

Identification of a Nuclear Export Signal in the Catalytic Subunit of AMP-activated Protein Kinase

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

INTRODUCTION

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

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Abbreviations used: AMPK, AMP-activated protein kinase; dAMPK, *Drosophila* AMPK; LMB, leptomycin B; NES, nuclear export sequence; NLS, nuclear localization sequence; NPC, nuclear pore complex; PKIA, protein kinase A inhibitor; Snf1, sucrose nonfermenting 1; SNUPN, snurportin 1.

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For most kinases, distinguishing in vivo from in vitro targets remains a difficult endeavor, as homogenized cells and tissues frequently used for in vitro kinase assays allow proteins that are normally either spatially or temporally separated to come into contact with each other. One means for regulating kinase access to targets in vivo is through such spatiotemporal control of each component. Although AMPK can be found both in the nucleus and the cytoplasm (Salt *et al.*, 1998), the exact mechanisms regulating its subcellular localization have not been fully elucidated. In yeast, only alkaline pH has been shown to induce movement of SNF1, the AMPK α orthologue in yeast, from the cytoplasm to the nucleus (Hong and Carlson, 2007). In mammalian cells, leptin (Suzuki *et al.*, 2007) and heat shock—possibly through MEK signaling (Kodiha *et al.*, 2007)—can also cause translocation of AMPK α subunits to the nucleus. In addition, isoform-specific AMPK subunits have also been shown to accumulate in the nucleus in a circadian manner (Lamia *et al.*, 2009), and nuclear translocation can also be induced in vivo in muscle cells after exercise stress (McGee *et al.*, 2003).

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

Primary amino acid sequence analysis can sometimes predict structure–function relationships for domains of a protein. For single subunit proteins, nuclear localization sequences and nuclear export sequences within the protein

itself help identify mechanisms for its localization. However, for multisubunit kinases like AMPK and the LKB1/STRAD/MO25 complex, such regulation is more complicated.

Although the AMPK α amino-terminus is highly conserved, containing the serine-threonine kinase domain, the AMPK α carboxy-terminus does not contain any known functional motifs outside of the β/γ binding sites. We previously noted that the final carboxy-terminal 20 amino acids of AMPK α are highly conserved across diverse species (Brenman and Temple, 2007). In this study, we utilized genetic and cell biological approaches to evaluate potential functions for the AMPK α C-terminus. We identify a critical new function for the carboxy-terminal amino acids of AMPK α *in vivo*, which affects AMPK α subcellular localization, phosphorylation, and ultimately organismal viability.

MATERIALS AND METHODS

Generating Transgenic Flies

Truncated *dAMPK α ΔC* was cloned into a pUAST vector as a BglII-EcoRI fragment of a *dAMPK α -RA* transcript (www.flybase.org) by inserting a stop codon after Proline 561 of wild-type *dAMPK α* using PCR-based mutagenesis. The GFP::*dAMPK α* and mCherry::*dAMPK α ΔC* fusion proteins were made using green fluorescent protein (GFP) or mCherry at the fused in-frame to the N-terminus of *dAMPK α* in the pUAST vector. Transgenes were introduced into a *w¹¹¹⁸* stock via P-element-mediated transformation by the Duke Animal Models Core facility.

Fly Stocks and Crosses

UAS-dAMPK α wt and *UAS-dAMPK α ΔC* alleles expressed in sensory neurons using a *l(2)109-80-GAL4* driver (Gao *et al.*, 1999) and recombined with *UAS-actin::GFP* to visualize sensory neurons, as described previously (Medina *et al.*, 2006). For rescue experiments, both constructs were expressed using the *Ubiquitin-Gal4* driver and crossed into *dampk α 3* null mutants. Adult males were scored for rescue by nonbar eye phenotype because of the lack of FM7 balancer chromosome. All flies were maintained at 25°C in yeast-cornmeal vials. Heat-shock experiments were performed by crossing mCherry and GFP constructs of wild-type *dAMPK α* to *Ubiquitin-Gal4*. Third instar larvae were subjected to heat shock for 1 h at 37°C and allowed to recover for 15 min at 25°C. Live larval imaging was performed as described (Mirouse *et al.*, 2007) using the 488-nm argon line for GFP and the 543-nm helium neon line for mCherry on a Zeiss LSM510 confocal microscope (Thornwood, NY). *UAS-GFP::APC2* flies were a gift from Dave Roberts and Mark Peifer (UNC-Chapel Hill).

Plasmid Construction

For cell culture studies, both wild-type and truncated versions of AMPK α 2 were amplified by PCR using Pfu DNA Polymerase (Stratagene, La Jolla, CA) from rat AMPK α 2 (AddGene, Cambridge, MA; plasmid 15991) and inserted into pEGFP-C1 (Clontech Laboratories, Palo Alto, CA). Mouse AMPK α 1 wild-type and truncated versions were generated using PCR amplification from mouse cDNA and inserted into pEGFP-C1. The SV40-NLS (nuclear localization signal; PKKKRKVG), AMPK α 2, and AMPK α 1 C-terminal tail tags were cloned into the C-terminus of the pEGFP-C1::GFP construct. For expressing the SV40NLS and the AMPK α 2 C-terminal tag together, the SV40NLS coding sequence was inserted into the N-terminal forward primer for amplification of the GFP-coding sequence. The SV40NLS::GFP amplicon was then inserted into a pEGFP-C2 plasmid containing the AMPK α 2 C-terminal tail-coding sequence at the C-terminus of that plasmid.

L546A and L550A substitutions in the AMPK α 2 C-terminal tail sequence were inserted into the reverse primer sequence and cloned using site-directed mutagenesis. Hemagglutinin (HA)-tagged human AMPK γ 1 and rat AMPK β 1 constructs were gifts from Reuben Shaw (UCSD), and mCherry::AMPK γ 1 was produced by inserting human AMPK γ 1 into a modified pEGFP-N1 vector with GFP replaced by Flag::mCherry (gift from Tom Maynard, UNC-Chapel Hill). Myc-tagged clones of wild-type AMPK α 2, AMPK α 2ΔC, and AMPK α 2^{L₅₄₆A, L₅₅₀A} constructs were all generated using standard site-directed mutagenesis and inserted into the pCMV-myc vector (Clontech, 631604). The RanQ69L clone was a gift from Andrew Wilde (University of Toronto), which was then inserted into a pmCherry-C1 plasmid for live cell visualization.

To generate AMPK-CRM1-NES (nuclear export sequence) fusions, sequences encoding residues Met1 to Val14 (human Snurportin-1) or Ser35 to Ile47 (human cAMP-dependent protein kinase inhibitor alpha [PKIA]) were added after Asp538 of rat AMPK α 2, replacing the C-terminal tail. Truncated AMPK α 2 missing only the last 14 amino acids (AMPK α 2ΔC⁵³⁸) was produced by introducing a UGA stop codon after Asp538. For GFP-tagged constructs, products were amplified using PCR, ligated into the pEGFP-C1 vector and

sequenced to verify fidelity. For myc-tagged constructs, the coding regions were amplified from the above GFP-AMPK α 2 plasmids and inserted into the pCMV-myc vector.

Immunohistochemistry

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

AMPK α Immunoprecipitation

Drosophila protein lysates for immunoprecipitation were prepared by collecting equal numbers of male and female flies (50 total) of each genotype in a 1.5-ml tube. One milliliter lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate 1 mM β -glycerolphosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 1:500 dilution of Sigma (St. Louis, MO) mammalian protease inhibitor cocktail) was added to each sample. Flies were then ground to homogeneity with a pestle, sonicated, and centrifuged at 16,000 × *g* for 10 min at 4°C to remove insoluble material and debris. Supernatants were collected, and the protein concentration was determined using the Bio-Rad Dc protein assay (Richmond, CA). Immune complexes were formed by incubation of 100 μ l of anti-dAMPK α mouse mAb (Abcam; ab51025) with 1 ml of 1 mg/ml fly lysate overnight at 4°C. Antibodies were precipitated by incubation with 20 μ l protein A/G agarose (Pierce Protein Research Products, Rockford, IL) for 3 h, followed by centrifugation and washing with lysis buffer. Proteins were eluted by 10-min incubation at 70°C in LDS-loading buffer (Invitrogen) and separated on 4–12% Bis-Tris NuPAGE gels along with 50 μ g of crude lysates. Electrophoresis was followed by Western blotting and probing for phospho-AMPK α (Cell Signaling Technology, Beverly, MA; 2535) and total dAMPK α , with anti-lamin C (LC28.26, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) used as a loading control.

AMPK $\alpha/\beta/\gamma$ Coimmunoprecipitation

HEK293 cells were transiently transfected with myc-tagged AMPK α 2, HA-tagged AMPK β 1, and FLAG-tagged Cherry-AMPK γ 1 in 10-cm dishes. Cells were harvested and lysed in 0.5 ml lysis buffer (see above), sonicated, and centrifuged at 16,000 × *g* for 10 min at 4°C. Protein concentrations of supernatants were determined followed by overnight incubation of 1 ml of each lysate at 1 mg/ml with 100 μ l of anti-myc antibody (9E 10, Developmental Studies Hybridoma Bank). Antibody precipitation and blotting were performed as described above. Primary antibody dilutions were anti-Myc (1:100), anti-HA (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, F-7), and anti-FLAG (1:1000, Sigma, M2). All blots were scanned and quantified using the Odyssey Infrared System (Li-Cor Biosciences, Lincoln, NE) using fluorescently-labeled secondary antibodies (Li-Cor; IRDye 680 anti-rabbit IgG and IRDye 800 anti-mouse IgG).

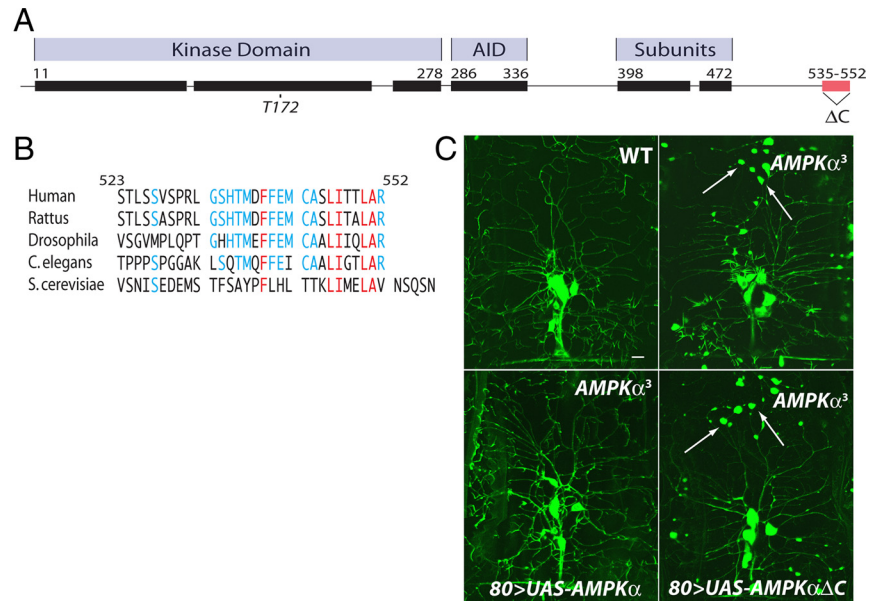
Cell Culture and Treatments

HEK293, HeLa, and CHO cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and penicillin-streptomycin. Cells were plated 24 h before transfection. DNA transfection of cells (1 μ g of DNA to 2 ml of medium) was performed using PolyJet DNA Transfection Reagent (SignaGen Laboratories, Ijamsville, MA), as directed by the supplier. LMB (10 ng/ml, Sigma) and cycloheximide (1 μ g/ml, Roche, Indianapolis, IN) were premixed in culture medium and added as indicated. Cells were treated with cycloheximide for 1 h before addition of leptomycin B (LMB) mix for indicated times.

Nuclear Localization Assay, Live Cell Imaging, and Tiling

HEK293 cells were transfected with the constructs indicated and scored 24 h later using fluorescence microscopy to observe the subcellular localization of GFP. 2xGFP alone was the negative control for localization and for treatment with LMB. For each GFP fusion, 200 cells were counted and scored as follows: predominantly nuclear, nuclear and cytoplasmic, or predominantly cytoplasmic localization. For imaging, cells were fixed and stained with DRAQ-5 (Biosstatus Limited, Shepshed, Leicestershire, United Kingdom) to visualize nuclei. For live cell imaging, 24 h after transfection the cells were treated with cycloheximide (1 μ g/ml) for 1 h followed by treatment with LMB (10 ng/ml). Pictures were taken every 10 min for GFP localization using an Olympus FV100 microscope (Melville, NY). Tiling images were also taken using the Olympus FV100 multiarea time-lapse imaging program followed by counting and deciphering of the subcellular localization of GFP as previously described (Henderson, 2000). To control for biased counting, 50 cells from each of the three classes (N, NC, and C) were selected and the fluorescence intensities

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



were quantified as previously described (Henderson, 2000). For cells identified as predominantly nuclear, the ratio of nuclear to cytoplasmic GFP level in a cell was greater than 2, for nuclear and cytoplasmic localization the same ratio was between 0.7 and 2, and for predominantly cytoplasmic localization the ratio was below 0.7.

RESULTS

The AMPK α Carboxy-Terminus Is Required for Function

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

To test the functional significance of the AMPK α carboxy-terminus, we generated transgenic *Drosophila* expressing full-length AMPK α lacking the final 22 amino acids (AMPK α Δ C) for reintroduction into AMPK α mutant or wild-type backgrounds. Although vertebrates contain two largely genetically redundant AMPK α genes, *Drosophila* encodes only a single AMPK α gene, thus greatly simplifying in vivo genetic analyses. Expression of full-length (amino terminally tagged or untagged) versions of AMPK α in a null background rescues the previously identified neuronal phenotype (Mirouse *et al.*, 2007; Figure 1C), whereas AMPK α Δ C versions do not (Figure 1C). Further, although both N-terminally GFP- or mCherry-tagged AMPK α rescue lethal null mutations to full viability at expected Mendelian ratios (data not shown), neither untagged nor tagged AMPK α Δ C rescue any mutant alleles to viability (scoring more than 1000 potential rescue events). These experiments indicate a crucial function for the carboxy-terminal 22 amino acids of *Drosophila* AMPK α in neuronal maintenance and viability.

The Carboxy-Terminal 22 Amino Acids of AMPK α Are Required for Normal Localization In Vivo

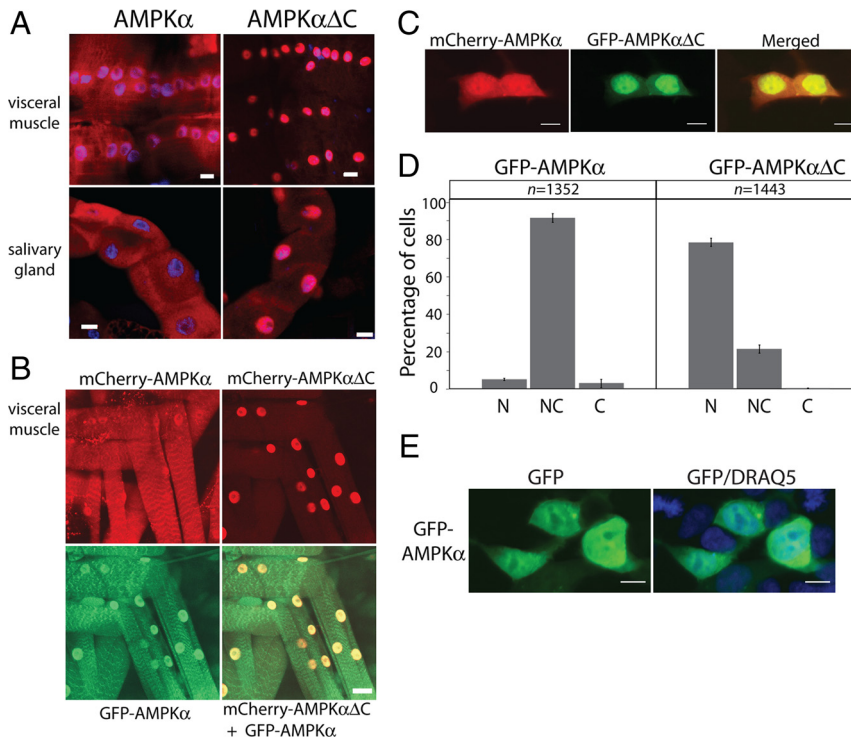
To explore why a 22-amino acid carboxy-terminal deletion of AMPK α fails to rescue AMPK α null mutants, we exam-

ined the localization of the truncated protein both by antibody immunohistochemistry and visualization of fluorescently tagged fusion protein. Expression of untagged AMPK α in transgenic animals and subsequent detection of AMPK α by immunohistochemistry revealed very clear subcellular localization differences between full-length and truncated protein (AMPK α Δ C; Figure 2A). AMPK α Δ C localizes predominantly in the nucleus, whereas full-length protein is both cytoplasmic and nuclear, using either immunohistochemistry of untagged protein (Figure 2A) or live animal images with fluorescently tagged proteins (Figure 2B). The observation that AMPK α Δ C is highly enriched in the nucleus was observed in diverse tissues including neurons, muscle, fat bodies, and salivary glands (Figure 2 and data not shown). Differential subcellular localization of AMPK α with and without the carboxy-terminus is most clearly demonstrated using a live transgenic animal simultaneously expressing both full-length and AMPK α Δ C in the same cells (Figure 2B). This observation was not restricted to *Drosophila* in vivo, as a conceptually similar result was observed in transiently transfected mammalian cells (HEK293) in vitro (Figure 2, C and E). Counting of transfected mammalian cells for subcellular localization demonstrated a clear difference between full-length AMPK α and AMPK α Δ C (Figure 2D).

The AMPK α Carboxy-Tail Functions as a LMB-sensitive NES

To elucidate whether the carboxy-terminus might act as a NES in AMPK α localization, we used a previously described mammalian cell assay to test for sequences that alter the subcellular localization of proteins (Frederick *et al.*, 2008). We fused the AMPK α carboxy-tail in frame at the C-terminus of two tandem GFP molecules (Figure 3A). Two consecutive fused GFPs (2xGFP, ~54 kDa) localize diffusely within the nucleus and the cytoplasm, as previously reported by Frederick *et al.* (2008) (see Figure 3B). Adding the 23-amino acid carboxy tail of rat AMPK α 2 (2xGFP- α 2 tail) leads to localization predominantly in the cytoplasm (Figure 3B).

Because a previous study demonstrated that endogenous (untagged) AMPK α protein in HeLa cells enriches in the nucleus upon treatment with LMB (Kodiha *et al.*, 2007), a



fluorescence for >50 cells in each group; N-C fluorescence ratios were >2.0, 0.7–2.0, and <0.7, for N, NC, and C, respectively. (E) Wild-type GFP-AMPK α control cells demonstrating roughly equal fluorescence in cytoplasm and nucleus. Nuclei were stained with DRAQ5 (blue). Bar, 10 μ m.

Figure 2. AMPK α lacking the carboxy-terminus localizes predominantly to nuclei in vivo and in vitro. (A) Indirect immunofluorescence of AMPK α (red) staining with anti-dAMPK α antibody on transgenic *Drosophila* 3rd instar larvae expressing either full-length wild-type AMPK α or the carboxy-truncated AMPK α (AMPK $\alpha\Delta C$). All transgenic proteins are expressed using the *Gal4-UAS* system, driven by *Ubiquitin-Gal4* (A and B). Nuclei were stained with ToPro-3 (blue). (B) Live animal images of larvae expressing amino-terminal mCherry-tagged wild-type (mCherry-AMPK α) or truncated (mCherry-AMPK $\alpha\Delta C$) AMPK α , GFP-tagged wild-type α (GFP-AMPK α) alone or in combination with mCherry-tagged truncated α (mCherry-AMPK $\alpha\Delta C$ +GFP-AMPK α). Bar, 10 μ m. (C) Co-expression of amino-terminal fluorescently tagged full-length AMPK $\alpha 2$ (mCherry-AMPK α , red) and truncated (GFP-AMPK $\alpha\Delta C$, green) in the same cotransfected mammalian cells (HEK293). (D) Scoring of transfected cells for subcellular localization of GFP-tagged full-length (GFP-AMPK α) or truncated (GFP-AMPK $\alpha\Delta C$) AMPK α as primarily nuclear (N), both nuclear and cytoplasmic (NC), or primarily cytoplasmic (C). Proper scoring for each group was confirmed by quantification of nuclear and cytoplasmic

specific inhibitor of CRM1-mediated nuclear export (Kutay and Guttinger, 2005), we tested whether LMB specifically inhibits this AMPK α tail-dependent nuclear export. Indeed, LMB treatment does result in altered localization of the 2xGFP- $\alpha 2$ tail from predominantly cytoplasmic (Figure 3B) to both nucleus and cytoplasm (Figure 3D). This effect of LMB can also be illustrated using time-lapse experiments, showing accumulation of proteins containing the carboxy tail of AMPK $\alpha 2$ in the nucleus within 10 min of LMB addition (Supplementary Figure 1).

Because CRM1 NESs are often leucine-rich (Kutay and Guttinger, 2005), we specifically mutated two conserved leucines in the carboxy tail (2xGFP- $\alpha 2^{L,L\rightarrow A,A}$; Figure 3D), which altered localization to both nuclei and cytoplasm similar to treatment of 2xGFP- $\alpha 2$ tail with LMB. Identical results were obtained using the carboxy tail of AMPK $\alpha 1$ (data not shown), which also contains the same conserved bulky hydrophobic residues (including leucines) at the same positions and suggests that any difference between AMPK $\alpha 1$ and AMPK $\alpha 2$ localization is not due to their tail sequences. Using a larger GFP-tagged construct containing full-length AMPK α with the dual leucine mutations (GFP-AMPK $\alpha^{L,L\rightarrow A,A}$) resulted in even more pronounced accumulation in the nucleus (Figure 3, E and F), perhaps because of diminished nondirectional diffusion through the nuclear pore or the presence of nuclear import signals elsewhere on AMPK.

Addition of the carboxy-terminal tail of AMPK $\alpha 2$ -2xGFP was even sufficient to overcome nuclear targeting via the SV40 NLS (SV40-NLS $\alpha 2$ tail, Figure 3B). This effect was not due to inactivation of the SV40 NLS, because treatment with LMB induced accumulation of the NLS-containing protein in the nucleus (Supplementary Figure 1). As the tail appears to act as a CRM1-dependent NES, we compared the AMPK α

tail sequence to known CRM1-dependent NESs (Figure 3C). Although the precise positioning of key residues for CRM1-dependent NESs vary, they are generally highly enriched for bulky hydrophobic amino acids (ϕ = leucine, isoleucine, phenylalanine, valine, and methionine) at specified spacings (ϕ -x-2/3- ϕ -x-2/3- ϕ -x- ϕ ; Kutay and Guttinger, 2005), generally consistent with an α helix. AMPK α carboxy tails are also enriched for bulky hydrophobic amino acids (Figure 1B), only one residue away from being a canonical NES (Figure 3C). (Yeast SNF1 does indeed match the consensus NES.) However, other proteins with defined NESs, including the nuclear-cytoplasmic shuttling heat-stress protein, HsfA2, also vary from the canonical consensus containing either four or five bulky hydrophobic residues at more flexible spacings (Heerklotz *et al.*, 2001). According to these more flexible criteria, AMPK α proteins in animals may also match the CRM1 consensus sequence.

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

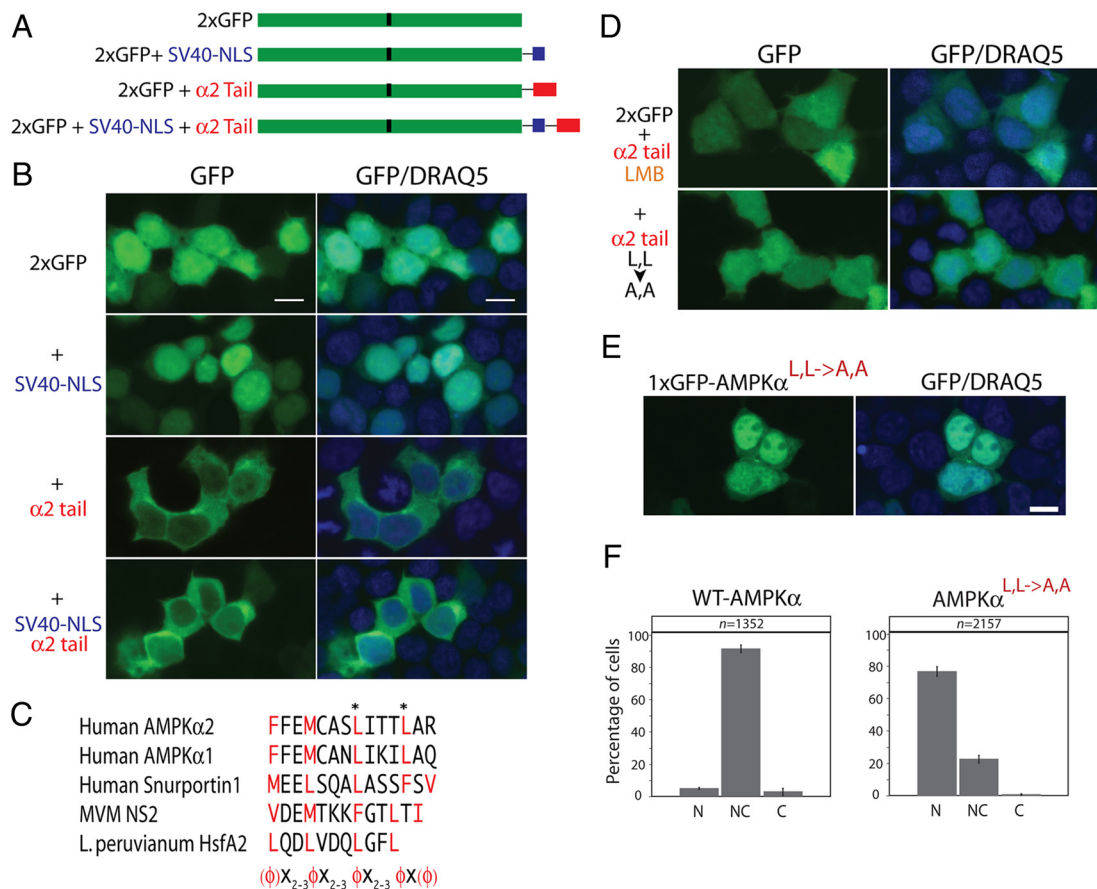


Figure 3. The carboxy-terminal tail of AMPK α functions as an NES. (A) Schematic representation of the four constructs containing two tandem GFP molecules (2xGFP) with or without the carboxy-terminal 23 amino acids of AMPK $\alpha 2$ (red) and/or the SV40 NLS (blue) used in B. (B) HEK293 cells transfected with constructs containing 2xGFP alone, 2xGFP with the SV40-NLS, 2xGFP with the AMPK $\alpha 2$ tail, or 2xGFP with both the SV40 NLS and the $\alpha 2$ tail. Nuclei were stained with DRAQ5 (blue). (C) Alignment of the AMPK $\alpha 2$ /AMPK $\alpha 1$ tail with confirmed CRM1-dependent NESs. The CRM1-NES consensus sequence is shown, consisting of either four or five bulky hydrophobic amino acids (ϕ , red) with variable spacing between them. Key leucine residues (often enriched in CRM1 NES sequences), L546 and L550, that were mutated for D are highlighted with asterisks (*). (D) Cells expressing either 2xGFP fused to the wild-type $\alpha 2$ tail and treated with the CRM1-specific inhibitor leptomycin B (LMB), or 2xGFP with L546A and L550A (L,L \rightarrow A,A) mutations in the $\alpha 2$ tail, to block CRM1-mediated nuclear export (compare to $\alpha 2$ tail in B). (E) Cells transfected with a GFP-tagged full-length AMPK $\alpha 2^{L,L\rightarrow A,A}$ mutant. (F) Scoring of cells for subcellular localization of GFP-AMPK α with the L,L \rightarrow A,A mutations to nucleus (N), nucleus and cytoplasm (NC) or cytoplasm (C; as in Figure 2D). Bar, 10 μ m.

AMPK α Cytoplasmic Localization Can Be Restored Using Other Defined NESs

Having identified sequences required for NES function at the carboxy-terminus of AMPK α , we wondered whether other well-documented NESs would function in lieu of the AMPK α carboxy-tail sequence. First, we further refined the putative AMPK α NES to within the carboxy-terminal 14 amino acids based on sequence alignment with other previously characterized CRM1-dependent NESs (Figure 3C) and confirmed the functional consequences of truncating only the final 14 amino acids (AMPK $\alpha\Delta C^{538}$), because this shorter truncation also localizes to the nucleus (Figure 4A). We then chose two well-characterized NESs to replace the putative AMPK α NES; there is a crystal structure of snurportin-1 (SNUPN) bound to CRM1 (Dong *et al.*, 2009), whereas PKIA contains a distinct but also well-characterized NES (Fornerod *et al.*, 1997). Both NESs served to restore the AMPK $\alpha\Delta C^{538}$ fusion protein to the cytoplasm (Figure 4A). The transplanted NESs did not act by disrupting nuclear import, as both constructs showed increased nuclear localization in the presence of LMB (data not shown). Additionally, the

SNUPN-NES and PKIA-NES AMPK α chimeric constructs retain significant affinity for β and γ by coimmunoprecipitation (Figure 4B), despite having no sequence identity with the AMPK α tail, indicating that these localization changes are not due to disruption of the AMPK heterotrimer.

As further confirmation that AMPK $\alpha/\beta/\gamma$ binding is not responsible for these α -tail-mediated changes in localization, we found that the construct which most strongly abolishes AMPK α cytoplasmic localization (AMPK $\alpha^{L,L\rightarrow A,A}$), contains the fewest amino acid changes, and retains essentially wild-type binding to the β and γ subunits (Figure 4B).

The Carboxy-Terminal AMPK α Tail Is Not Required for Binding β/γ Subunits

Although the carboxy tail of AMPK α appears to act as a NES, previous immunoprecipitation experiments with transfected cells mapped the β -binding site to amino acids 313–473 of AMPK α (Iseli *et al.*, 2005), indicating that the carboxy tail of AMPK α is not required for association with the β/γ subunits. The AMPK heterotrimer crystal structures also indicate that the γ subunit has minimal contact

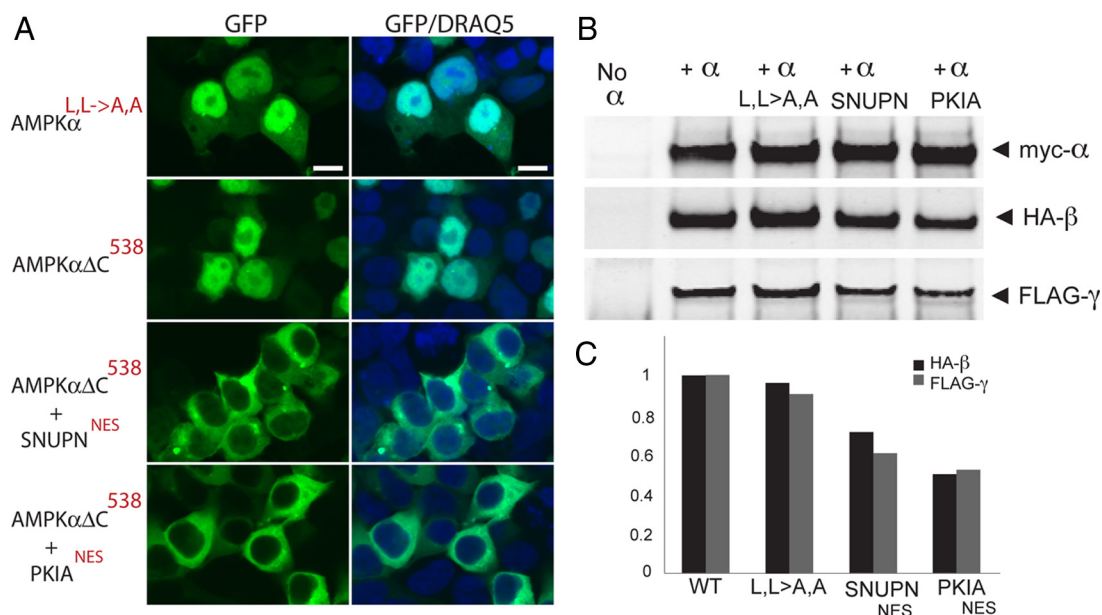


Figure 4. Previously identified NESs can substitute for the carboxy-terminus of AMPK α to restore cytoplasmic localization. (A) HEK 293 cells transiently transfected with GFP-tagged AMPK α 2 constructs containing the L546A and L550A mutations (AMPK $\alpha^{L,L\rightarrow A,A}$), a 14-amino acid C-terminal deletion (AMPK $\alpha\Delta C^{538}$), or with the 14 C-terminal residues of AMPK α 2 replaced with the CRM1-dependent NESs from human snurportin-1 (SNUPN, MEELSQLASSFSV, 14 amino acids) or cyclic-AMP-dependent protein kinase inhibitor α (PKIA, SNELALKLAGLDI, 13 amino acids). Bar, 10 μ m. (B) AMPK α , β , and γ subunits were coimmunoprecipitated with anti-myc antibody from HEK cell lysates either expressing HA-tagged AMPK β 1 and FLAG-tagged AMPK γ 1 alone (first lane), or coexpressed with myc-tagged wild-type AMPK α , L546A/L550A (L,L \rightarrow A,A) AMPK α , or SNUPN and PKIA AMPK α -NES chimeras. (C) The relative association of β and γ subunits with myc-tagged wild-type, L,L \rightarrow A,A mutant, or NES chimera AMPK α variants normalized to AMPK α wild-type levels (WT), calculated from the protein band intensities in B.

with the carboxy tail of AMPK α /SNF1 (Amodeo *et al.*, 2007; Xiao *et al.*, 2007). Using coimmunoprecipitation experiments, we also confirmed that the carboxy tail is not required for AMPK α / β / γ association in transfected cells, as β and γ both still associate with AMPK $\alpha\Delta C$ (Supplementary Figure 2). However, the tail may increase complex association, affinity and/or stability (Iseli *et al.*, 2005 and Supplementary Figure 2).

AMPK α Nuclear Entry Is Dependent on Ran

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

Several signaling proteins, including ERK2 and MEK1, have a conserved sequence motif (TPT or SPS), in which phosphorylation of these residues will induce translocation into the nucleus in a Ran-independent manner (Chuderland *et al.*, 2008). Although the AMPK α isoforms contain similar sequences (TPS in α 1 and TPT in α 2), mutation of these residues to alanine or phosphomimetic glutamic acid residues failed to affect the localization of the truncated form of AMPK α (data not shown).

To further distinguish between the possible import pathways responsible for AMPK translocation, we examined the effect of a Ran mutant (RanQ69L) that halts Ran-dependent nuclear import and export by blocking its GTP hydrolysis (Bischoff *et al.*, 1994). Transiently transfecting cells with this

Ran mutant along with GFP-tagged AMPK α , we see that AMPK α is restricted to the cytoplasm (2nd row, Figure 5), as is its upstream activator LKB1 (Dorfman and Macara, 2008; 4th row, Figure 5). Even the carboxy-terminal AMPK α truncation (AMPK $\alpha\Delta C$), which is normally strongly nuclear, localizes similarly to full-length AMPK α in the presence of RanQ69L (3rd row, Figure 5), indicating that AMPK α is normally basally imported via a Ran-GDP-dependent pathway.

Conventional NLSs are typically enriched for a single cluster of basic amino acids (K/R; e.g., SV40 NLS) or are separated in a bipartite manner by a linker region of 10–12 residues (Leung *et al.*, 2003). AMPK α 2 but not AMPK α 1 has been proposed to contain a K-K/R-x-K/R NLS within the kinase domain that is activated by leptin in C2C12 cells (Suzuki *et al.*, 2007), allowing differential localization between AMPK α 1 and AMPK α 2. Although it is not known whether HEK cells respond to leptin, we did not observe any difference in localization between AMPK α 1 and AMPK α 2 with or without mutations in this putative kinase domain NLS (data not shown), indicating that this leptin-stimulated nuclear translocation does not function in HEK cells. Along with our findings using RanQ69L and the nuclear localization of the truncated AMPK α in several cell types in *Drosophila*, these results ultimately suggest that AMPK contains another Ran-dependent NLS, either elsewhere in α , or in the β or γ subunits that is basally active.

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

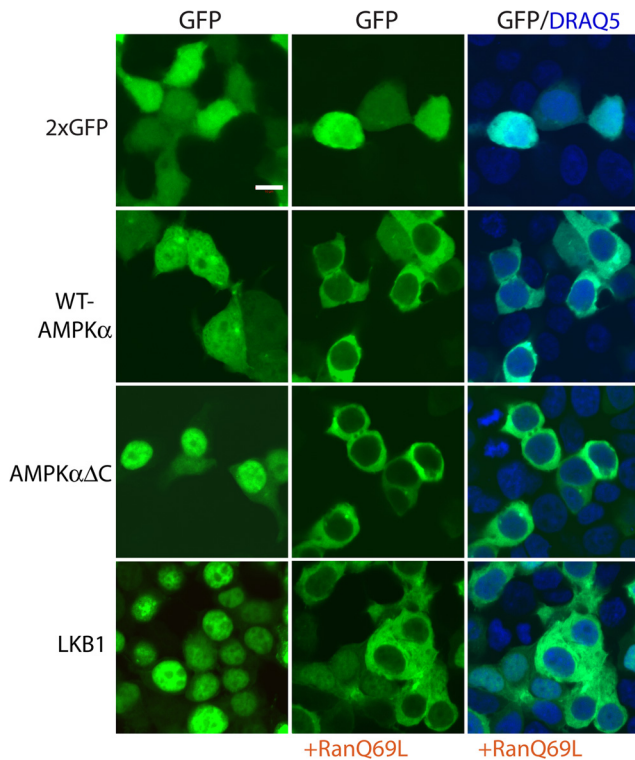


Figure 5. AMPK α , like LKB1, requires Ran-GTP hydrolysis for nuclear import. HEK 293 cells transiently transfected with constructs expressing two fused GFP molecules (2xGFP), GFP-tagged full-length AMPK α 2, truncated AMPK α 2 (AMPK α Δ C) or LKB1, with or without the GTP-hydrolysis defective Ran mutant RanQ69L. Nuclei are stained with DRAQ5 (blue). Bar, 10 μ m.

Heat Shock Increases Nuclear AMPK α In Vivo

Numerous in vitro studies have suggested changing cellular environments and conditions may change AMPK α localization, including alkaline pH, heat shock and oxidative stress, and leptin stimulation (Hong and Carlson, 2007; Kodiha *et al.*, 2007; Suzuki *et al.*, 2007). We wondered whether these

stressors that affect AMPK α localization in vitro, might also affect AMPK α localization in vivo. Indeed, using live transgenic animal (larvae) imaging, we found that heat-shock induced nuclear enrichment of AMPK α in vivo (Figure 6). Although both GFP- and mCherry-tagged AMPK α increased nuclear enrichment upon heat shock (Figure 6, D and E), other GFP-tagged proteins, including APC2, which contains both NES (Rosin-Arbesfeld *et al.*, 2000) and NLS (Zhang *et al.*, 2000) sequences, did not localize to the nucleus under heat shock (Figure 6F). AMPK α without the carboxy-terminal NES did not appear to change localization significantly (data not shown) because it already appears nuclear (Figure 6C). Other stressors, including inducing oxidative stress by feeding larvae paraquat and food starvation, did not alter AMPK α localization as they had in vitro (data not shown).

The Truncated Nuclear AMPK α Isoform Shows Reduced Phosphorylation In Vivo

Although there are clear differences in AMPK α localization dependent on the carboxy-terminal putative NES, we wondered what downstream consequences might be elicited. For example, LKB1, an upstream activator of AMPK α , requires cytoplasmic localization for activation (Baas *et al.*, 2003; Boudeau *et al.*, 2003a). To determine whether or not the differentially localized AMPK α , with or without the putative NES, might also affect the phosphorylation of the invariant Thr172 (Thr184 in *Drosophila*) that is required for AMPK activity (Lizcano *et al.*, 2004), we measured the phosphorylation levels of Thr184 in transgenic *Drosophila* animals expressing either wild-type or truncated AMPK α Δ C by Western blot. Quantification of phospho-AMPK α (pAMPK α) in either total lysates (Figure 7A) or immunoprecipitated AMPK α , normalized to total AMPK α levels (Figure 7B), indicates that only ~20% of the truncated nuclear-enriched protein is phosphorylated relative to wild-type full-length protein.

We wondered whether the physiological stress of heat shock that causes AMPK α translocation into the nucleus (Figure 6) would have any consequences for AMPK function based on the well-known requirement that a conserved threonine in the AMPK α activation loop must be phosphor-

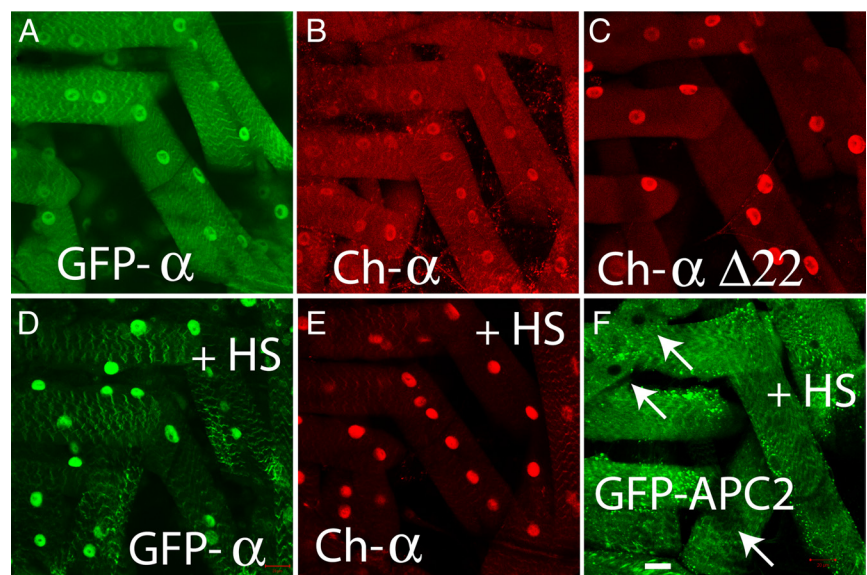


Figure 6. Heat-shocked transgenic larvae demonstrate increased nuclear localization of AMPK α in vivo. Live animal images of 3rd instar *Drosophila* larvae expressing GFP-tagged (A and D) or mCherry-tagged wild-type AMPK α (B and E), mCherry-tagged truncated AMPK α Δ C (C), or GFP-tagged APC2 (containing both NLS and NES sequences). Animals in D–F were subjected to heat shock at 37°C for 1 h, followed by a 15-min recovery at 25°C. All transgenic proteins are expressed using the *Gal4-UAS* system, driven by *Ubiquitin-Gal4*. Bar, 20 μ m.

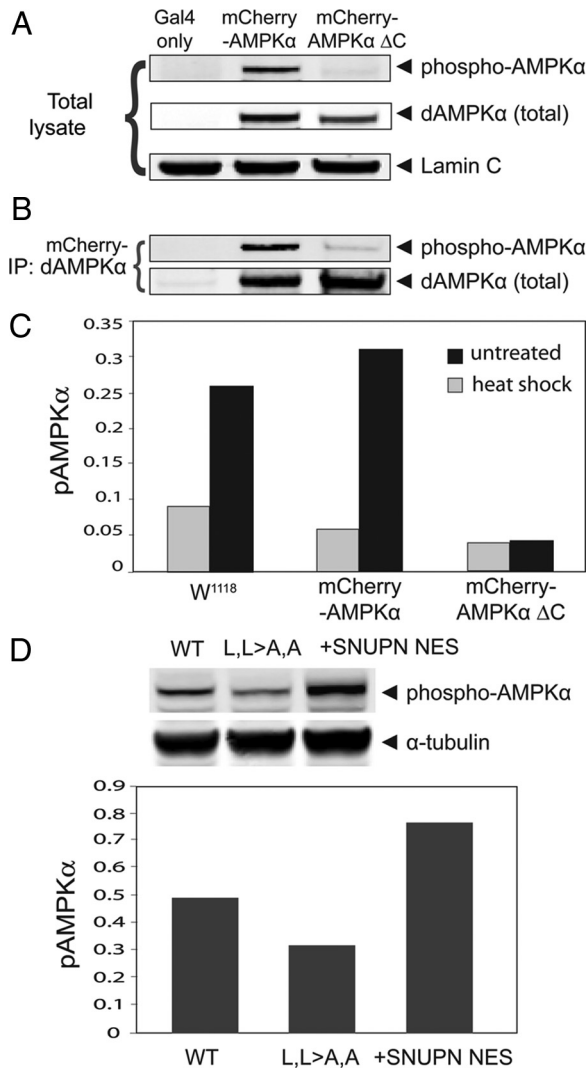


Figure 7. Phosphorylation of the activation loop threonine (T184) AMPK α is reduced in a nuclear enriched form in *Drosophila* in vivo and increased in the cytoplasmic form in human cells in vitro. (A) Western blot of total lysates from transgenic *Drosophila* flies expressing Gal4 alone, mCherry-tagged full-length AMPK α , or mCherry-tagged truncated AMPK α (AMPK α ΔC) probed for phospho-AMPK α , total AMPK α , and Lamin C (loading control). (B) Western blot of proteins immunoprecipitated with anti-AMPK α antibody from fly lysates from the above fly lines, probed for phospho- and total AMPK α . Both AMPK α protein constructs are overexpressed using the Gal4-UAS system, driven by *Ubiquitin-Gal4*. (C) Heat-shock stress (■) on live larvae induces increased activation loop threonine phosphorylation (pAMPK α) of wild-type (mCherry-AMPK α) and endogenous (W¹¹¹⁸) AMPK α but not AMPK α missing the NES (AMPK α ΔC). Standardized to Lamin C as a loading control. (D) Cytoplasmic AMPK α isoforms, wild-type (WT), and WT substituted with the snurportin (SNUPN) NES, demonstrate increased activation loop threonine phosphorylation compared with the nuclear (L,L>A,A) isoform. Standardized to tubulin as a loading control.

ylated for AMPK activity (Lizcano *et al.*, 2004). Indeed, although both endogenous untagged and wild-type mCherry-tagged AMPK α displayed dramatic increases in phospho-AMPK α upon heat shock, the version missing the NES showed no change in phospho-AMPK α (Figure 7C). Importantly, decreased phosphorylation of predominantly

nuclear localized AMPK α appears to be highly conserved as cytoplasmic localization of AMPK α isoforms in human embryonic kidney (HEK) cells show increased phospho-AMPK α compared with the nuclear-enriched AMPK α isoform (Figure 7D). The importance of phospho-AMPK α regulation as an indicator of AMPK activity is demonstrated by the tight regulation between introduced transgenic/exogenous phospho-AMPK α and resulting in decreased endogenous phospho-AMPK α levels (Supplementary Figure 3).

DISCUSSION

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

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

Previous studies have elucidated both nuclear and cytoplasmic targets of AMPK. In the cytoplasm, AMPK most notably phosphorylates and inhibits ACC, a rate-limiting enzyme required for fatty acid synthesis (Carling *et al.*, 1989). Conversely, there are several known nuclear targets of AMPK (Leff, 2003; Bronner *et al.*, 2004; Jager *et al.*, 2007; Narkar *et al.*, 2008), including PGC1 α and PPAR α / γ / δ , which regulate transcription in the nucleus. Furthermore, AMPK accumulation in or dispersion from the nucleus can be regulated by exercise, cellular stress, and circadian rhythms. In one study, exercise increased induced nuclear translocation of AMPK α 2 in skeletal muscle (McGee *et al.*, 2003), where AMPK is known to activate PGC1 α and subsequent gene transcription (Jager *et al.*, 2007). Another more recent study has demonstrated that AMPK α 1 in the nucleus fluctuates in a circadian manner, regulating the circadian clock by inducing degradation of cryptochrome 1 (Lamia *et al.*, 2009). Clearly, mechanisms that regulate AMPK subcellular localization are widely utilized to modulate its access to downstream substrates.

The nuclear pore complex (NPC) plays a key role as a molecular sieve to help compartmentalize proteins between nucleus and cytoplasm. Indeed, many nucleocytoplasmic shuttling proteins contain signals to direct them in and/or out through the NPC (Yasuhara *et al.*, 2009). An AMPK trimer would far exceed the generally accepted nuclear pore diffusional cutoff size of 40 kDa (Gorlich and Kutay, 1999) and would thus also need such NPC shuttling signals. Despite distinct AMPK targets in both the nucleus and cyto-

plasm, the mechanisms for regulating its localization remain unclear, particularly in organisms that have only single $\alpha/\beta/\gamma$ subunits (e.g., *Drosophila*), where localization models based on different genetically encoded isoforms are not applicable.

One previous model for nuclear AMPK localization proposes that AMPK α 2, but not AMPK α 1, contains an NLS in the kinase domain that becomes functional only upon addition of leptin (Suzuki *et al.*, 2007). Because not all organisms encode leptin and we did not observe any localization differences for AMPK α 2 when mutating key residues in the proposed leptin-stimulated NLS in transfected HEK293 cells (data not shown), regulation of AMPK localization is likely cell type-dependent. This can also be seen in the differential localization between AMPK α 1 and AMPK α 2 isoforms in insulinoma cells (Salt *et al.*, 1998), in contrast to HEK cells, where α 1 and α 2 localize similarly (data not shown). These effects are also seen with the β subunit, because only AMPK β 1 enriches in the nucleus upon mutation of two phosphorylation sites in HEK cells (Warden *et al.*, 2001), whereas AMPK complexes containing β 2 preferentially localize in the nucleus in C2C12 cells under leptin treatment (Suzuki *et al.*, 2007). Distinct SNF1 β subunits are also thought to promote differential subcellular localization in yeast (Vincent *et al.*, 2001). Altogether, these results suggest that cells differentially regulate AMPK localization, and thus activity, through multiple pathways, depending on their unique metabolic requirements and hormonal responses.

The findings herein identify amino acids at the carboxy-terminus of AMPK α that modulate its nuclear export (Figures 1 and 3) that are nearly universal, with these sequences found across phyla (Figures 1 and 3), closely matching the consensus sequence for the leucine-rich CRM1-dependent NESs (la Cour *et al.*, 2004). Further, we identify a stress treatment in vivo, heat shock, which causes nuclear translocation of AMPK α , which requires the NES for increased phospho-AMPK α under this stressor. Because phosphorylation of the activation loop is required for AMPK activity, this mechanism might be expected to be beneficial for surviving physiological stress.

As C-terminally truncated AMPK α localizes to the nucleus in vitro in HEK cells and in vivo in *Drosophila* under unstressed conditions, this suggests that AMPK α is basally imported to the nucleus and that regulation of AMPK localization in response to stress would predominantly be affected through modulation of the export pathway. Adding further complexity, localization of the AMPK complex and partitioning of specific subunits may also be both cell type- and context-dependent. For instance, in multicellular organisms certain tissues (e.g., fat) provide energy to other tissues/organs (e.g., muscle) at their own expense. In these cases, AMPK activation likely leads to different physiological outcomes between cell types, such as increased lipid mobilization in fat cells versus increased lipid uptake in muscle cells. In these situations, differential localization of AMPK in distinct cell types could be used to generate these different cellular responses.

A further avenue of inquiry in regulation of AMPK localization is in the possible effects of posttranslational modification of AMPK subunits on the accessibility of the carboxy-tail of AMPK α . As the AMPK α carboxy-tail folds into a pocket formed by the α and β subunits after a long flexible loop (Amodeo *et al.*, 2007; Xiao *et al.*, 2007), altering the strength of these interactions could change its accessibility to CRM1, thus activating or inhibiting nuclear export. Although there are conserved residues that could be posttranslationally modified in the AMPK α carboxy-tail adjacent to

the putative NES, we have so far been unable to identify residues flanking the NES that change the subcellular localization of AMPK α in vitro or are required for genetic rescue in vivo, as described earlier. One tantalizing possibility is that the potential phosphoserine mutations in β 1 increase nuclear localization of AMPK by enhancing β interactions with the AMPK α tail, thus blocking nuclear export.

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

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