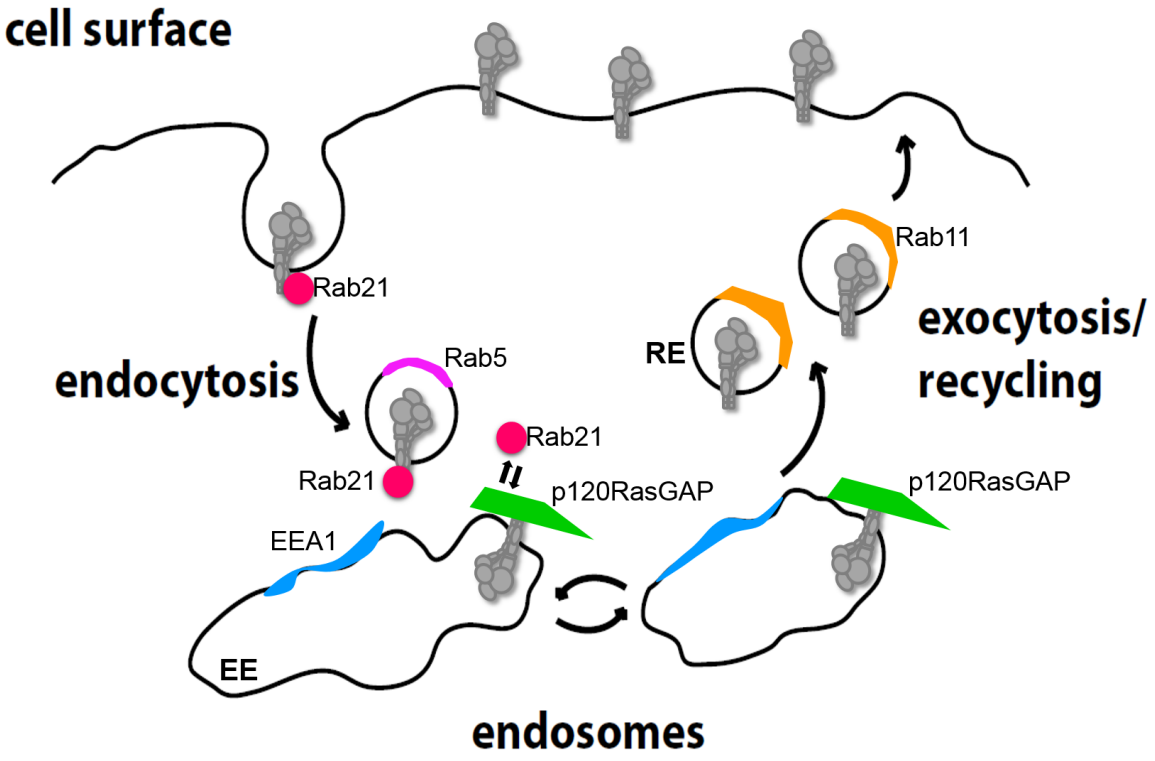


Figure 7 **Integrin heterodimers containing β1-integrin are recycled in a Rab21/ p120RasGAP/Rab11-dependent manner (Figure adapted from Mai et al., 2011).**

Integrin signalling

Since integrins are expressed uniquely on different cell types, they maintain the integrity of organs and tissues by preventing cell survival in improper environments (Desgrosellier & Cheresh, 2010). Integrins are also known to be highly implicated in intracellular signalling and can perform "inside-out" signalling as well as "outside-in" signalling (Desgrosellier & Cheresh, 2010). "Inside-out" signalling describes when integrin function is controlled from within the cell. For example, association of the β-integrin cytoplasmic tail with FERM domain-containing proteins such as talin and kindlin switches the integrin heterodimer from a low-affinity conformation to a high-affinity active conformation (Harburger, Bouaouina, & Calderwood, 2009; Wegener et al., 2007). "Outside-in" signalling initiated by integrins involves recruiting signalling moieties (such as focal adhesion kinase) that provide signals to the migratory and proliferative machinery of the cell (P. T. Caswell et al., 2009).

Systemic analysis of cell surface proteome

There are hundreds to thousands of proteins at the cell surface. The cell-surface proteome holds a wide diversity of proteins that contribute to numerous cellular processes. Experimental approaches that are hypothesis-driven about specific proteins are limited and do not allow for a systemic "snapshot" of how the cell-surface proteome is regulated by a specific stimulus.

Some previous studies have successfully purified the cell-surface proteome and quantified changes in cell surface proteins in response to changing stimuli or environments. For example, Rugg-Gunn and partners used cell-surface protein biotinylation to isolate membrane proteins and

found changes in the cell-surface proteome upon differentiation of stem cells (Rugg-Gunn et al., 2012).

In a study of the cell-surface proteome in human mesenchymal stem cells in response to the growth factor, bFGF, Lee and associates (2013) used functional Gene Ontology (GO) analyses to identify functional protein groups or clusters that undergo regulation (Lee et al., 2013). GO includes three structured, controlled vocabularies that describe gene products in terms of their associated molecular functions, cellular components, and biological processes (D. W. Huang, Sherman, & Lempicki, 2009). GO analyses allow for an understanding of the biological meaning and relevance of the results of a cell-surface proteome isolation and identification (D. W. Huang et al., 2009). Isolation of the cell-surface proteome coupled with quantitative analysis of changes of certain functional groups as denoted by GO allows for a systematic and biologically relevant understanding of how an entire cell compartment may react and adapt to changing conditions.

Rationale and hypothesis

AMPK is emerging as a key regulator of cell physiology by allowing cell survival and homeostasis under conditions of energy stress. A response to energy stress is likely to involve a coordinated remodeling of cell surface proteins that would effect a reduction in energy expenditure and increased energy intake. Which cell surface proteins AMPK may regulate as well as the mechanism by which AMPK may control cell surface membrane traffic are poorly understood. Elucidating the regulation by AMPK of the cell surface proteome may allow for a better understanding of how the cell responds to metabolic stress and may lead to improved characterization of human cancers and diseases allowing for the discovery of potential treatment avenues.

I hypothesize that the cell surface proteome is significantly altered during metabolic stress through the activation of AMPK. Using mass spectrometry, I determined how AMPK activation regulates the cell surface proteome. Of the several groups of proteins regulated at the cell surface by AMPK, I identified cell adhesion and cell migration molecules as key targets of AMPK regulation. I examined the mechanism of regulation of adhesion molecule membrane traffic by AMPK and found that β1-integrin abundance is depleted at the cell surface following AMPK activation. This effect is not seen with transferrin receptor and appears to be related to the changed recruitment of Dab2 to CCPs upon AMPK activation. Therefore AMPK regulates the cell surface proteome in response to metabolic stress potentially through the regulation of Dab2 dependent CME of specific cargoes.

Materials and Methods

RPE Cell Culture and AMPK Activation

Human non-immortalized, non-transformed Retinal Pigment Epithelial (AREP-19) cells were obtained from ATCC (henceforth RPE cells). Populations of RPE cells stably expressing clathrin light chain a fused to enhanced green fluorescent protein (RPE -GFP-CLCa) or to red fluorescent protein (RPE -RFP-CLCa) were previously generated (Aguet, Antonescu, Mettlen, Schmid, & Danuser, 2013). All RPE cells were maintained in 10cm culture dishes in DMEM F12 supplemented with 10% fetal bovine serum (FBS) and 5% streptomycin/penicillin in a humidified incubator at 37°C and 5% CO_2. For experiments requiring AMPK activation, RPE-GFP-CLCa- were washed with PBS and then cultured in DMEM F12 supplemented with 0.1% FBS and 100µM A-769662 purchased from Abcam (Cambridge, MA) or 2 mM AICAR purchased from Cell Signaling Technology (Danvers, MA). After treatment for 10, 20, 30, 60, or 90 minutes, the cells were washed with PBS and harvested.

cDNA transfection

The engineering and validation of a mCherry-Dab2 expression cDNA construct was previously described (Mettlen et al., 2010). Transfection of RPE cells with Dab2-mCherry cDNA or mCherry only cDNA (control) was performed using FuGene HD transfection reagent (Promega, Madison, WI) as per the manufacturer's instructions. Briefly, RPE-GFP-CLCa cells were transfected using 1.1µg DNA and 3.3µL FuGene HD reagent in a total volume of 1mL (per well of a six-well plate) for 4 hours.

Cell Surface Protein Labelling and Purification

In order to selectively and covalently modify cell-surface proteins with a biotin moiety, cells were ectopically labelled with 0.5mg/mL Sulfo-NHS-SS-biotin (**Figure 8**) in PBS for 30 minutes at 16°C and then quenched with 50mM Tris-HCl (Bioshop, Burlington, ON) for 10 minutes at 16°C. Cells were then homogenized in modified RIPA buffer (50mM Tris-Cl pH 7.4; 0.25% Na deoxycholate; 150 mM NaCl, 1 mM EDTA; 50mM n-octylglucoside) supplemented with protease inhibitors. Homogenized cells were incubated under constant rotation at 4°C for 2 hours and then centrifuged at 13000rpm for 15 minutes. After centrifugation, the pellet was discarded and the protein concentration was measured. Equal amounts of protein were incubated under constant rotation with streptavidin-conjugated sepharose beads overnight at 4°C. Intracellular proteins were collected in the supernatant and after rigorous RIPA washes, cell-surface proteins were eluted with 100mM DTT (Bioshop, Burlington, ON).

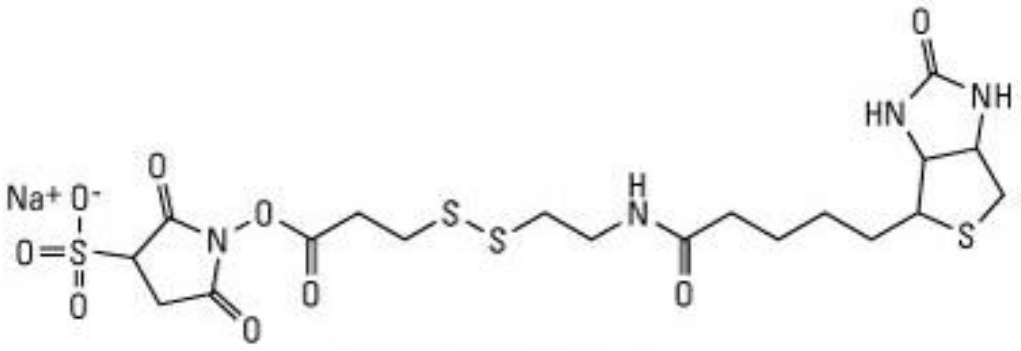


Figure 8 **Sulfo-NHS-SS-Biotin was purchased from Pierce and used to ectopically label cell surface proteins. The sulfonate group on the NHS gives the reagent a negative charge which prevents permeability of the PM, thus allowing labelling of cell surface proteins. The NHS group reacts with exposed primary amines and forms a stable amide conjugate. The long spacer arm on this reagent prevents stearic hindrance but also contains a disulphide bond which can be easily cleaved using a reducing reagent such as DTT**

Trypsin Digest of Cell Surface Proteins, Zip-Tip Preparation, LC-ESI-MS/MS, and Protein Pathway Analysis

Cell-surface proteins were incubated with digestion buffer (1M Tris; 2M Urea; 50% Acetonitrile; pH8.8) and 1µL trypsin (Roche, Indianapolis, IN) at 37°C overnight. Samples were incubated in 2mM DTT for 1h at 50°C. After brought to room temperature, samples were incubated with 1µL of trypsin (Roche, Indianapolis, IN) for 1h at 37°C followed by 5% formic acid solution. LC-ESI-MS/MS analysis was performed after solid-phase extraction of peptides using Zip-Tip® C18 (EMD Millipore, Billerica, MA) stationary phase for small-scale sample cleanup.

LC-ESI-MS/MS data for charge states 1, 2, 3, with a minimum peptide length of 4 were run against 4 search engine algorithms (Sequest, Tandem, Mascot, and Omssa) set for maximum 2 missed cleavages to identify proteins. All proteins present in background samples were subtracted from the total list of results. I found the ratio of proteins found in control samples to proteins found in samples treated with the AMPK-activator, A-769662, and set the following cut-offs: Control/AMPK-activated total protein identifications ≥ 2 = Proteins depleted at the cell surface after AMPK-activation; Control/AMPK-activated total protein identifications ≤ 0.5 = Proteins enriched at the cell surface after AMPK-activation. GO annotation and clustering of the identified proteins was obtained for each category (Proteins depleted vs enriched at the cell surface after AMPK-activation) using DAVID bioinformatics resources (version 6.7) see http://david.abcc.ncifcrf.gov/summary.jsp (D. W. Huang et al., 2009).

Immunofluorescence

To detect cell-surface proteins, intact cells were washed in PBS supplemented with 1 mM $CaCl_2$ and 1mM $MgCl_2$ (PBS+) and blocked for 15 minutes on ice in a solution of PBS+ containing 3% BSA before primary antibody to an ectopic epitope (recognizing Transferrin receptor, CD71, from Santa Cruz Biotechnologies, Santa Cruz, CA or β1-integrin from EMD Millipore, Darmstadt, Germany) was added for 1h at 4°C. Coverslips were washed in PBS+ before fixing in a solution of PBS+ containing 4% PFA for 30 minutes at room temperature. After quenching with a solution of PBS+ containing 100mM Glycine and washing with PBS, cells were blocked with a PBS solution containing 3% BSA for 15 minutes and secondary antibodies were added to the coverslips for 1 hour at room temperature. Secondary antibody used was goat anti-mouse cy3.

To visualize total (cell-surface and intracellular) protein content, cells were fixed in a PBS solution containing 4% PFA for 30 minutes at room temperature, quenched with a PBS solution containing 100mM glycine, and permeabilized with 0.1% Triton X-100 in PBS and 100mM glycine for 10 minutes at room temperature. Cells were washed in PBS and blocked in a PBS solution containing 3% BSA for 20 minutes at room temperature before primary antibody was added for 1 hour at room temperature. Coverslips were rinsed in PBS before the addition of secondary antibodies for 1h at room temperature. After several PBS washes, coverslips were mounted in Dako fluorescent mounting media (Dako, Carpinteria, CA). Secondary antibody used was goat anti-mouse cy3.

Epifluorescence Imaging

Fixed cells on 22mm by 22mm coverslips mounted on glass slides using Dako fluorescent mounting media (Dako, Carpinteria, CA) were visualized using a 63x/numerical aperture 1.49 oil objective on a Leica DM5000 B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) with a X-Cite 120 fluorescence illumination system light source (Lumen Dynamics Group, Missassauga, ON)**.** Images were recorded using fixed camera settings (LEICA CTR 5000, Leica Microsystems, Wetzlar, Germany). Images were acquired using Adobe Photoshop (San Jose, CA) and all exposure times and image scaling were equal within an experiment.

Quantification of cell surface staining and total staining intensity

Surface β1-integrin or TfR or total β1-integrin in each cell was quantified using ImageJ software (National Institutes of Health). Regions of interest corresponding to entire cells were overlaid onto micrographs in ImageJ (National Institutes of Health) and mean intensity of fluorescence was measured and average background intensity was subtracted from these means. Images were 16-bit and typical intensity ranges were between 10-30000 units ensuring pixel intensity saturation did not occur.

Measurements of β1-integrin cell surface abundance under three conditions (Control, AICAR, A-769662) were subject to one-way analysis of variance (ANOVA) with Newman-Keuls post test, with $p<0.05$ as a threshold for significant difference among conditions. Measurements of TfR cell surface abundance under two conditions (Control, A-769662) were subject to a Student's t-test, with $p<0.05$ as a threshold for significant difference among conditions.

TIRF Microscopy Imaging and analysis

Fixed cells on 22mm by 22mm coverslips were mounted on two double-strips of double-sided tape on a glass slide and sealed with VALAP (Antonescu et al., 2011). The cells were visualized using a 60x/numerical aperture 1.49 oil objective on an Olympus IX81 microscope (Olympus, Tokyo, Japan) equipped with individual CellTIRF modules illuminated with lasers of wavelengths 491nm and 561nm and emission filters of 520nm ± 35nm and 624nm ± 40nm. Images were recorded using fixed camera settings (C9100-13 EM-CCD, Hamamatsu). Images were acquired using Volocity (PerkinElmer, Waltham, MA) and all exposure times and image scaling were equal within an experiment.

CCPs are diffraction limited objects whose light patterns are approximated by a point-spread function. I detected CCPs using a Gaussian model approach as per *Aguet et al 2013* to identify the position of CCPs and the intensity of GFP-LCa and of mCherry-Dab2 fluorescence at each identified CCP (Aguet et al., 2013).

Western Blotting

Lysates from whole-cell extracts, cell-surface proteins, or intracellular proteins were subjected to gel electrophoresis. Proteins were then transferred to PVDF membranes (Bio-Rad, Burlington, ON). The membranes were blocked in a solution of TBS-T containing 4% BSA or 3% milk for 30 minutes at room temperature and then incubated at room temperature with the primary antibody for 1h. Rabbit anti-CD71 (1:1000), rabbit anti-Glut1 (1:1000), and rabbit anti-EGFR (1:1000) were purchased from GeneTex (Irvine, CA), goat anti-EGFR, mouse anti-TfR (1:400, 1:1000), and mouse anti-CHC (1:1000) were purchased from Santa Cruz Biotechnology

(Santa Cruz, CA). Rabbit anti-β1 integrin (1:1000), rabbit anti-pAMPK (1:500), rabbit anti-pACC (1:500), rabbit anti-pan-actin (1:1000), and rabbit anti-total ERK (1:1000) were purchased from Cell Signaling Technology (Danvers, MA). Mouse anti-β1-integrin (1:400) was purchased from Calbiochem (Billerica, MA). Secondary antibodies were goat anti-rabbit, goat anti-mouse, (Jackson Immunoresearch, West Grove, PA). Streptavidin-POD (1:1000) was purchased from Roche.

Results

Systematic analysis of the regulation of abundance of proteins requires a method for purifying cell surface proteins. I established this methodology using limited biotinylation of cell surface proteins. Subsequently, to study how AMPK regulates cell surface membrane traffic, I sought to optimize conditions for AMPK activation. Following this, I combined these methods to isolate cell surface proteins from control and AMPK-activated cells. These samples were subjected to mass spectrometry analysis, allowing identification of cell surface proteins in control and AMPK-activated cells. The proteins identified by this method were then subjected to Gene Ontology (GO) clustering to identify cellular processes involving cell surface proteins that are regulated by AMPK activation. This approach is described below.

Results Part 1: Regulation of the cell-surface proteome by AMPK

Validation and optimization of cell-surface protein purification assay

In order to determine regulatory effects of metabolic stress on the cell surface proteome, I developed an assay to selectively purify cell surface proteins. I labeled cell surface proteins using Sulfo-NHS-SS-Biotin (5 mg/mL; Pierce) at 16°C for 30 minutes (**Figure 8**). The sulfonate group on the NHS gives the reagent a negative charge which confers cell impermeability, thus allowing for labelling of cell surface proteins exclusively. The NHS group on the reagent reacts with exposed primary amines and forms a stable amide conjugate. The long spacer arm on this reagent prevents stearic hindrance but also contains a disulphide bond which can be easily cleaved using a reducing reagent such as DTT. After incubation of biotinylated cells with streptavidin labeled

sepharose beads, I eluted the biotinylated proteins with 50mM DTT and performed SDS-PAGE followed by silverstain. When cells were not labeled with the biotinylation reagent, few to no proteins were eluted from the streptavidin beads (**Figure 9**, *left lane*) whereas when the cells were labeled with the biotinylation reagent, many proteins were eluted (**Figure 9**, *2 right lanes*). Hence, this biotinylation assay successfully and cleanly (i.e. with little background non-specific binding of unbiotinyalted proteins to beads) extracts proteins from RPE cells; whether this assay is selective for cell surface proteins or whether the reagent was off-target labelling cytosolic proteins remained to be determined.

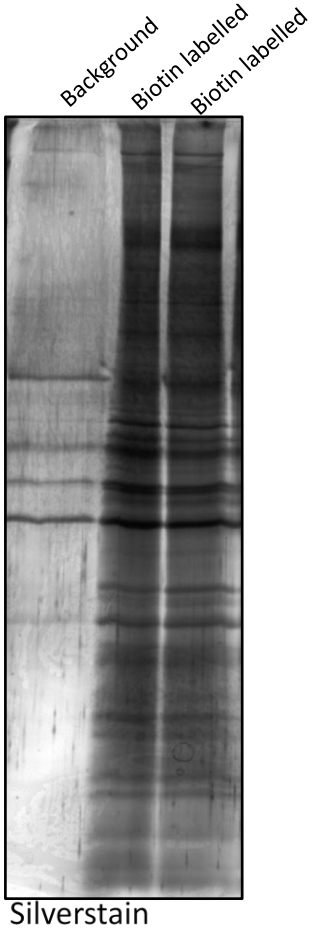


Figure 9 **Silverstain gel image of cells either unlabelled (Background) or labelled with Sulfo-NHS-SS-biotin (Biotin labelled) and subjected to purification using streptavidin-conjugated sepharose beads. The silverstain only detected proteins from samples of cells labelled with Sulfo-NHS-SS-Biotin. Therefore, only biotinylated proteins can be purified with these beads.**

I next sought to confirm that the purification assay selectively labels cell surface proteins with an exofacial biotin attachment. To ensure that the purification assay selectively labels and isolates cell surface proteins rather than intracellular proteins, I performed SDS-PAGE with extracts from my assays and performed a Western blot with antibodies specific to integral membrane proteins or intracellular proteins which will act as markers for these types of proteins (**Figure 10**). The integral membrane protein EGFR which has an exofacial domain of 588 amino acids harboring 35 lysine resides was successfully extracted from cells and found in the pull-down fraction. Erk and actin are cytosolic proteins that are known to interact highly with cell-surface proteins (Burridge et al., 1990; DeMali, Wennerberg, & Burridge, 2003; Kang & Banfield, 2010), therefore I expected to see some detection of these proteins in the pull-down fraction.

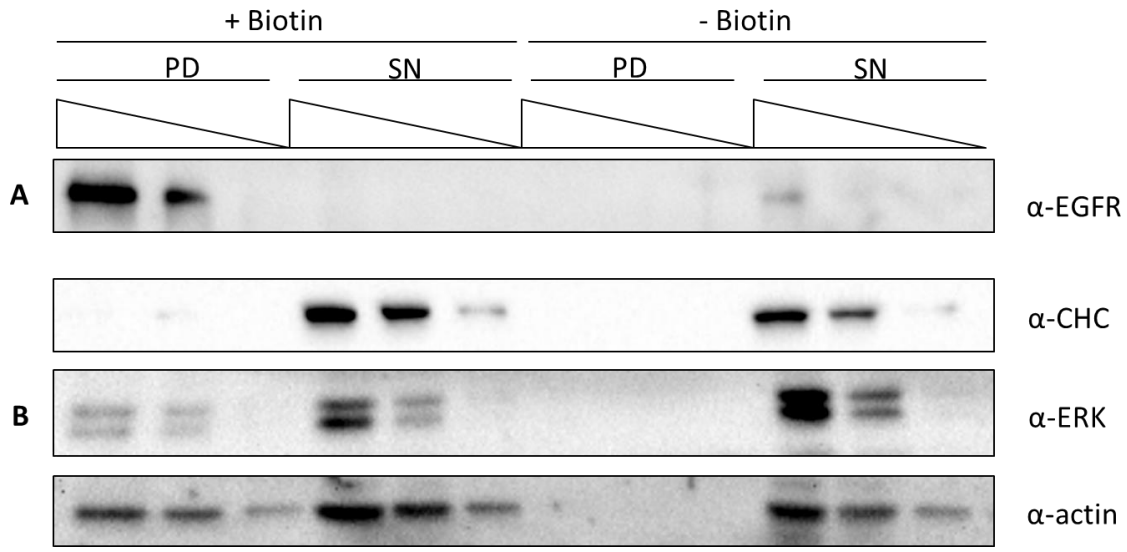


Figure 10 **Western blot images showing clean purification of cell surface proteins with little cytosolic content. Pull-down (PD) and Supernatant (SN) fractions were loaded in decreasing concentrations (left to right). A) EGFR is an integral membrane protein that is found in the PD fraction of biotinylated cells suggesting effective selective cell-surface protein purification. B) Cytosolic protein, clathrin heavy chain (CHC), is found predominantly in the SN fraction of both biotinylated and unlabeled cells. Cytosolic, but highly membrane protein-interacting, proteins Erk and actin were detected primarily in the SN fraction with some low-level detection in the PD fractions. No proteins were detected in the PD fraction when cells were not biotinylated.**

CHC is the best candidate for a cytosolic protein because it has no direct contacts with integral membrane proteins and can only form low affinity interactions with membrane-associated complexes. Furthermore, of the 1675 amino acid residues in CHC, 94 are lysines so I would expect CHC to be heavily biotinylated if the reagent was permeable to the cell surface.

Intracellular proteins CHC, Erk, and actin were found primarily in the supernatant fraction, with Erk and actin appearing faintly in the pull-down fraction as well. When I did not biotinylate cells, EGFR, CLC, Erk, and actin were all found exclusively in the supernatant fraction. The purification assay that I developed successfully selectively labels and extracts cell-surface proteins and intracellular proteins can be retrieved from the supernatant fraction. This assay uses a reducing reagent, DTT, to elute the proteins from the beads. Whether the elution of protein was specific to a reducing environment or not was necessary to next elucidate.

I tested several different elution conditions to determine the ideal conditions for maximal recovery of biotinylated proteins without also recovering proteins that may have stable, non-specific associations with the beads (**Figure 11**). I designed the biotinylation and purification assay so that the only way to elute biotinylated cell-surface proteins from the streptavidin labelled beads is to introduce a reducing environment to cleave the disulphide bond contained within the sulfo-NHS-SS-biotin reagent. High salt concentrations can interrupt electrostatic interactions between proteins and beads. I do not expect to see proteins eluted by high salt concentration in the cell-surface purification assay because the beads used were highly specific to bind biotinylated proteins. A high concentration of free biotin may be expected to elute cell surface proteins from streptavidin labeled beads by outcompeting and displacing current biotin-streptavidin linkages. Biotin and streptavidin bind with such high affinity and their off-rate is so low, however, that this method of elution is extremely unlikely to successfully elute biotinylated

cell-surface proteins. Only when I used DTT to elute the proteins were there any proteins labelled on the silverstain gel, an analysis method used to detect any protein even in very low concentrations. RIPA buffer and LSB were both suitable solvents for this assay when combined with the reducing reagent DTT. High salt and high free biotin concentrations were not successful in eluting the proteins from the beads, as expected. Elution of proteins from streptavidin labelled sepharose beads was only successful in the presence of a reducing reagent such as DTT.

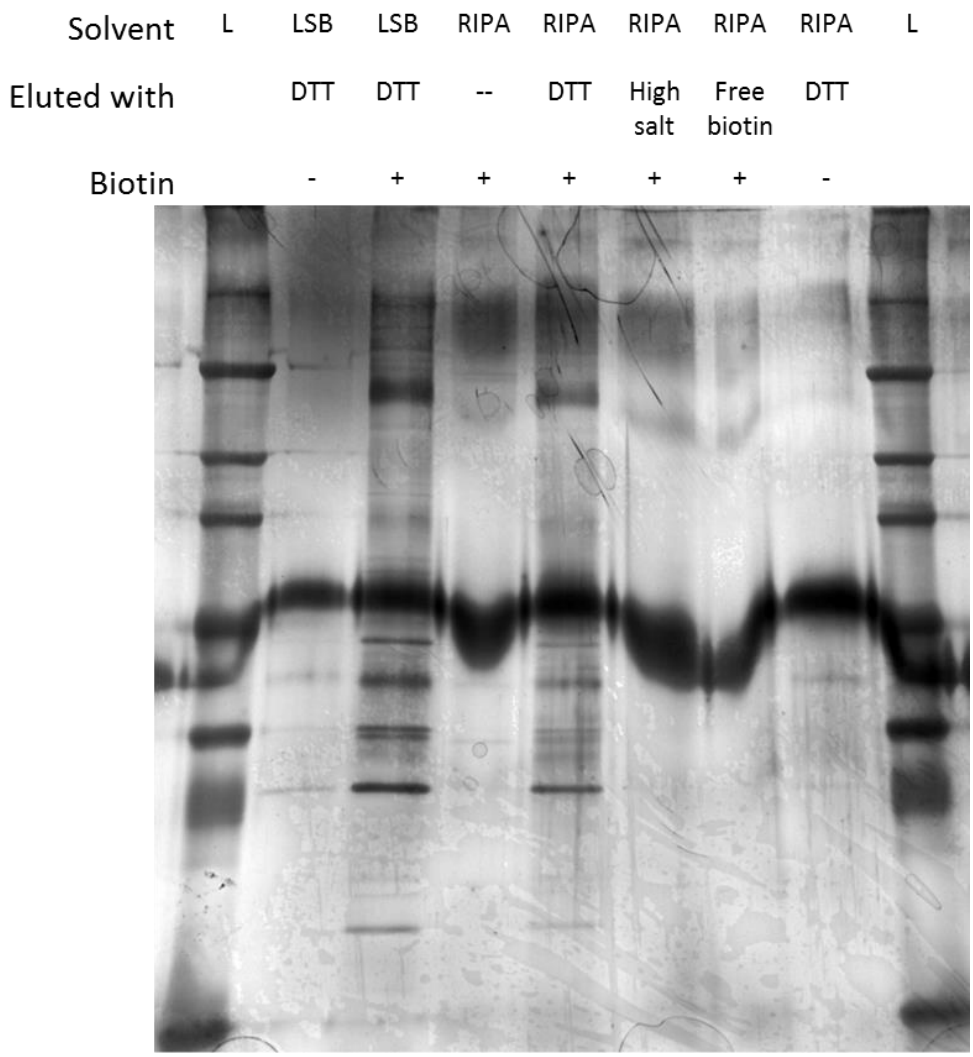


Figure 11 **Three different eluting agents were tested to determine the ideal conditions for maximal elution of biotinylated proteins with as little contamination as possible. Only DTT was successful in eluting biotinylated proteins from streptavidin conjugated sepharose beads. Neither high concentration of salt nor an excess of free biotin were successful in eluting the biotinylated proteins.**

AMPK is activated by chemical analogues of AMP

AMPK is the metabolic stress sensor of the cell and biologically is activated upon AMP binding its gamma subunit displacing ATP. To study the effects of AMPK activation and not of other processes that also may be affected by increased levels of AMP in the cell, I sought to determine if AMPK can be experimentally activated with the AMP analogues AICAR and A-769662 (**Figure 12**).

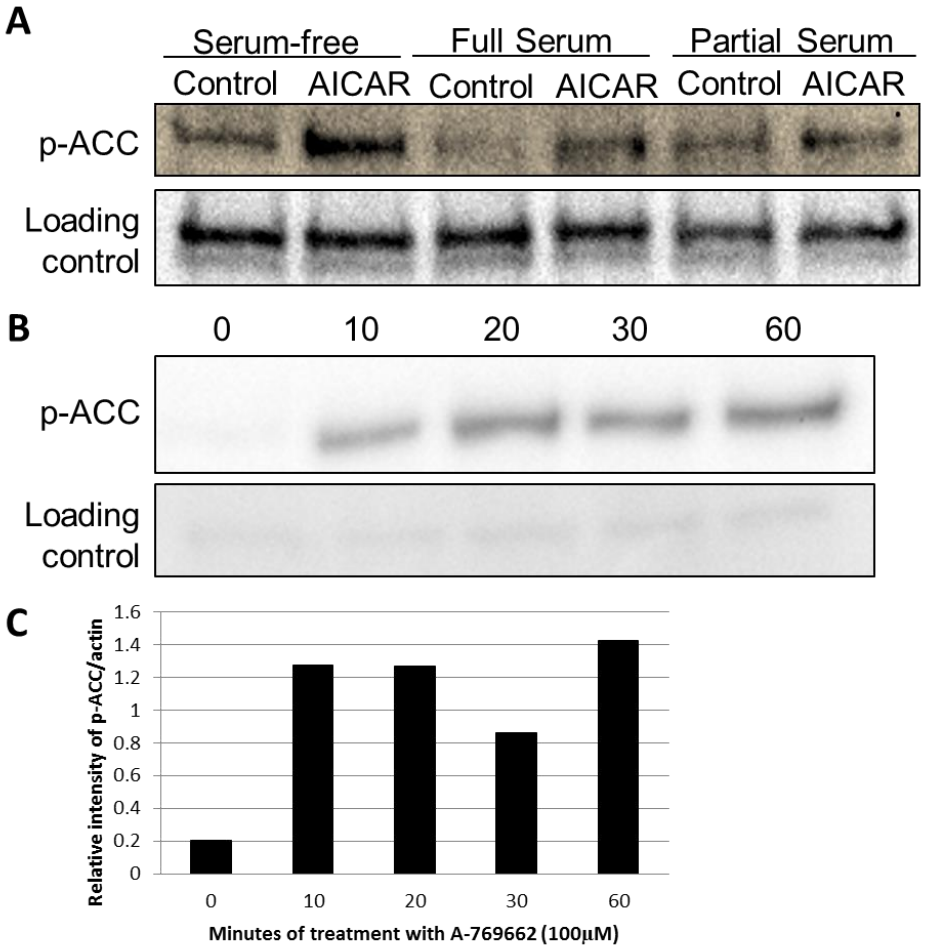


Figure 12 **A) A western blot for pACC shows the activation of AMPK in basal cells or cells treated with AICAR. Serum-free conditions lead to AMPK activation in untreated cells as expected in starvation conditions. AICAR treatment in full serum conditions lead to a repressed p-ACC signal. Partial serum AICAR treatment shows some p-ACC detection in control cells and a detectable increase in p-ACC after AICAR treatment. B) p-ACC detection over time with A-769662 treatment. AMPK is activated within 10 minutes of treatment and the p-ACC detection increases over time (quantification in C).**

Serum starvation is used extensively in biochemical experiments because it allows for observation of acute responses and eliminates the possibility of the growth factors from the serum having an effect on the proteins or pathways of interest. Serum starvation would be helpful for studying the effect of AMPK activation because too much serum could inhibit AMPK. However, AMPK is activated in response to metabolic stress and the complete absence of serum may result in high basal AMPK activation, with little ability of added AMP analogues to further activate AMPK. Conceptually, a low serum concentration is the ideal condition – enough serum to prevent high basal AMPK activation and not enough serum so that AMPK is inhibited.

To determine the best conditions for AMPK activation, I performed SDS-PAGE followed by a Western blot for p-ACC, an indirect measure of AMPK activation (**Figure 12**a). I performed the stimulation of cells with AICAR for 90 minutes in the absence of serum, with 10% serum, or with 0.1% serum. Basal activation of AMPK was highest when grown in serum-free media. Activation of AMPK was greater in full serum with AICAR stimulation compared to control. Partial serum was also an effective media solution for the experimental activation of AMPK (**Figure 12**a). I confirmed that low levels of serum would be ideal for future experiments focused on changes after AMPK activation.

I next sought to select a time point of AMPK activation for my cell-surface proteome analysis at which AMPK will have been active for a sufficient time to allow changes in cell-surface abundance of proteins to occur. To determine the time course of AMPK activation, I treated cells with A-769662 for 0, 10, 20, 30, or 60 minutes in partial serum and performed a western blot with an antibody recognizing p-ACC to measure AMPK activation indirectly (**Figure 12**b). AMPK activation reaches near maximum intensity after 10 minutes and remains

relatively consistent over 60 minutes (**Figure 12**c). Since AMPK is activated as early as 10 minutes following A-769662 treatment, performing the cell-surface purification assay at 90 minutes after treatment allows 80 minutes for changes in cell surface abundance to manifest themselves.

Regulation of the cell surface proteome by AMPK activation

The cell surface proteome is a dynamically regulated compartment of the cell highly involved in many important processes. AMPK is the metabolic stress sensor of the cell and its activation allows cells to respond to changes in their environment to maintain energetic homeostasis. To investigate the effect on the cell surface proteome upon AMPK activation I prepared samples of cell surface proteins using the cell-surface protein purification assay described above (n=4); samples were either 1) proteins eluted from beads after cells were not biotinylated (Background), 2) untreated cell-surface proteins (Control), or 3) cell surface proteins treated with 100µM A-769662 for 90 minutes (AMPK-activated). Once I eluted the cell surface proteins I subjected them to tryptic digestion and prepared them for mass spectrometry injection. Each sample was injected into the LC-ESI-MS/MS. Data for charge states 1, 2, 3, with a minimum peptide length of 4 were run against 4 search engine algorithms (Sequest, Tandem, Mascot, and Omssa) set for a maximum of 2 missed cleavages to identify proteins in each sample. I performed this experiment 4 times with 3 treatments each to make 12 total samples and MS runs. Approximately 30000 peptides and over 400 unique protein identifications were found.

While mass spectrometry is very sensitive and allows detection of proteins often at low abundance, it is possible that either identification of individual peptides/proteins may be

incorrect. To increase my confidence in the identification of cellular processes that are regulated by AMPK activation, I employed an unbiased clustering approach. This clustering allowed for identification of Gene Ontology (GO) classifications that are collectively up- or down-regulated at the cell surface by AMPK (**Figure 13**).

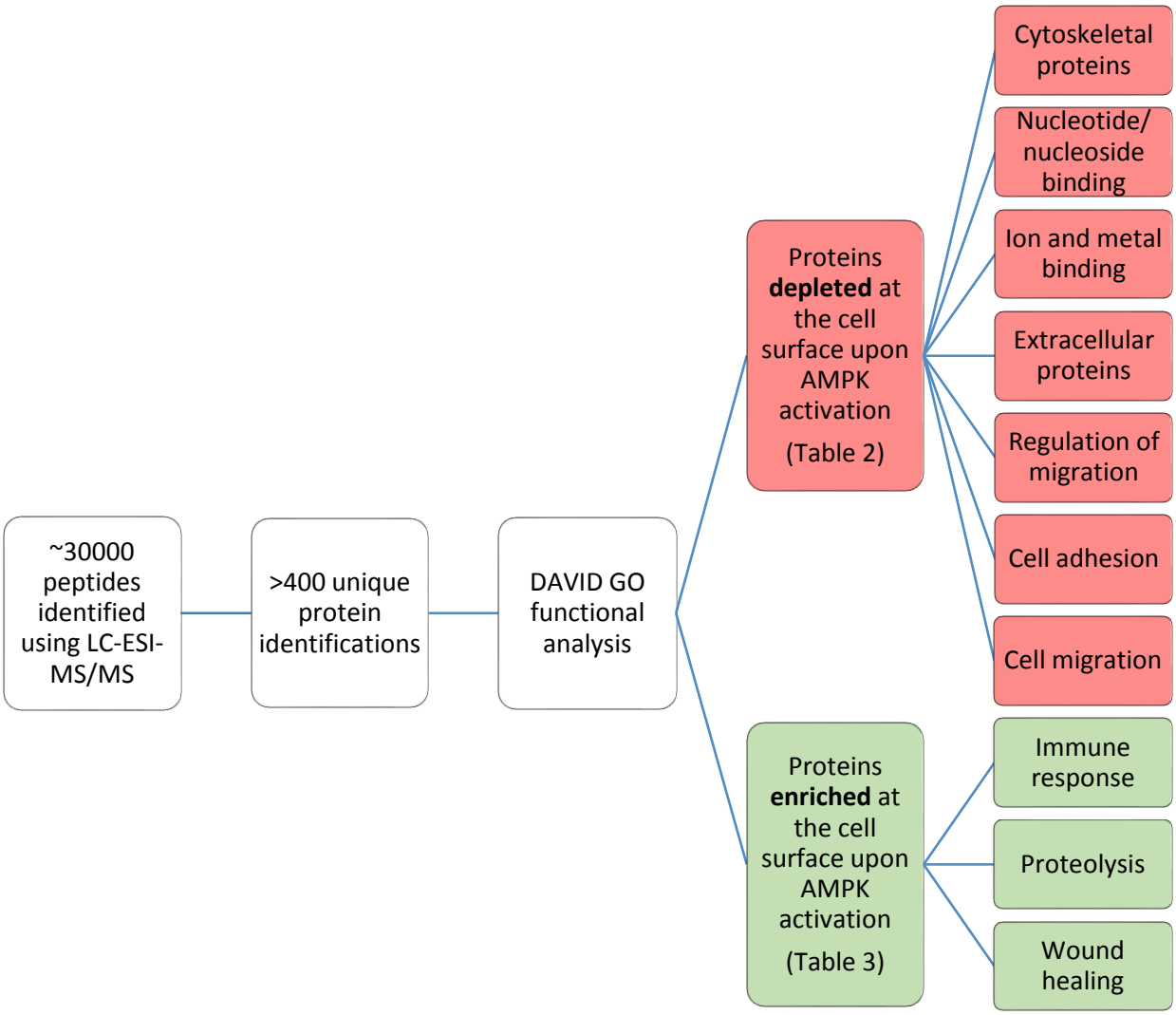


Figure 13 **I employed an unbiased clustering approach (D. W. Huang et al., 2009) to proteins identified from the cell-surface biotinylation mass spectrometry screen. This clustering allowed for identification of Gene Ontology (GO) classifications that are collectively up- or down-regulated at the cell surface by AMPK.**

The DAVID bioinformatics resource functional annotation clustering tool (version 6.7; see http://david.abcc.ncifcrf.gov/summary.jsp) measures relationships between GO annotation terms on the basis of the degree of their co-association with genes within the inputted list to create clusters of functional annotation groups (D. W. Huang et al., 2009). This allows for interpretation of MS results at the biological module level (D. W. Huang et al., 2009). It is advantageous to monitor how AMPK regulates biological modules instead of individual proteins, as it is possible that any single peptide or protein is identified incorrectly by the mass spectrometry analysis; the likelihood of large clusters of proteins of similar GO annotation being incorrectly identified is vanishingly small.

The DAVID GO analysis software provides functional annotation clusters and their enrichment scores, p-values, and Benjamini scores. Enrichment scores rank overall enrichment of annotation term groups and is the geometric mean of all the enrichment p-values of each annotation term in the cluster; Greater enrichment scores mean group members are playing more enriched roles in the given study or pathway and scores greater than or equal to 1.3 are considered of note (D. W. Huang et al., 2009). The Benjamini score is a testing correction technique used to globally correct enrichment p-values to control family-wide false discovery rates; Benjamini values that remain significant by the same standards set for p-values indicate a strong relationship (D. W. Huang et al., 2009).

I used the DAVID GO analysis software to cluster identified proteins into functional categories and found several groups of proteins that were depleted or enriched at the cell surface after AMPK-activation (**Figure 13**).

Using the GO functional analysis, I found the following functional clusters down-regulated at the cell surface by AMPK activation: cytoskeletal proteins, nucleotide/nucleoside

binding, ion and metal binding, extracellular proteins, and interestingly, proteins involved in regulation of migration, cell adhesion, and cell migration, which had enrichment scores of 6.76, 4.48, 3.48, 3.06, 2.71, 2.6, and 2.59 respectively (Table 2). The significant clusters enriched at the cell surface after AMPK activation were: immune response, proteolysis, and wound healing, which had enrichment scores of 3.06, 1.81, and 1.8, respectively (Table 3). Of note, cell adhesion and migration were found with a high degree of confidence (enrichment scores above 1.3) to be regulated by AMPK; 58 of the proteins that were identified are involved in cell adhesion and are regulated at the cell surface by AMPK activation (Table 4). 49 cell surface proteins involved in cell adhesion were down-regulated at the PM upon AMPK activation while 9 were enriched at the PM when AMPK was activated.

Table 2 DAVID GO functional clustering analysis results for proteins depleted at the cell-surface after AMPK activation.

Functional Annotation Clusters from David Database				
Proteins depleted at the cell-surface after AMPK activation		classification stringency: medium		
cytoskeletal proteins	**Enrichment Score: 6.76**	**Count**	**P_Value**	**Benjamini**
GOTERM_CC_FAT	cytoskeleton	165	2.3E-08	1.3E-05
GOTERM_CC_FAT	non-membrane-bounded organelle	271	1.5E-07	4.3E-05
GOTERM_CC_FAT	intracellular non-membrane-bounded organelle	271	1.5E-07	4.3E-05
nucleotide/nucleoside binding	**Enrichment Score: 4.48**	**Count**	**P_Value**	**Benjamini**
GOTERM_MF_FAT	adenyl nucleotide binding	190	2.3E-06	2.7E-03
GOTERM_MF_FAT	purine nucleoside binding	192	2.8E-06	1.6E-03
GOTERM_MF_FAT	nucleoside binding	193	2.9E-06	1.2E-03
GOTERM_MF_FAT	ATP binding	175	1.8E-05	5.3E-03
GOTERM_MF_FAT	adenyl ribonucleotide binding	175	3.8E-05	8.8E-03
GOTERM_MF_FAT	nucleotide binding	246	7.2E-05	1.4E-02
GOTERM_MF_FAT	purine nucleotide binding	214	8.3E-05	1.4E-02
GOTERM_MF_FAT	ribonucleotide binding	199	7.7E-04	7.3E-02
ion and metal binding proteins	**Enrichment Score: 3.48**	**Count**	**P_Value**	**Benjamini**
GOTERM_MF_FAT	ion binding	429	1.4E-04	2.0E-02
GOTERM_MF_FAT	cation binding	422	2.0E-04	2.6E-02
GOTERM_MF_FAT	metal ion binding	418	2.2E-04	2.5E-02
GOTERM_MF_FAT	transition metal ion binding	291	4.1E-04	4.3E-02
GOTERM_MF_FAT	zinc ion binding	241	1.6E-03	1.2E-01
extracellular proteins	**Enrichment Score: 3.06**	**Count**	**P_Value**	**Benjamini**
GOTERM_CC_FAT	extracellular region part	110	4.9E-05	7.0E-03
GOTERM_CC_FAT	extracellular space	75	3.2E-03	9.8E-02
GOTERM_CC_FAT	extracellular region	190	4.0E-03	1.1E-01
Regulation of migration	**Enrichment Score: 2.71**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	regulation of locomotion	33	1.8E-04	2.5E-02
GOTERM_BP_FAT	regulation of cell migration	30	2.1E-04	2.9E-02
GOTERM_BP_FAT	regulation of cell motion	32	4.4E-04	4.2E-02
GOTERM_BP_FAT	positive regulation of cell migration	17	3.3E-03	1.4E-01
GOTERM_BP_FAT	positive regulation of locomotion	18	3.6E-03	1.4E-01
GOTERM_BP_FAT	negative regulation of locomotion	13	4.8E-03	1.6E-01
GOTERM_BP_FAT	negative regulation of cell migration	12	7.9E-03	2.1E-01
GOTERM_BP_FAT	positive regulation of cell motion	17	8.5E-03	2.2E-01
Cell Adhesion	**Enrichment Score: 2.6**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	cell adhesion	87	3.2E-04	3.6E-02
GOTERM_BP_FAT	biological adhesion	87	3.4E-04	3.8E-02
GOTERM_BP_FAT	cell-cell adhesion	30	1.4E-01	7.5E-01
Cell migration	**Enrichment Score: 2.59**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	cell motility	43	1.5E-03	8.9E-02
GOTERM_BP_FAT	localization of cell	43	1.5E-03	8.9E-02
GOTERM_BP_FAT	cell migration	38	3.9E-03	1.5E-01
GOTERM_BP_FAT	cell motion	58	5.2E-03	1.7E-01

Table 3 DAVID GO functional clustering analysis results for proteins enriched at the cell-surface after AMPK activation.

Functional Annotation Clusters from David Database				
Proteins enriched at the cell-surface after AMPK activation		classification stringency: medium		
Immune response	**Enrichment Score: 3.06**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	acute inflammatory response	10	5.6E-07	8.1E-04
GOTERM_BP_FAT	activation of plasma proteins involved in acute inflammatory response	7	4.0E-06	2.9E-03
GOTERM_BP_FAT	complement activation, classical pathway	6	9.0E-06	4.4E-03
GOTERM_BP_FAT	humoral immune response mediated by circulating immunoglobulin	6	1.3E-05	4.6E-03
GOTERM_BP_FAT	positive regulation of response to stimulus	12	2.4E-05	7.0E-03
GOTERM_BP_FAT	activation of immune response	8	4.2E-05	1.0E-02
GOTERM_BP_FAT	immune effector process	9	5.8E-05	1.2E-02
GOTERM_BP_FAT	complement activation	6	5.8E-05	1.1E-02
GOTERM_BP_FAT	protein processing	8	1.3E-04	2.1E-02
GOTERM_BP_FAT	humoral immune response	7	1.4E-04	2.0E-02
GOTERM_BP_FAT	immunoglobulin mediated immune response	6	2.0E-04	2.6E-02
GOTERM_BP_FAT	protein maturation by peptide bond cleavage	7	2.2E-04	2.6E-02
GOTERM_BP_FAT	leukocyte mediated immunity	7	2.2E-04	2.6E-02
GOTERM_BP_FAT	protein maturation	8	2.2E-04	2.4E-02
GOTERM_BP_FAT	B cell mediated immunity	6	2.3E-04	2.4E-02
GOTERM_BP_FAT	complement activation, alternative pathway	4	4.1E-04	3.9E-02
GOTERM_BP_FAT	positive regulation of immune response	8	6.2E-04	5.5E-02
GOTERM_BP_FAT	lymphocyte mediated immunity	6	6.6E-04	5.5E-02
GOTERM_BP_FAT	response to wounding	15	8.3E-04	6.2E-02
GOTERM_BP_FAT	adaptive immune response	6	1.0E-03	7.2E-02
GOTERM_BP_FAT	somatic recombination of immune receptors	6	1.0E-03	7.2E-02
GOTERM_BP_FAT	inflammatory response	11	1.6E-03	1.0E-01
GOTERM_CC_FAT	membrane attack complex	3	1.6E-03	1.9E-01
GOTERM_BP_FAT	innate immune response	7	2.6E-03	1.5E-01
GOTERM_BP_FAT	positive regulation of immune system process	9	2.7E-03	1.5E-01
GOTERM_BP_FAT	cytolysis	3	1.8E-02	5.0E-01
GOTERM_CC_FAT	extracellular space	13	1.9E-02	4.0E-01
GOTERM_CC_FAT	extracellular region part	16	2.3E-02	3.6E-01
GOTERM_BP_FAT	immune response	13	4.3E-02	6.8E-01
GOTERM_BP_FAT	defense response	12	4.4E-02	6.8E-01
Proteolysis	**Enrichment Score: 1.81**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	proteolysis	23	7.2E-04	5.7E-02
GOTERM_MF_FAT	exopeptidase activity	5	6.6E-03	5.1E-01
GOTERM_MF_FAT	peptidase activity	13	9.9E-03	5.5E-01
GOTERM_MF_FAT	peptidase activity, acting on L-amino acid peptides	12	1.8E-02	6.2E-01
GOTERM_MF_FAT	carboxypeptidase activity	3	5.0E-02	6.5E-01
GOTERM_MF_FAT	metalloexopeptidase activity	3	5.2E-02	6.2E-01
GOTERM_MF_FAT	metallopeptidase activity	5	1.0E-01	7.4E-01
Wound healing	**Enrichment Score: 1.8**	**Count**	**P_Value**	**Benjamini**
GOTERM_BP_FAT	response to wounding	15	8.3E-04	6.2E-02
GOTERM_BP_FAT	activation of caspase activity	5	2.0E-03	1.3E-01
GOTERM_BP_FAT	positive regulation of peptidase activity	5	2.8E-03	1.5E-01
GOTERM_BP_FAT	positive regulation of caspase activity	5	2.8E-03	1.5E-01
GOTERM_BP_FAT	coagulation	6	3.5E-03	1.8E-01
GOTERM_BP_FAT	hemostasis	6	4.5E-03	2.2E-01
GOTERM_BP_FAT	regulation of caspase activity	5	8.0E-03	3.3E-01
GOTERM_BP_FAT	regulation of endopeptidase activity	5	9.1E-03	3.6E-01
GOTERM_BP_FAT	wound healing	7	1.2E-02	4.3E-01
GOTERM_BP_FAT	regulation of body fluid levels	6	1.3E-02	4.3E-01
GOTERM_BP_FAT	positive regulation of hydrolase activity	6	3.4E-02	6.3E-01
GOTERM_BP_FAT	cell death	13	5.6E-02	7.0E-01
GOTERM_BP_FAT	death	13	5.8E-02	7.0E-01
GOTERM_BP_FAT	regulation of hydrolase activity	7	1.2E-01	8.1E-01
GOTERM_BP_FAT	apoptosis	10	1.5E-01	8.5E-01
GOTERM_BP_FAT	positive regulation of catalytic activity	9	1.5E-01	8.5E-01
GOTERM_BP_FAT	programmed cell death	10	1.6E-01	8.6E-01

Table 4 Proteins involved in cell adhesion regulated at the cell surface by AMPK activation. The majority of cell surface adhesion proteins were found to be depleted at the cell surface after AMPK activation.

Depleted at the Cell-Surface after AMPK Activation	Depleted at the Cell-Surface after AMPK Activation	Elevated at the Cell-Surface after AMPK Activation
ADAM metallopeptidase with thrombospondin type 1 motif, 13	lysyl oxidase-like 2	D4 molecule
FAT tumor suppressor homolog 1 (Drosophila)	mucin 16, cell surface associated	PDZ domain containing 2
FAT tumor suppressor homolog 2 (Drosophila)	mucin 5AC, oligomeric mucus/gel-forming; similar to hCG1778310	cadherin 4, type 1, R-cadherin (retinal)
FAT tumor suppressor homolog 4 (Drosophila)	myosin binding protein C, cardiac	collagen, type XI, alpha 1
LY6/PLAUR domain containing 3	neural cell adhesion molecule 1	collagen, type XXII, alpha 1
NEL-like 1 (chicken)	neural cell adhesion molecule 2	contactin associated protein-like 3; contactin associated protein-like 3B
NEL-like 2 (chicken)	neurexin 1	
SCO-spondin homolog (Bos taurus)	neurexin 2	discoidin domain receptor tyrosine kinase 2
SRY (sex determining region Y)-box 9	neuropilin 1	similar to Afadin (Protein AF-6); myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4
amyloid beta (A4) precursor protein-binding, member 1	nidogen 2 (osteonidogen)	
bone morphogenetic protein 1	poliovirus receptor-related 3	
catenin (cadherin-associated protein), alpha-like 1	polycystic kidney and hepatic disease 1 (autosomal recessive)	tuberous sclerosis 1
catenin (cadherin-associated protein), delta 1	protein tyrosine phosphatase, receptor type, F	
chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	protein tyrosine phosphatase, receptor type, M	
collagen, type VI, alpha 3	protein tyrosine phosphatase, receptor type, S	
collagen, type XI, alpha 2	protocadherin 19	
collagen, type XIV, alpha 1	protocadherin 7	
discs, large homolog 1 (Drosophila)	protocadherin beta 10; protocadherin beta 9	
fer (fps/fes related) tyrosine kinase	protocadherin gamma subfamily B, 3	
fermitin family homolog 3 (Drosophila)	sema domain, seven thrombospondin repeats (type 1 and type 1-like), (semaphorin) 5A	
fibronectin type III domain containing 3A	sialic acid binding Ig-like lectin 7	
integrin, alpha 11	sorbin and SH3 domain containing 1	
integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	spleen tyrosine kinase	
intercellular adhesion molecule 5, telencephalin	sushi, nidogen and EGF-like domains 1	
trophinin	thrombospondin 2	

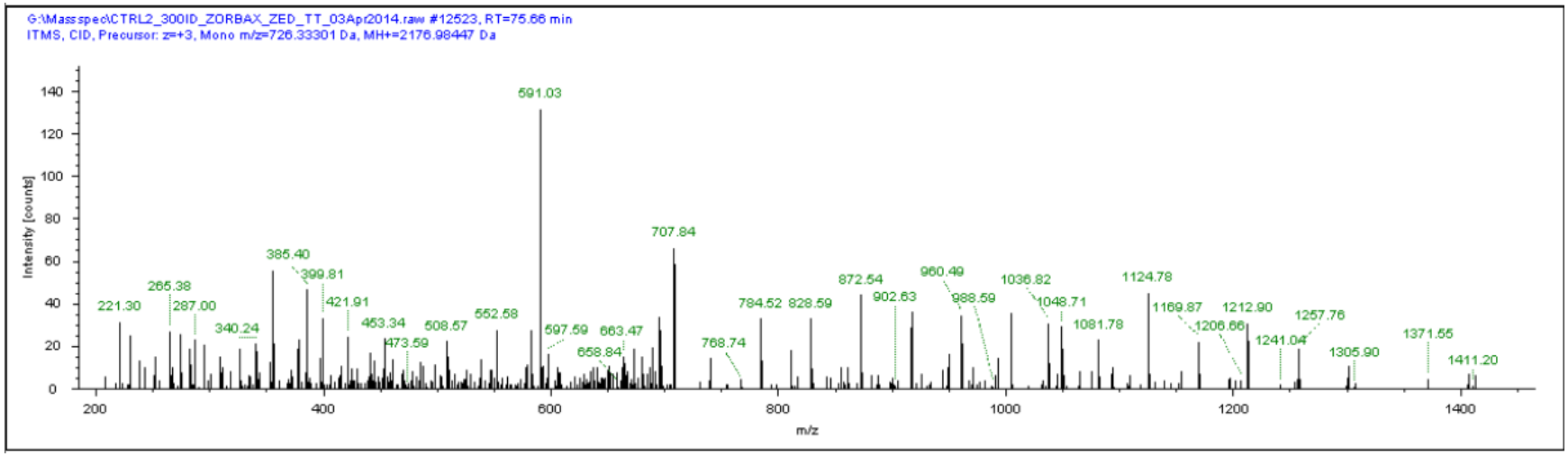


Figure 14 **Spectrum of integrin alpha 11 as detected by LC-ESI-MS/MS.**

Integrins are central players for cell adhesion and cell migration. Notably, Integrin α4 and Integrin α11 were two of the proteins involved that were down-regulated at the cell surface by AMPK activation. Shown in **Figure 14** is a sample spectrum of integrin α11, demonstrating the suitable signal-to-noise for detection of peptide fragments for this protein.

In summary thus far, I have developed a method that allows systematic identification of cell surface proteins by selective purification and mass spectrometry. I have identified several functional categories of proteins regulated at the cell surface by AMPK activation. Particularly, I identified cell adhesion and migration as processes regulated by AMPK activation. The next question is whether we can confirm that cell adhesion proteins such as integrins are indeed regulated at the level of cell surface abundance by AMPK.

Results Part II: The specific regulation of β1-integrin membrane traffic by AMPK

β1-integrin cell surface content is reduced upon AMPK activation

Mass spectrometry experiments and functional clustering analysis revealed a connection between AMPK activation and changes in cellular adhesion and migration. β1-integrin is the major binding partner for integrins α4 and α11 (identified in the screen) and for other alpha integrins not identified in the screen but known binding partners. β1-integrin is, therefore, a candidate target protein to be regulated by AMPK upon energetic stress.

In order to determine if AMPK activation regulates β1-integrin on the cell surface, as was found in the mass spectrometry analysis with the α4- and α11-integrins, I fluorescently labeled cell surface β1-integrin in intact control and AMPK-activated cells with an antibody that recognizes an exofacial epitope on β1-integrin (**Figure 15**a). Notably, this antibody does not complete with binding to ECM ligands and does not discriminate between active and inactive integrin, and as such as an optimal reporter of total cell surface β1-integrin levels (Jimenez-Soto et al., 2009). In cells treated with A-769662 or AICAR there was less β1-integrin on the cell surface compared to untreated cells (**Figure 15**b).

To confirm that cell surface β1-integrin levels are indeed regulated by AMPK activation by a third method, I performed the cell surface protein purification assay characterized above and performed SDS-PAGE with the cell surface proteins (pull down) and with the intracellular proteins (supernatant), followed by a western blot with an antibody against β1-integrin. Cells treated with AICAR had decreased β1-integrin on the cell surface and increased intracellular β1-integrin compared to untreated cells (**Figure 15**c). Cells treated with EGF as a negative control,

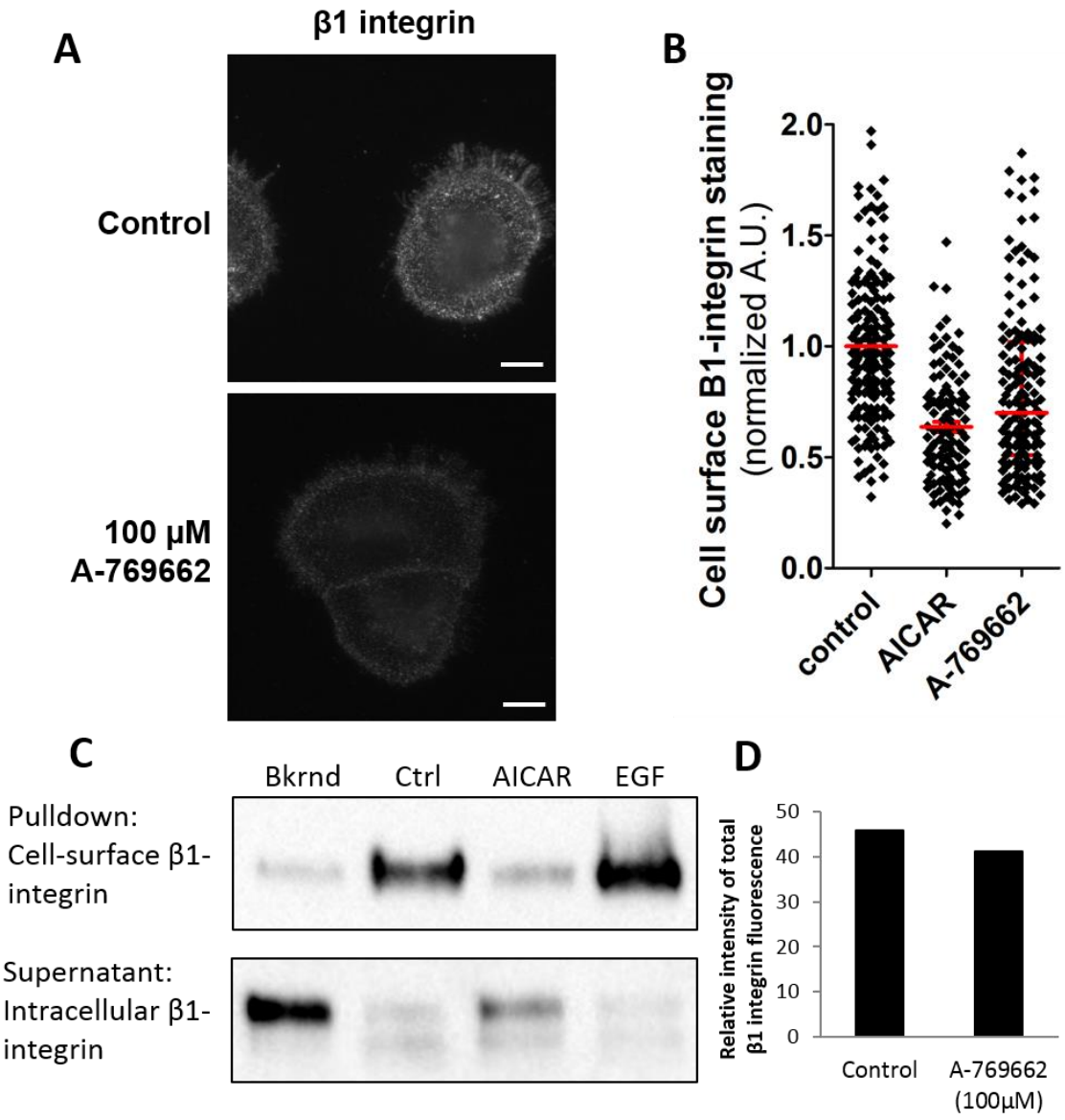


Figure 15 **β1-integrin cell surface content is reduced upon AMPK activation. A) Micrographs of cells either treated with 100µM A-769772 or untreated, labelled with an antibody specific to an ectopic β1-integrin residue (scale bars = 5 µm; Quantification in B). C) Western blot with an antibody specific to β1-integrin. Protein in the pull-down fraction is cell-surface β1-integrin, whereas protein in the supernatant reflects intracellular β1-integrin. AMPK activation causes a decrease in β1-integrin at the cell surface and an increase in intracellular β1-integrin. D) Total levels of β1-integrin do not change when AMPK is activated by A-769662.**

showed no decrease in cell surface β1-integrin, showing the specificity of regulation of cell surface β1-integrin by AMPK activation.

A reduction in cell surface β1-integrin could be due to translocation of cell surface β1-integrin to intracellular membrane compartment(s), or degradation of β1-integrin. To determine if AMPK activation effected a reduction in total levels of β1-integrin, I fluorescently labelled all the β1-integrin in permeabilized cells treated with A-769662 and in untreated cells. There was no change in total β1-integrin levels in cells with activated AMPK (**Figure 15**d). Hence, the abundance of β1-integrin is regulated at the cell surface by AMPK activation mainly through directing membrane traffic to intracellular membrane compartments that do not result in the degradation of β1-integrin in the timescale of this experiment (90 min). Whether other cell surface proteins were regulated by AMPK or if this effect was specific to the cell adhesion receptor, β1-integrin, remained to be determined.

Transferrin receptor is not regulated at the cell surface by AMPK activation

To investigate the regulation of other cell surface proteins by AMPK activation, I fluorescently labeled cell surface TfR in intact control and AMPK-activated cells using an antibody to transferrin receptor that recognizes an exofacial epitope (**Figure 16**a). There was no difference in TfR cell-surface content between control cells and those treated with A-769662 (**Figure 16**b). Unlike β1-integrin, TfR is not regulated at the cell surface by AMPK activation.

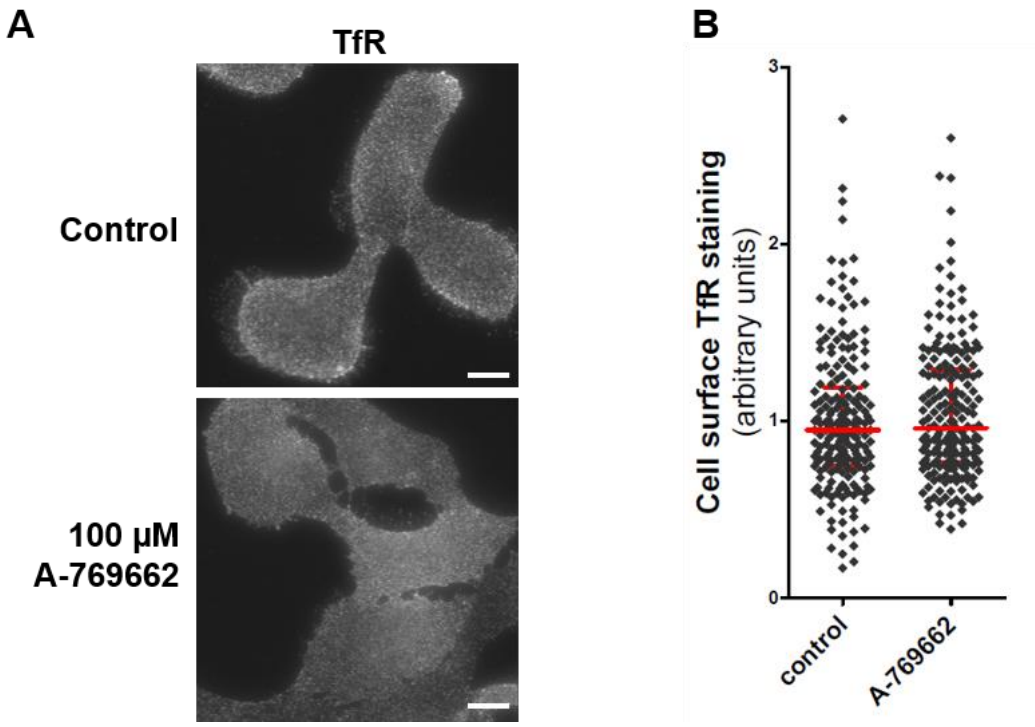


Figure 16 **AMPK activation does not regulate TfR abundance at the cell surface. A) Micrographs of RPE cells fluorescently labeled with an antibody recognizing an exofacial epitope on TfR. Cells were untreated or treated with 100µM A-769662 for 90 minutes. TfR abundance does not change at the cell surface when AMPK is activated (Scale bars = 5 µm; Quantification in B).**

Hence, although β1-integrin and TfR both internalize primarily through CME followed by intracellular traffic through early and recycling endosomes, the mechanism that effects regulation of β1-integrin translocation to the cell interior (e.g. increased endocytosis, decreased recycling or both) is specific and does not control TfR endomembrane traffic.

AMPK activation alters assembly of Dab2-contating CCPs

CME is the principle route of internalization of membrane proteins. CCPs are diffraction limited puncta and Dab2 is a CLASP that is required for the CME of β1-integrin but not that of TfR. Does AMPK regulate β1-integrin on the cell surface through manipulation of Dab2

recruitment to CCPs? I examined the relationship between Dab2 recruitment to CCPs and AMPK activation using dual-colour TIRF microscopy imaging of RPE cells expressing eGFP-CLCa and mCherry-Dab2 (**Figure 17**a). Diffraction-limited GFP-CLCa puncta were detected by a Gaussian model approach as per *Aguet et al. 2013. Dev. Cell. 26:279-91*. At each clathrin punctum, the intensity of GFP-CLCa and of mCherry-Dab2 was measured and 2D histograms were created of the GFP-CLC versus mCherry-Dab2 fluorescence intensities (mean values per cell) (**Figure 17**b). The frequency value of each bin between the control and A-769662 treated conditions were also plotted (**Figure 17**c). After 60 minutes of A-769662 treatment, Dab2 is recruited to smaller CCPs than in untreated cells. Therefore, AMPK activation alters assembly of Dab2-containing CCPs.

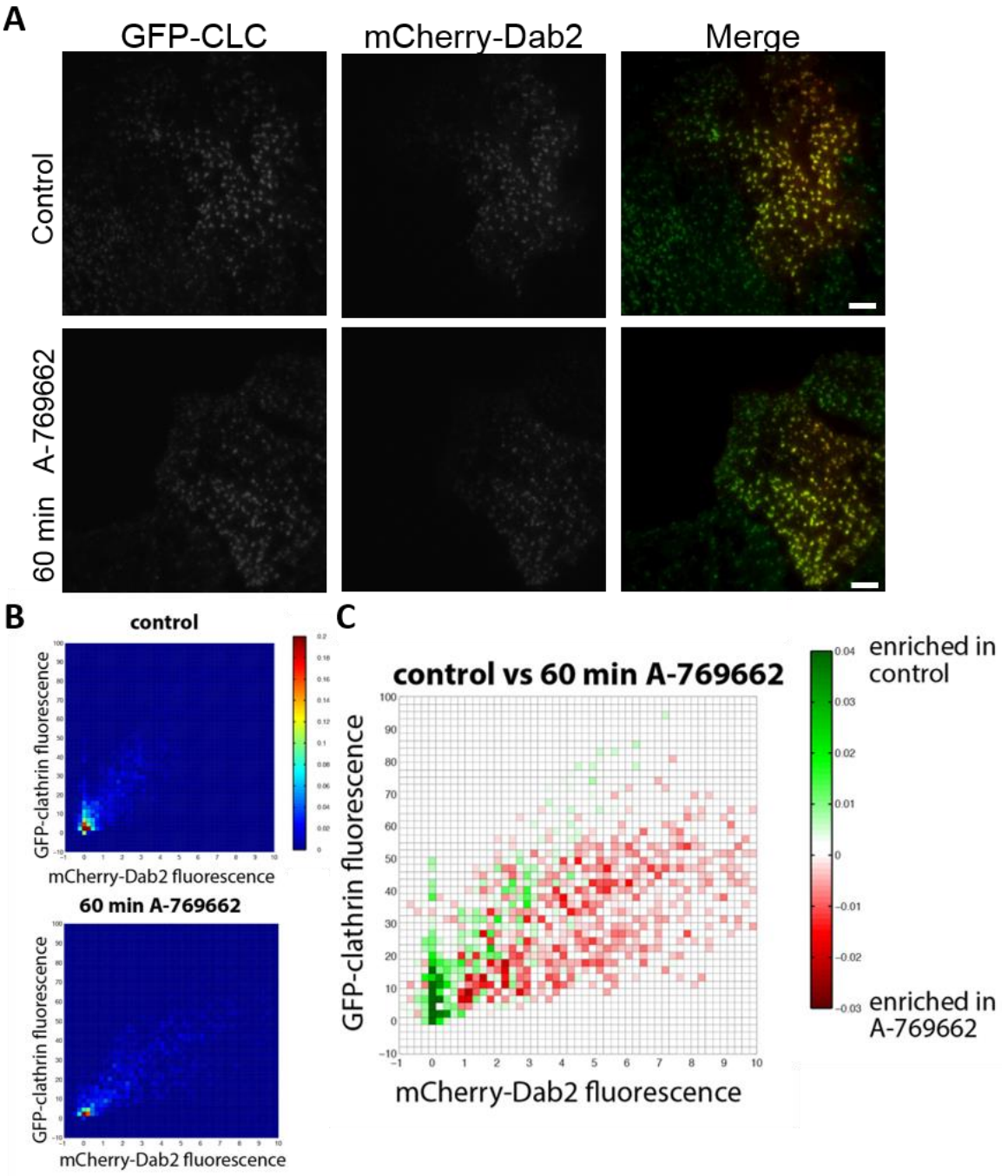


Figure 17 **RPE cells stably expressing GRP fused to clathrin light chain (GFP-CLC) and transfected with mCherry-Dab2 were treated with 100µM A-769662 for 60 minutes or left untreated. A) Shown are representative micrographs obtained by total internal reflection fluorescence microscopy. Scale 5 µm. B) Diffraction-limited GFP-CLC puncta were detected by a Gaussian model approach, as described in Aguet et al. 2013. Dev. Cell. 26:279-91. Shown are 2D histograms of the GFP-CLC versus mCherry-Dab2 fluorescence intensity (mean values per cell). C) Also shown is the difference in the frequency value of each bin between the control and A-769662 treated conditions.**

Discussion

Understanding how metabolic signaling regulates the cell surface proteome provides important information on mechanisms of membrane traffic and on how cells adapt to changing environments. In mammalian cells, nutrient availability fluctuates at the cellular and tissue level with diet, blood flow, and exercise (Gowans et al., 2013). In addition, nutrient availability also fluctuates at the subcellular level, as has been shown to occur at presynaptic terminus of neurons that undergo ATP stress during synaptic transmission (Rangaraju, Calloway, & Ryan, 2014). Thus, both rare, dramatic depletion ATP events as well as more frequent, smaller fluctuations of ATP concentration occur in a wide variety of cell types under normal physiological conditions. How these fluctuations in ATP levels controls cell physiology has remained poorly understood.

AMPK is known as the "metabolic stress sensor of the cell" because it recognizes energy limited environments and its activation leads to increasing energy supply and reducing anabolism (Chen et al., 1999). Many targets of AMPK kinase activity are known and well characterised such as the translocation of GLUT-1 (Wu et al., 2013) and GLUT-4 (Klip, Schertzer, Bilan, Thong, & Antonescu, 2009) to the PM upon AMPK activation to allow for increased glucose uptake in energy-limited environments. Active AMPK also phosphorylates ACC, thereby inactivating it and inhibiting fatty acid synthesis and promoting fatty acid oxidation. The cell surface is a dynamic compartment of the cell and the profile of proteins on the surface of a cell can be remodelled to adapt to different cellular environments.

In this present study, I identified a novel regulation of the cell-surface proteome by AMPK activation. I demonstrated that several functional groups of proteins are either up- or down-regulated in abundance at the plasma membrane as a result of AMPK activation. Of

primary interest was the discovery that proteins involved in cell adhesion are significantly down-regulated in their abundance at the PM upon AMPK activation. I confirmed the regulation of adhesion molecule membrane traffic by AMPK activation by focusing my investigation on β1-integrin and its changes in abundance at the cell surface. I showed that upon AMPK activation, β1-integrin cell surface levels are decreased and that this regulation correlates with alterations in the assembly of clathrin-coated pits containing Dab2, endocytic structures that specialize in the endocytosis of a subset of cell surface proteins which include β1-integrin (Teckchandani et al., 2012).

Regulation of the cell surface proteome by AMPK

My AMPK-regulated cell-surface protein screen identified cytoskeletal proteins as a functional group regulated by AMPK. From **Figure 10**b, we can say that any proteins in the "cell-surface" elution are true cell-surface proteins or proteins with tight interactions with cell-surface proteins. Therefore, identifying cytoskeletal proteins in the screen tells us a number of things. First, this identification indicates that AMPK may be regulating either the cytoskeletal proteins identified or, importantly, the cell-surface proteins to which they bind. Integrins are a perfect example of proteins that this identification may point to – they are cell surface proteins that bind ECM and cytoskeletal proteins to form focal contacts and adhesions.

The identification of regulation of cytoskeletal proteins by AMPK also supports evidence that AMPK regulates cell polarity and dynamics (Nakano & Takashima, 2012; Nakano et al., 2010) and that AMPK induces cytoskeleton reorganization in epithelial cells (Miranda et al., 2010). Establishing polarity is an integral process for several important events such as

development. Mice that do not express the AMPK α1 and α2 subunits are early embryonic lethal (Viollet et al., 2009). Nakano *et al* (2010) identified a cytoplasmic linker protein, CLIP-170, in an *in vitro* screen for AMPK substrates. CLIP-170 is a microtubule plus end protein that binds endocytic vesicles to microtubules (Nakano et al., 2010). AMPK regulation results in alteration of CLIP-170 dynamics on the plus ends of microtubules (Nakano et al., 2010). An interesting phenotype of AMPK depletion that was shown in these studies was a marked enhancement of microtubule stabilization in migrating cells and that AMPK depletion impaired the polarized stabilization of microtubules (Nakano et al., 2010). AMPK has also been shown to induce actin cytoskeleton remodeling in epithelial cells particularly uncoupling from paxillin, predominance of cortical f-actin, and shortening or radiation of stress fibres induced upon AMPK activation (Miranda et al., 2010).

Analysis of changes in the cell surface proteome

To identify proteins regulated in abundance at the cell surface after AMPK activation, I found the ratio of proteins found in control samples to proteins found in samples treated with A-769662 (AMPK-activated) and set the following cut-offs: Control/AMPK-activated total protein identifications ≥2 = Proteins depleted at the cell surface after AMPK activation; Control/AMPK-activated total protein identifications ≤ 0.5 = Proteins enriched at the cell surface after AMPK activation. This threshold was arbitrary and served as a first approach to identifying changes in abundance of cell surface proteins that is perhaps only suitable to examine changes in clusters of proteins with similar GO annotations, and not of individual proteins.

Statistical analyses have been described that will allow systematic and unbiased comparison of protein levels in Control versus AMPK-activated conditions based on the number of peptides detected in each condition (Jankowski, Zhu, & Marshall, 2008). This will be the work of future students and researchers that will use the dataset that I have found for further analyses of changes in the cell-surface proteome as a response to metabolic stress.

There exist several other methods that allow quantification of protein levels between samples including multidimensional protein identification technology (MudPIT) and stable isotope labeling by amino acids in cell culture (SILAC) (S.-E. Ong, 2002; Shao-En Ong, Foster, & Mann, 2003; Shao-En Ong & Mann, 2006; Washburn, Wolters, & Yates, 2001). The disadvantages of these methods are that each requires either specialized instrumentation, specialized proprietary analysis software and/or growing cells in isotope-specific media (for SILAC) which is labour and resource intensive (Shao-En Ong et al., 2003; Shao-En Ong, 2012; Washburn et al., 2001).

The mass spectrometry approach that I enlisted was indeed successful because I identified a GO annotation cluster (cell adhesion and migration) that changes with AMPK activation and I was able to validate this regulation with two other independent methods. Hence, while there are other possibilities to expand upon my approach in the future, my body of work has successfully identified novel candidate cell surface proteins and functions that are regulated by AMPK.

Coverage of the cell surface proteome by lysine-reactive biotinylation purification strategy

At the beginning of this study I expected to find certain proteins in the screen to use as positive controls. For example, I expected to find GLUT-1 enriched in control cells versus A-769662-treated cells because of previous research showing increased GLUT-1 abundance at the PM after AMPK activation (Wu et al., 2013). Considering that it is a 12-membrane pass protein (Salas-Burgos, Iserovich, Zuniga, Vera, & Fischbarg, 2004), it is not surprising that GLUT-1 was not present in my screen. I used RIPA buffer in my homogenization process instead of Laemmli sample buffer because it is better for the solubilisation of membrane proteins however proteins with many membrane spanning domains are notoriously difficult to solubilize (Seddon, Curnow, & Booth, 2004). Hence, while the method that I employed for purification of membrane proteins allowed the identification of the presence and regulation of many integral membrane proteins, this method did not allow detection of all membrane proteins.

Another possible reason why certain cell-surface proteins were not detected is that the reagent used to label cell surface proteins was amine-reactive and therefore, detection of proteins relies on accessible extracellular amines. Furthermore, preparation of mass spectrometry samples involved tryptic digestion and trypsin recognizes positively charged residues and cleaves peptide chains at the carboxyl side of lysine or arginine. Labeling of cell-surface proteins with the amine reactive sulfo-NHS-SS-biotin effectively masks the positive charge on lysines thereby limiting tryptic cleavages sites. Proteins such as β1-integrin that have a very large extracellular domain and a very small intracellular domain would have few available tryptic cleavage sites making that protein difficult to detect peptides of this protein by mass spectrometry. Alternate labelling reagents (such as EZ-Link Hydrazide-PEG4-Biotin from Pierce) that bind to carbohydrates are

available and can be utilized to create another screen to complement the one I described today. The list of proteins identified by my cell-surface proteome assay is not a complete proteome, however the changes that I detected using consistent labeling methods allowed for identification of processes regulated by AMPK. In this study I focused on the regulation of adhesion molecule membrane traffic by AMPK but many other interesting results may come from exploring the other identified functional groups of proteins identified.

Benefits of LC-ESI-MS/MS in the quantification of peptide identifications

There are many analytical tools available for the identification of peptides and in this study I chose to analyze my tryptic peptides using LC-ESI-MS/MS. The three main events during MS analysis are ion production, ion transmission, and ion detection (Bakhtiar & Tse, 2000). Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are complementary ionization approaches that are both sensitive and allow observation of intact polymers with a molecular weight of 100000 Da or higher (Bakhtiar & Tse, 2000). MALDI uses pulses of laser light to desorb the analyte from a solid phase surface and yield gaseous ions (Bakhtiar & Tse, 2000). ESI produces gaseous ions that are singly or multiply charged directly from solution by creating a fine spray of highly charged droplets in the presence of a strong electric field (Bakhtiar & Tse, 2000). ESI ionized molecules from a liquid state whereas MALDI ionizes from a solid state.

Liquid chromatography followed by tandem mass spectrometry has been shown to be more sensitive compared to solid-substrate based methods for protein identification and may detect hundreds of protein in a single experiment (Jankowski et al., 2008; J. Marshall et al., 2004). I

chose to use ESI for my samples so they could be ionized after liquid chromatography and analysed by tandem mass spectrometry. Another alternative to ESI is nanospray ionization. I did not use nanospray ionization because the distribution of m/z detected does not follow a normal distribution and is, therefore, much more challenging to quantify (J. Marshall, personal communication). I based my LC-ESI-MS/MS protocols off of past studies that have successfully quantified changes in peptide abundances using this method (Bowden, Beavis, & Marshall, 2009; Jankowski et al., 2008; J. Marshall et al., 2004).

My MS analysis identified over 30000 peptides with greater than 400 unique protein identifications (**Figure 13**). Many of the proteins identified were indeed cell-surface proteins, however I identified a subset of proteins that were putative transcription factors, nuclear pore proteins, and other nuclear contaminants (Appendices 1 and 2). If future MS analyses are required to confirm findings from this study, some strategies to eliminate nuclear contaminations include the addition of calcium and magnesium chelators as well as the addition of RNAse and DNAse crystals to the homogenization buffer.

Although the list of proteins identified through my cell-surface protein assay and LC-ESI-MS/MS is not an exhaustive one, it will provide many avenues for further research. I pursued the regulation of cell adhesion and migration by AMPK but there are many other functional clusters that I have identified to be regulated at the PM by AMPK that can be explored.

Regulation of migration by AMPK activation

Epithelial cell migration is a critical cellular process involved in development, tissue homeostasis, and wound healing. Emerging evidence suggests that cellular energy stress is a key

regulator of cell adhesion and migration. Activation of AMPK reduces cell adhesion in endothelial progenitor cells (Kaiser et al., 2012), and reduces cell migration in vascular smooth muscle cells (Liang et al., 2008), U937 monocytes (Kanellis et al., 2006), and glioblastoma cells (Ferla et al., 2012). My data support the role of AMPK in regulating adhesion and migration and provide a novel understanding of the mechanism of this regulation. I showed that AMPK regulates cell-surface levels of proteins involved in adhesion and migration and that AMPK activation results in altered assembly of Dab2-containing clathrin coated pits. How AMPK regulates β1-integrin levels at the cell surface remains to be uncovered.

β1-integrin is present in many integrin complexes and is a critical adhesion molecule involved in multiple cellular processes of adhesion, migration, intracellular signalling, angiogenesis, and invasion. Integrins are regulated by substrate binding and by intracellular signaling, leading to regulation of integrin conformation, and/or membrane traffic (P. T. Caswell et al., 2009). Dab2 is an adaptor protein involved in β1-integrin endocytosis. AMPK is emerging as a regulator of membrane traffic (Antonescu et al., 2014) and this present study provides some clues as to how AMPK controls the cell surface proteome. In this study I observed a change in the assembly of Dab2-containing CCPs as a result of AMPK activation. The observed regulation of β1-integrin at the cell surface may be a result of changed Dab2-dependent CME.

Regulation of β1-integrin membrane traffic by AMPK

AMPK is a kinase that phosphorylates sites that have at least one basic side chain (R, H, or K) either 3 or 4 residues N-terminal with respect to the serine or threonine residue phosphorylated. It also requires hydrophobic side chains 4 or 5 residues N-terminal of the

residue to be phosphorylated (D. G. Hardie, 2011). There are some proteins phosphorylated by AMPK that do not exactly abide by these patterns however AMPK's substrate recognition motifs are very well-defined compared to most protein kinases studied to date (D. G. Hardie, 2011). It is not currently known whether Dab2 is directly phosphorylated by AMPK or not but predictions based on consensus sequence searches indicate that it is not a direct downstream target for the kinase. Dab2 is, however, known to be phosphorylated by a number of kinases including the cyclin-dependent kinase CDC, and protein kinase C (PKC) (He, Xu, Xu, & Hall, 2003).

Phosphorylation of Dab2 results in changes in subcellular location during mitosis. Dab2 is negatively regulated in mitosis by progressive displacement from the PM paralleled by a loss of co-localization with clathrin, culminating in metaphase/anaphase, and partial recovery in cytokinesis (Chetrit et al., 2011). Negative regulation of Dab2 may be part of an accommodation of the cell to the altered physicochemical conditions prevalent in mitosis (Chetrit et al., 2011). Dab2 is regulated under a variety of context and therefore I expect Dab2 to be highly regulated in varying environments. Dab2 regulation to control endocytosis in response to varying cellular conditions and environments may be a more ubiquitous mechanism of regulation than previously thought.

In this study I showed an interesting correlation between changes in β1-integrin abundance at the cell surface and changes in regulation of Dab2-recruitment to CCPs as a result of AMPK activation. These results may be evidence of a pathway that links AMPK and Dab2 (**Figure 18**), but the opposite conclusion may be suggested as well – perhaps AMPK is directly regulating β1-integrin at the cell surface and changes in Dab2-recruitment to CCPs are just a reporter of β1-integrin regulation. β1-integrin is phosphorylated at S785 and this phosphorylation in both F9 and GD25 cells results in promoted adhesion but inhibited cell spreading and directed cell

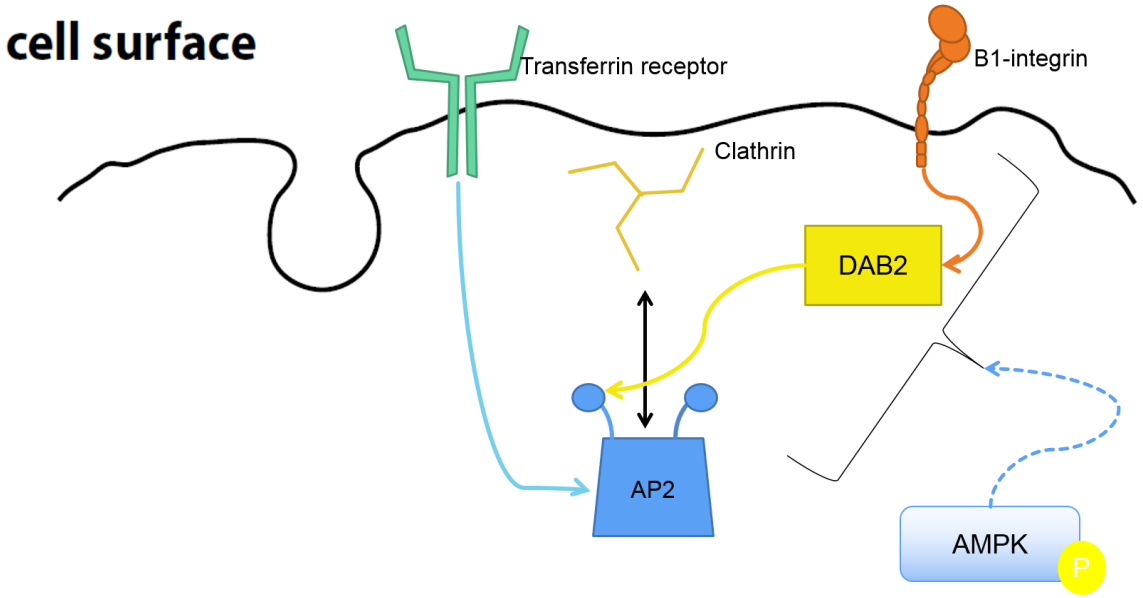


Figure 18 **Dab2 is a CLASP that is required for the internalization of β1-integrin but not that of Transferrin receptor. My research has shown that AMPK activation results in altered Dab2 recruitment to CCPs. I also showed that AMPK activation regulates adhesion molecules at the PM. AMPK may be regulating adhesion molecule membrane traffic by targeting Dab-2-dependent CME.**

migration (Mulrooney, Hong, & Grabel, 2001). There is a possibility that β1-integrin may be phosphorylated by AMPK in order to regulate its endocytosis.

While AMPK can directly phosphorylate a series of substrates, another possible mechanism by which AMPK may regulate cell surface β1-integrin is to do so indirectly, through the activation of the p38 MAPK. Activated p38 MAPK has been shown to facilitate the CME of certain PM proteins such as EGFR (Tomas, Futter, & Eden, 2013; Zwang & Yarden, 2006) and AMPK activation has been linked to phosphorylation of p38 MAPK (Bae et al., 2011). Treatment of NCI-H292 cells with the AMPK activator metformin activated phosphorylation of p38 MAPK and treatment with compound C, an inhibitor of AMPK, inhibited metformin-activated phosphorylation of p38 MAPK (Bae et al., 2011). There is a close dependence between AMPK and p38 MAPK in the phosphorylation cascade that leads to energetic stress-dependent apoptosis executed by p53 (Filomeni, Cardaci, Da Costa Ferreira, Rotilio, & Ciriolo, 2011).

Furthermore, p38 MAPK has been linked to phosphorylation of β1-integrin (Sato et al., 2005). Perhaps p38 MAPK also mediates the response of the cell surface proteome to energetic stress and phosphorylates β1-integrin to regulate adhesion at the PM in response to activation by AMPK.

AMPK may also regulate β1-integrin cell-surface abundance through regulation of its recycling. Upon internalization, β1-integrin localizes to Rab21-positive early endosomes where it is retained by interaction with Rab21 (Pellinen et al., 2006). P120RasGAP displaces the Rab21/integrin interaction and is required for β1-integrin exit from Rab21-endosomes (Mai et al., 2011). Overexpression of Rab21 stimulates cell migration and cancer cell adhesion to collagen and human bone, whereas knock down of Rab21 impairs integrin-mediated cell adhesion and motility (Pellinen et al., 2006). Perhaps AMPK regulates the recycling of integrins through phosphorylation of Rab21, p120RasGAP, or β1-integrin.

Regulation of AMPK activation during cell migration

AMPK is activated in response to detection of increased AMP levels as a result of ATP depletion. Considering that ATP is such an important and highly used building block within the cell, massive global decreases in ATP levels are unlikely to be frequent events in many tissues and cells. However, fluctuations or depletions in ATP concentration occur at localized regions within the cell, phenomena tied to highly-energy consuming cellular events are more likely to occur physiologically. For example, ATP is highly consumed during synaptic transmission at the pre-synaptic portion of a neuron, which requires a high rate of local ATP synthesis (Rangaraju et al., 2014).

Cell migration can potentially create energy-limited domains within the cell. The leading edge of a migrating cell is highly metabolically active because of several processes that occur; rapid membrane traffic of adhesion molecules for disassembly of focal contacts, actin polymerization and depolymerisation events facilitating formation and travel of lamellipodia, and ATPase function of f-actin. It is likely that ATP is locally depleted at the leading edge of migrating cells and that the role I demonstrated for AMPK in regulating adhesion molecules membrane traffic at the cell surface may be highly physiologically relevant here. Migrating cells require coordinated and polarized regulation of their cell surface proteomes. Local ATP-depletion at the leading edge may be one signal to the cell to regulate its adhesion properties by activating AMPK leading to a changed cell-surface distribution of integrins and other cell adhesion proteins.

Specificity of AMPK activation by nucleoside analogues

In this study, I used the AMP mimetics AICAR and A-769662 to activate AMPK (See Appendix 3). All effects of AMPK activation observed from AICAR treatment were confirmed with A-769662 treatment. There is a strong movement supporting the use of A-769662 instead of AICAR treatment to activate AMPK both in *in vitro* studies and in pursuit of possible therapeutic avenues for these compounds (Göransson et al., 2007). AICAR is internalized by adenosine transporters (Gadalla et al., 2004) and is converted by adenosine kinase to the AMP mimetic ZMP within the cells (Corton et al., 1995). Nucleoside transporters (such as adenosine transporters) are integral membrane proteins that mediate and facilitate the cellular intake of nucleosides and nucleobases, nucleoside analog drugs used in the treatment of some cancers and

viral infections, and they are the target of certain compounds used for cardiovascular disease treatment (Rose & Coe, 2008). The regulation of the cell surface proteome that I have attributed to the activation of AMPK could possibly be related to the binding of AICAR to nucleoside transporters and the purine signalling that would ensue, however I observed the same effects using A-769662 which is not internalised by nucleoside transporters (Göransson et al., 2007) to activate AMPK.

Another problem with the use of AICAR is that ZMP has been found to regulate other AMP-sensitive enzymes such as glycogen phosphorylase (Longnus et al., 2003), while A-769662 has not been found to have off-target effects (Cool et al., 2006). Furthermore, ZMP, while not an agonist or an antagonist of adenosine receptors, does compete with adenosine for re-uptake into cells (Gadalla et al., 2004) which could have an effect on overall AMP, ADP, and ATP levels. All of my findings have been confirmed with A-769662 and future experiments in my research group will primarily involve the use of A-769662 instead of AICAR. Further studies with AMPK silencing will support the evidence I found for regulation of the cell-surface proteome as a result of AICAR and A-769662 treatment.

Regulation of integrins and cell migration during cancer: possible role of AMPK

Why does AMPK activation result in a pronounced redistribution of adhesion molecules on the cell surface? What are the implications of this finding? Disseminated disease is the most common cause of death in cancer patients (Brooks, Lomax-Browne, Carter, Kinch, & Hall, 2010). Metastasis is a complex and poorly understood cascade that allows for tumour cells from a primary focus to colonize a secondary location for proliferation and formation of secondary

tumours (Brooks et al., 2010). A metastatic tumour cell must complete the entire metastasis cascade (**Figure 19**) in order to be successful and the vast majority of cancer cells are unable to do so (Brooks et al., 2010).

Metastatic cells colonize different target organs using the bloodstream but can also utilize the lymphatic system (Felding-Habermann, 2003). Metastatic disease cannot progress without the formation of a blood supply to the solid primary tumour mass (Bergers & Benjamin, 2003) so the metastatic cascade begins with angiogenesis – the development of new blood vessels directed to the site of the primary tumour (Brooks et al., 2010). After a blood source to the tumour is formed, the tumour cells must detach from each other and from their substrate (Brooks et al., 2010). This allows the tumour cells to begin their invasion of and migration through the basement membrane (BM) and ECM and to subsequently invade the BM of the blood vessel endothelium (Brooks et al., 2010). Intravasation follows and the tumour cells can travel through the blood system (Brooks et al., 2010).

A large number of tumour cells make their way to the blood stream but few of them give rise to metastases – survival within the vasculature is difficult considering the shear forces generated by blood flow (Felding-Habermann, 2003). A similarly difficult step in the metastasis cascade is the adhesion of cancer cells to endothelial cells within the blood vessel which prevents the cells from being cleared from circulation and allows for extravasation (Brooks et al., 2010). Integrin supported adhesion of circulating tumour cells is considered the first critical step toward target organ colonization (Abdel-Ghany, Cheng, Elble, & Pauli, 2002). Once outside of the blood vessel, the tumour cells colonize the target organ site and proliferate to develop a secondary tumour (Brooks et al., 2010).

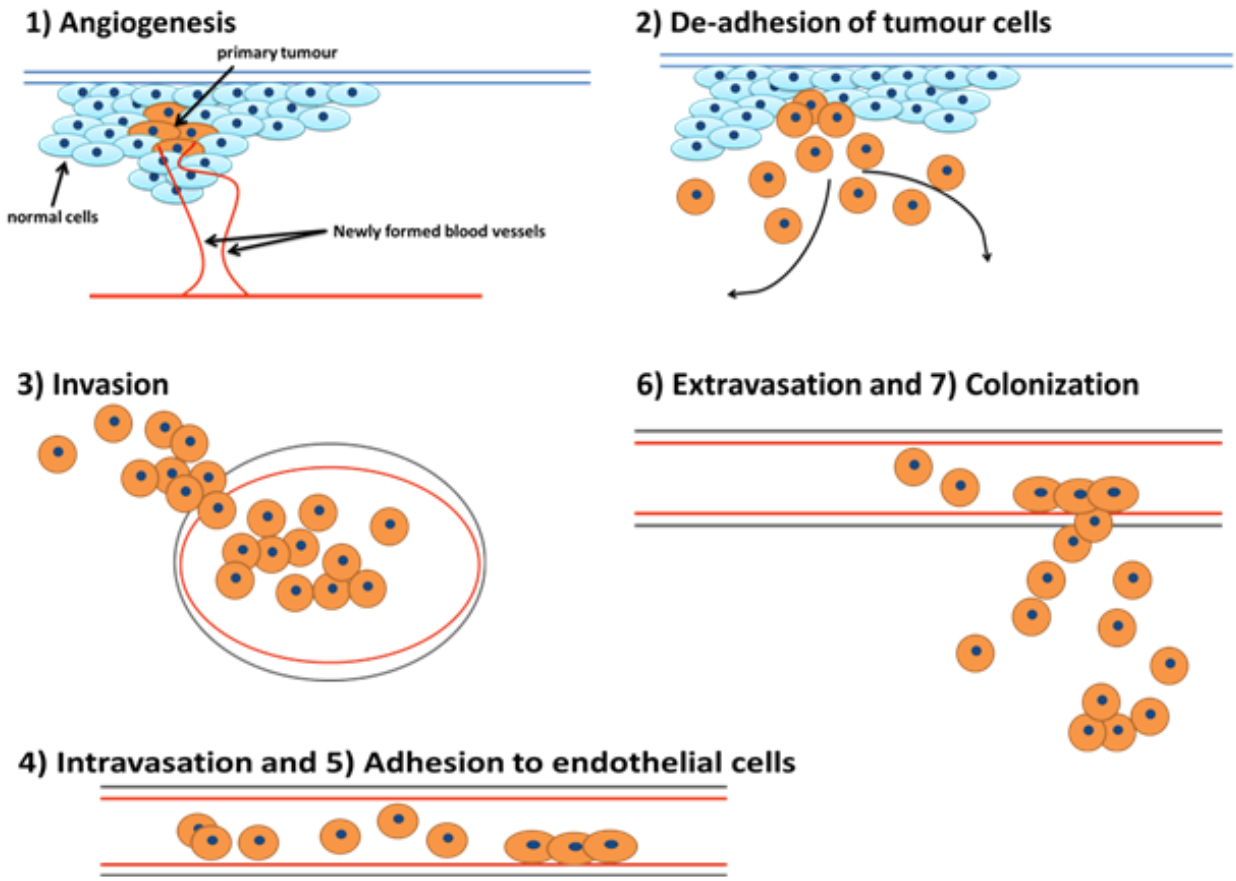


Figure 19 **The steps in the metastatic cascade include: 1) Angiogenesis, 2) De-adhesion of tumour cells from the primary location, 3) Invasion, 4) Intravasation, 5) Endothelial adhesion, 6) Extravasation, and 7) Colonization of a secondary location. There are important roles for integrins and other adhesion molecules at each of these steps (adapted from Felding-Habermann, 2003).**

p53 is a tumour suppressor gene that is mutated in 50% of all human cancers (Muller et al., 2009). The mutation results in the loss of tumour suppressor function but still allows for the production of a full length p53 with a point mutation (Muller et al., 2009). Mutant p53 promotes the recycling of integrins and ErbB2, thereby controlling invasion and metastasis (Muller et al., 2009). Interesting, AMPK phosphorylates p53 at serine 15 thereby stabilizing/up-regulating the tumour suppressor protein (D. G. Hardie, 2011; R. G. Jones et al., 2005). AMPK induces a p53-dependent metabolic checkpoint by causing G1-S-phase cell cycle arrest (R. G. Jones et al., 2005). Phosphorylation of p53 at serine 15 by AMPK is essential for mediating the effects of

AMPK on p53-dependent cell-cycle arrest (R. G. Jones et al., 2005). Further studies may investigate the role of p53 in the regulation of cell adhesion by AMPK activation and the effects of loss of p53 function in this system.

Integrins are known to have multiple distinct roles in tumour cell metastasis. Integrin expression in tumour tissue can be very different from normal tissue (Desgrosellier & Cheresh, 2010). Changes in recycling may affect the profile of integrins on the cell surface and may have implications in tumour cell metastasis progression. For example, integrins αvβ3, α5β1, and αvβ6 are normally expressed at low or undetectable levels in most adult epithelia but can be highly up-regulated in some tumours (Desgrosellier & Cheresh, 2010).

Up-regulation of αvβ3 is highly implicated in increased invasiveness partly due to its recruitment of matrix metalloproteinases (MMPs) (Koshiba et al., 1998). MMPs degrade the ECM and, therefore, prepare the path for tumour cells to migrate, invade and spread to distant secondary areas (Bourboulia & Stetler-Stevenson, 2010). Some integrins, such as α2β1, have decreased expression in tumour cells potentially increasing tumour cell dissemination (Kren et al., 2007).

Studies that have found correlations between integrin expression levels in human tumours and pathological outcomes have identified several integrins that might have crucial roles in cancer progression and may be therapeutic targets (Desgrosellier & Cheresh, 2010). Integrin antagonists can inhibit tumour growth by affecting tumour cells and also tumour-associated host cells such as the angiogenic endothelium (Desgrosellier & Cheresh, 2010). Controlling the profile of integrins on the cell surface by changes in their endocytosis may contribute to observed typical integrin over- or under-expression on different cancer cells.

Although there are clinical trials in progress assessing the safety of function-blocking antibodies against certain integrins known to be overexpressed in proliferating tissues (Avraamides et al., 2008), this type of therapy may present some of the same issues as others that target proliferating cells (such as chemotherapy).

Metformin is an AMPK-activating drug that is commonly administered to treat diabetes. Diabetics treated with metformin had a significantly lower incidence of cancer than those on other treatments (Evans, Donnelly, Emslie-Smith, Alessi, & Morris, 2005) suggesting that AMPK may be a therapeutic target in cancer treatment and prevention. Supporting this suggestion, treatment of a tumor-prone mouse model with three different AMPK activators – metformin, phenformin (a more potent sister drug to metformin), or A-769662 – significantly delayed tumor development (X. Huang et al., 2008). Perhaps the role of AMPK activation protecting against tumor development is attributed or associated with its role in regulating cell surface adhesion molecules.

A better understating of the regulation of the cell-surface proteome and the cellular mechanisms that lead to its remodelling may be crucial in the development of targeted therapies in the treatments of certain cancers.

Conclusion

In summary, I have developed an assay to selectively label and purify cell surface proteins for the identification of changes in the cell surface proteome as a result of metabolic stress. Using this assay I identified a novel regulation of the cell-surface proteome by AMPK activation. I demonstrated that several functional groups of proteins are either up- or down-regulated in abundance at the plasma membrane as a result of AMPK activation. Of primary interest was the discovery that proteins involved in cell adhesion are significantly down-regulated in their abundance at the PM upon AMPK activation. I confirmed the regulation of adhesion molecule membrane traffic by AMPK activation by focusing my investigation on β1-integrin and its changes in abundance at the cell surface. I showed that upon AMPK activation, β1-integrin cell surface levels are decreased and that this regulation correlates with alterations in the assembly of clathrin-coated pits containing Dab2. Discovering the mechanisms by which AMPK regulates the cell surface proteome will allow for a better understanding of how cells respond to their environments in physiological and pathological states and may lead to the development of new therapeutic targets.

Appendix 1 – Top 100 proteins identified in the cell-surface assay to be depleted at the cell-surface after AMPK activation

Accession	Total Protein Id Count	Total AMPK-activated	Total Control	AMPK-activated/Control	Description
gi|000044580	74	0	74	0.00	ZNF142
gi|000044581	74	0	74	0.00	zinc finger protein 142
gi|000044582	74	0	74	0.00	zinc finger protein 142 (clone pHZ-49) isoform CRA_a
gi|000165626	73	0	73	0.00	Uncharacterized protein
gi|000165627	73	0	73	0.00	zinc finger protein 142 (clone pHZ-49) isoform CRA_b
gi|000105717	68	0	68	0.00	PREDICTED: hypothetical protein
gi|000196646	41	0	41	0.00	methylcrotonoyl-Coenzyme A carboxylase 1 (alpha) isoform CRA_b
gi|000196647	41	0	41	0.00	methylcrotonoyl-CoA carboxylase subunit alpha, mitochondrial precursor
gi|000183660	35	0	35	0.00	Myosin-XVIIIb
gi|000069900	30	0	30	0.00	LOC100292035
gi|000046342	25	0	25	0.00	adenylate cyclase type 6 isoform b
gi|000046343	25	0	25	0.00	adenylate cyclase type 6 isoform a
gi|000104447	25	0	25	0.00	hCG1989975
gi|000021948	24	0	24	0.00	Uncharacterized protein TTN
gi|000113364	23	0	23	0.00	CDNA FLJ46154 fis, clone TESTI4001348
gi|000113365	23	0	23	0.00	DCDC5
gi|000113366	23	0	23	0.00	Doublecortin domain-containing protein 5
gi|000032273	22	0	22	0.00	vacuolar protein sorting 13C (yeast) isoform CRA_i
gi|000032277	22	0	22	0.00	vacuolar protein sorting 13C (yeast) isoform CRA_d
gi|000032271	22	0	22	0.00	vacuolar protein sorting-associated protein 13C isoform 2A
gi|000114461	20	0	20	0.00	Uncharacterized protein
gi|000032279	20	0	20	0.00	vacuolar protein sorting-associated protein 13C isoform 1A
gi|000032281	20	0	20	0.00	vacuolar protein sorting 13C (yeast) isoform CRA_c
gi|000032282	20	0	20	0.00	vacuolar protein sorting 13C (yeast) isoform CRA_a

Accession	Total Protein Id Count	Total AMPK-activated	Total Control	AMPK-activated/Control	Description
gi|000032276	19	0	19	0.00	vacuolar protein sorting 13C (yeast) isoform CRA_e
gi|000032272	19	0	19	0.00	vacuolar protein sorting 13C (yeast) isoform CRA_g
gi|000032267	19	0	19	0.00	Uncharacterized protein
gi|000032268	19	0	19	0.00	VPS13C
gi|000032269	19	0	19	0.00	vacuolar protein sorting-associated protein 13C isoform 2B
gi|000032270	19	0	19	0.00	VPS13C
gi|000085712	19	0	19	0.00	PREDICTED: OTU domain containing 1
gi|000059134	18	0	18	0.00	histone H1.5
gi|000032274	18	0	18	0.00	vacuolar protein sorting 13C (yeast) isoform CRA_b
gi|000032275	18	0	18	0.00	vacuolar protein sorting 13C (yeast) isoform CRA_f
gi|000098208	17	0	17	0.00	PREDICTED: hypothetical protein
gi|000098209	17	0	17	0.00	PREDICTED: hypothetical protein
gi|000032278	17	0	17	0.00	vacuolar protein sorting-associated protein 13C isoform 1B
gi|000032280	17	0	17	0.00	vacuolar protein sorting 13C (yeast) isoform CRA_h
gi|000057480	17	0	17	0.00	USP39
gi|000057482	17	0	17	0.00	Uncharacterized protein
gi|000057483	17	0	17	0.00	U4/U6.U5 tri-snRNP-associated protein 2
gi|000057485	17	0	17	0.00	Uncharacterized protein
gi|000127310	17	0	17	0.00	protein kinase DNA-activated catalytic polypeptide isoform CRA_d
gi|000185093	17	0	17	0.00	ENSP00000313420
gi|000185094	17	0	17	0.00	protein kinase DNA-activated catalytic polypeptide isoform CRA_c
gi|000185096	17	0	17	0.00	DNA-dependent protein kinase catalytic subunit isoform 1
gi|000185095	16	0	16	0.00	DNA-dependent protein kinase catalytic subunit isoform 2
gi|000185091	16	0	16	0.00	PREDICTED: similar to protein kinase, DNA-activated, catalytic polypeptide
gi|000185092	16	0	16	0.00	Uncharacterized protein

Accession	Total Protein Id Count	Total AMPK-activated	Total Control	AMPK-activated/Control	Description
gi|000048604	16	0	16	0.00	cDNA FLJ54020, highly similar to Heterogeneous nuclear ribonucleoprotein U
gi|000048605	16	0	16	0.00	Heterogeneous nuclear ribonucleoprotein U
gi|000048606	16	0	16	0.00	heterogeneous nuclear ribonucleoprotein U isoform a
gi|000048607	16	0	16	0.00	heterogeneous nuclear ribonucleoprotein U isoform b
gi|000048608	16	0	16	0.00	Uncharacterized protein
gi|000019368	16	0	16	0.00	Heterogeneous nuclear ribonucleoprotein U (Scaffold attachment factor A) (Fragment)
gi|000148282	16	0	16	0.00	HNRPU protein
gi|000172891	16	0	16	0.00	homeodomain-interacting protein kinase 3 isoform 1
gi|000070997	16	0	16	0.00	Uncharacterized protein
gi|000056996	16	0	16	0.00	coiled-coil domain containing 102A
gi|000056997	16	0	16	0.00	coiled-coil domain-containing protein 102A
gi|000119930	16	0	16	0.00	DENN/MADD domain containing 4C isoform CRA_c
gi|000119931	16	0	16	0.00	DENN/MADD domain containing 4C isoform CRA_d
gi|000119932	16	0	16	0.00	DENN/MADD domain containing 4C isoform CRA_a
gi|000119933	16	0	16	0.00	DENN/MADD domain containing 4C isoform CRA_b
gi|000103877	15	0	15	0.00	Mucin-16
gi|000158092	15	0	15	0.00	Aldehyde oxidase
gi|000158093	15	0	15	0.00	aldehyde oxidase 1 isoform CRA_a
gi|000158094	15	0	15	0.00	aldehyde oxidase
gi|000158097	15	0	15	0.00	ENSP00000260930
gi|000175598	15	0	15	0.00	Uncharacterized protein
gi|000050112	15	0	15	0.00	Ponsin
gi|000117272	15	0	11	0.00	Uncharacterized protein
gi|000064026	14	0	14	0.00	cDNA FLJ51005, highly similar to Aldehyde oxidase (EC 1.2.3.1)
gi|000175242	14	0	14	0.00	desmoglein-2 preproprotein

Accession	Total Protein Id Count	Total AMPK-activated	Total Control	AMPK-activated/Control	Description
gi|000098609	14	0	14	0.00	mitogen-activated protein kinase-activated protein kinase 2 isoform CRA_g
gi|000067793	14	0	14	0.00	PREDICTED: hypothetical protein
gi|000151760	14	0	14	0.00	NFX1-type zinc finger-containing protein 1
gi|000151763	14	0	14	0.00	zinc finger NFX1-type containing 1 isoform CRA_b
gi|000072324	13	0	13	0.00	ARFGAP3
gi|000151793	13	0	13	0.00	PREDICTED: similar to Protein MICAL-3
gi|000170463	13	0	13	0.00	biorientation of chromosomes in cell division protein 1-like
gi|000038950	13	0	10	0.00	Solute carrier family 12 member 8
gi|000038951	13	0	10	0.00	Solute carrier family 12 member 8
gi|000038953	13	0	10	0.00	solute carrier family 12 member 8
gi|000101754	13	0	13	0.00	protein phosphatase 1J
gi|000101755	13	0	13	0.00	hCG1997303 isoform CRA_c
gi|000101756	13	0	13	0.00	hCG1997303 isoform CRA_f
gi|000206075	13	0	13	0.00	LOC646976
gi|000056608	13	0	13	0.00	hemicentin 1 isoform CRA_a
gi|000101266	13	0	10	0.00	SLC12A8
gi|000101267	13	0	10	0.00	ZNF148
gi|000008646	13	0	10	0.00	SLC12A8
gi|000147794	12	0	12	0.00	cDNA FLJ75603
gi|000130433	12	0	12	0.00	zinc finger RAN-binding domain containing 3 isoform CRA_c
gi|000130434	12	0	12	0.00	zinc finger RAN-binding domain containing 3 isoform CRA_f
gi|000068277	12	0	12	0.00	cDNA, FLJ96929, highly similar to Homo sapiens taste receptor, type 1, member 1 (TAS1R1), mRNA
gi|000051151	12	0	12	0.00	topoisomerase (DNA) II binding protein 1 isoform CRA_b
gi|000046164	12	0	12	0.00	USP54
gi|000103526	12	0	12	0.00	taste receptor type 1 member 1 isoform b precursor

Appendix 2 – Top 100 Proteins identified in the cell-surface assay to be enriched at the cell-surface after AMPK activation

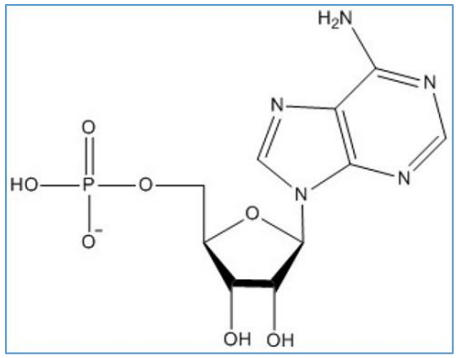
Accession	Total Protein Id Count	Total AMPK-activated	Total Control	Control/ AMPK-activated	Description
gi|000166661	18	16	0	0.00	homeobox D12
gi|000166663	18	16	0	0.00	Homeobox protein Hox-D12
gi|000084178	8	8	0	0.00	latent-transforming growth factor beta-binding protein 3 isoform 2 precursor
gi|000084179	8	8	0	0.00	latent-transforming growth factor beta-binding protein 3 isoform 1 precursor
gi|000149607	7	6	0	0.00	ubiquitin carboxyl-terminal hydrolase 12
gi|000149608	7	6	0	0.00	ubiquitin specific peptidase 12 isoform CRA_b
gi|000149609	7	6	0	0.00	ubiquitin-specific protease 12-like 1
gi|000143814	7	7	0	0.00	GOLGA4
gi|000138841	7	7	0	0.00	golgi autoantigen golgin subfamily a 4 isoform CRA_a
gi|000138842	7	7	0	0.00	Golgin subfamily A member 4 isoform 1
gi|000138836	7	7	0	0.00	Golgin subfamily A member 4 isoform 2
gi|000138837	7	7	0	0.00	GOLGA4
gi|000138838	7	7	0	0.00	GOLGA4
gi|000202964	7	6	0	0.00	USP12
gi|000138834	7	7	0	0.00	golgi autoantigen golgin subfamily a 4 isoform CRA_c
gi|000138835	7	7	0	0.00	GOLGA4
gi|000022138	7	7	0	0.00	ENSP00000405842
gi|000150329	6	6	0	0.00	myosin light polypeptide kinase isoform CRA_g
gi|000050144	6	6	0	0.00	Putative uncharacterized protein DKFZp451A123
gi|000050146	6	6	0	0.00	myosin-2
gi|000127760	5	5	0	0.00	hCG1778643
gi|000127761	5	5	0	0.00	CDNA FLJ13262 fis, clone OVARC1000912
gi|000077458	5	5	0	0.00	Uncharacterized protein
gi|000077459	5	5	0	0.00	cDNA FLJ58079, highly similar to Homo sapiens SH3 multiple domains 1 (SH3MD1), mRNA
gi|000048683	5	5	0	0.00	nuclear receptor corepressor 1 isoform 1
gi|000048694	5	5	0	0.00	Uncharacterized protein
gi|000048685	5	5	0	0.00	NCOR1
gi|000048686	5	5	0	0.00	NCOR1

Accession	Total Protein Id Count	Total AMPK-activated	Total Control	Control/ AMPK-activated	Description
gi|000048515	5	5	0	0.00	Nuclear receptor co-repressor
gi|000162372	5	5	0	0.00	MYLK
gi|000194262	4	4	0	0.00	hCG27698 isoform CRA_d
gi|000194257	4	4	0	0.00	probable ATP-dependent RNA helicase DDX47 isoform 2
gi|000194258	4	4	0	0.00	DEAD box protein
gi|000194259	4	4	0	0.00	probable ATP-dependent RNA helicase DDX47 isoform 1
gi|000102569	4	4	0	0.00	GOLGB1
gi|000039245	4	4	0	0.00	ENSP00000409619
gi|000023593	4	4	0	0.00	YLP motif-containing protein 1
gi|000023595	4	4	0	0.00	hCG22358 isoform CRA_c
gi|000023596	4	4	0	0.00	YLPM1
gi|000023597	4	4	0	0.00	YLP motif-containing protein 1
gi|000066309	4	4	0	0.00	Uncharacterized protein
gi|000129133	4	4	0	0.00	PREDICTED: similar to Serine/threonine-protein kinase Pim-3
gi|000075557	4	4	0	0.00	PREDICTED: hypothetical protein
gi|000045500	4	4	0	0.00	ENSP00000295971
gi|000045501	4	4	0	0.00	RNA-binding protein 47
gi|000045502	4	4	0	0.00	RNA-binding protein 47 isoform a
gi|000045503	4	4	0	0.00	RNA-binding protein isoform CRA_c
gi|000045504	4	4	0	0.00	RNA-binding protein isoform CRA_a
gi|000045505	4	4	0	0.00	RNA binding motif protein 47
gi|000149991	4	4	0	0.00	cDNA FLJ61302, highly similar to Glutamate receptor, ionotropic kainate 1
gi|000043699	4	4	0	0.00	cDNA FLJ30924 fis, clone FEBRA2006521, highly similar to Junctophilin-3
gi|000186245	4	4	0	0.00	Uncharacterized protein
gi|000101718	4	4	0	0.00	MAP1D
gi|000125651	4	4	0	0.00	high affinity cGMP-specific 3,5-cyclic phosphodiesterase 9A isoform o
gi|000125653	4	4	0	0.00	high affinity cGMP-specific 3,5-cyclic phosphodiesterase 9A isoform a
gi|000051819	4	4	0	0.00	Hqp0256 protein
gi|000016938	4	4	0	0.00	PDE9A
gi|000025623	4	4	0	0.00	apoptotic chromatin condensation inducer in the nucleus isoform 3
gi|000025624	4	4	0	0.00	Uncharacterized protein

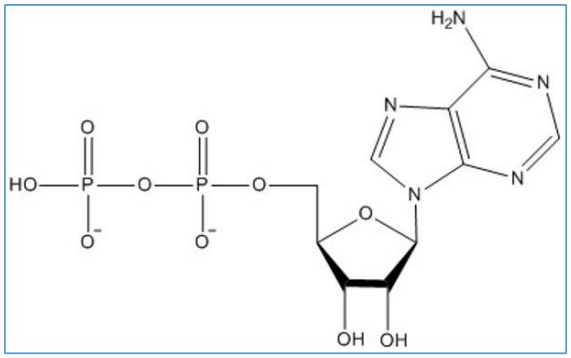
Accession	Total Protein Id Count	Total AMPK-activated	Total Control	Control/ AMPK-activated	Description
gi|000025625	4	4	0	0.00	apoptotic chromatin condensation inducer in the nucleus isoform 2
gi|000025626	4	4	0	0.00	apoptotic chromatin condensation inducer in the nucleus isoform 1
gi|000025627	4	4	0	0.00	ENSP00000451328
gi|000025628	4	4	0	0.00	Apoptotic chromatin condensation inducer in the nucleus
gi|000143772	4	4	0	0.00	Uncharacterized protein
gi|000049856	4	4	0	0.00	junctophilin-3
gi|000093136	4	4	0	0.00	polycystic kidney disease 2-like 1 isoform CRA_b
gi|000051971	4	4	0	0.00	cDNA FLJ60694, highly similar to Deubiquitinating protein VCIP135 (EC 3.4.22.-)
gi|000051972	4	4	0	0.00	deubiquitinating protein VCIP135
gi|000032614	4	4	0	0.00	hypothetical protein FLJ14668 isoform CRA_b
gi|000164401	4	4	0	0.00	high affinity cGMP-specific 3,5-cyclic phosphodiesterase 9A isoform m
gi|000164403	4	4	0	0.00	high affinity cGMP-specific 3,5-cyclic phosphodiesterase 9A isoform n
gi|000193929	4	4	0	0.00	methionine aminopeptidase 1D, mitochondrial precursor
gi|000209331	4	4	0	0.00	DEAD (Asp-Glu-Ala-Asp) box polypeptide 47 isoform 1 variant (Fragment)
gi|000183578	3	2	0	0.00	Uncharacterized protein
gi|000183579	3	2	0	0.00	cDNA FLJ54283, highly similar to Heat shock 70 kDa protein 1
gi|000183580	3	2	0	0.00	cDNA FLJ54407, highly similar to Heat shock 70 kDa protein 1
gi|000183581	3	2	0	0.00	cDNA FLJ54389, highly similar to Heat shock 70 kDa protein 1
gi|000183586	3	2	0	0.00	cDNA FLJ54328, highly similar to Heat shock 70 kDa protein 1
gi|000183587	3	2	0	0.00	Uncharacterized protein
gi|000183588	3	2	0	0.00	Uncharacterized protein
gi|000183589	3	2	0	0.00	cDNA FLJ54392, highly similar to Heat shock 70 kDa protein 1
gi|000183590	3	2	0	0.00	cDNA FLJ54408, highly similar to Heat shock 70 kDa protein 1

Accession	Total Protein Id Count	Total AMPK-activated	Total Control	Control/ AMPK-activated	Description
gi|000183591	3	2	0	0.00	heat shock 70kDa protein 1B
gi|000183592	3	2	0	0.00	heat shock 70 kDa protein 1A/1B
gi|000183593	3	2	0	0.00	heat shock 70kDa protein 1A
gi|000183594	3	2	0	0.00	cDNA FLJ54303, highly similar to Heat shock 70 kDa protein 1
gi|000183595	3	2	0	0.00	Uncharacterized protein
gi|000183597	3	2	0	0.00	Uncharacterized protein
gi|000183599	3	2	0	0.00	Heat shock 70kDa protein 1A
gi|000183600	3	2	0	0.00	cDNA FLJ38698 fis, clone KIDNE2002015, highly similar to HEAT SHOCK 70 kDa PROTEIN 1
gi|000183601	3	2	0	0.00	cDNA FLJ54370, highly similar to Heat shock 70 kDa protein 1
gi|000197488	3	3	0	0.00	diacylglycerol kinase delta 130kDa isoform CRA_a
gi|000197489	3	3	0	0.00	diacylglycerol kinase delta isoform 2
gi|000038127	3	3	0	0.00	beta-1,4 N-acetylgalactosaminyltransferase 2 isoform b
gi|000036282	3	2	0	0.00	ubiquitin carboxyl-terminal hydrolase 46 isoform 1
gi|000036283	3	2	0	0.00	USP46
gi|000036284	3	2	0	0.00	ENSP00000390102
gi|000118432	3	3	0	0.00	cDNA FLJ75863, highly similar to Homo sapiens zinc finger protein 432 (ZNF432), mRNA
gi|000118433	3	3	0	0.00	zinc finger protein 432

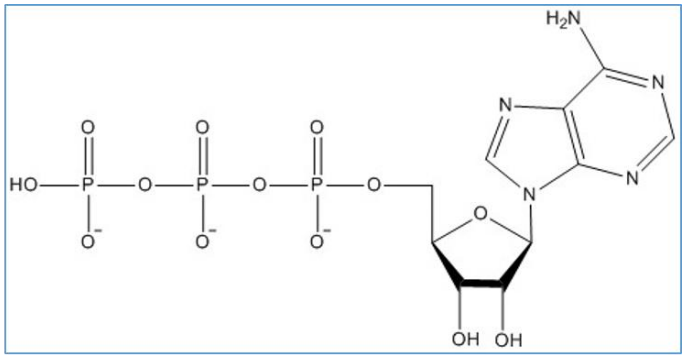
Appendix 3 – Structural diagrams of AMP, ADP, ATP, AICAR, ZMP, and A-769662



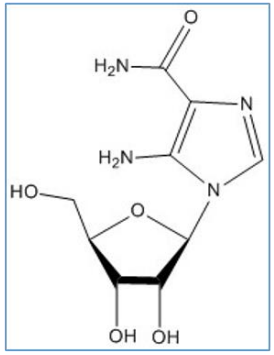
AMP



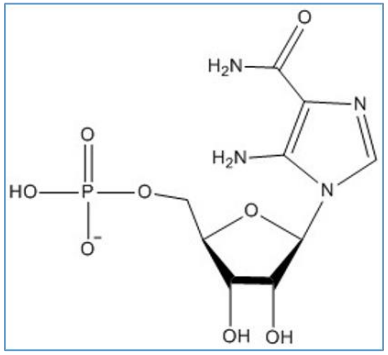
ADP



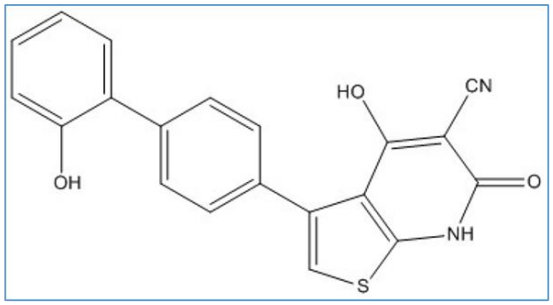
ATP



AICAR



ZMP



A-769662

Structures made using ChemDraw Std (PerkinElmer).

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