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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 (AMPKα). When this sequence is modified AMPKα shows increased nuclear localization via a Ran-dependent import pathway. Cytoplasmic localization can be restored by substituting well-defined snurportin-1 or protein kinase A inhibitor (PKIA) CRM1-binding NESs into AMPKα. We demonstrate a functional requirement in vivo for the AMPKα carboxy-terminal NES, as transgenic Drosophila expressing AMPKα lacking this NES fail to rescue lethality of AMPKα 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 AMPKα via its C-terminal NES provides an additional mechanism for cells to use in the regulation of AMPK activity and localization.

INTRODUCTION

AMP-activated protein kinase (AMPK) consists of a trimer containing a catalytic serine-threonine kinase subunit (α) and two regulatory subunits (β and γ; Davies et al., 1994; Stapleton et al., 1994; Gao et al., 1996). Many cellular stressors activate AMPK, including oxidative stress, heat shock, and low energy levels (low ATP-AMP ratios). In response, AMPK restores energetic balance by inhibiting anabolic processes that consume energy, whereas activating catabolic processes that produce energy. Thus, modulation of 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 AMPKα orthologue in yeast, from the cytoplasm to the nucleus (Hong and Carlson, 2007). In mammalian cells, leptin (Suzuki et al., 2007) and heat shock—possibly through MEK signaling (Kodih et al., 2007)—can also cause translocation of AMPKα subunits to the nucleus. In addition, isoform-specific AMPK subunits have also been shown to accumulate in the nucleus in a circadian 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-1α (PGC-1α), 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 AMPKα amino-terminus is highly conserved, containing the serine-threonine kinase domain, the AMPKα carboxy-terminus does not contain any known functional motifs outside of the βγ binding sites. We previously noted that the final carboxy-terminal 20 amino acids of AMPKα are highly conserved across diverse species (Brennan and Temple, 2007). In this study, we utilized genetic and cell biological approaches to evaluate potential functions for the AMPKα C-terminus. We identify a critical new function for the carboxy-terminal amino acids of AMPKα in vivo, which affects AMPKα subcellular localization, phosphorylation, and ultimately organiational viability.

MATERIALS AND METHODS

Generating Transgenic Flies

Truncated dAMPKαC was cloned into a pUAST vector as a BglII-EcoRI fragment inserted into the transcription (www.bflybase.org) by inserting a stop codon after Proline 561 of wild-type dAMPKα using PCR-based mutagenesis. The GFP-dAMPKα and mCherry-dAMPKα fusion proteins were made using green fluorescent protein (GFP) or mCherry at the fused in-frame to the N-terminal and C-terminal 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-dAMPKα and UAS-dAMPKαC alleles expressed in sensory neurons using a B2090-80GAL4 driver (Gao et al., 1996) and recombined with UAS-actin:GFP to visualize sensory neurons, as described previously (Meditino et al., 2006). For rescue constructs, both rescues were constructed using the Ubiquitin-Gal4 driver and crossed into dmpkα null mutants. Adult males were scored for rescue by nanyth element 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 constructs and GFP constructs of wild-type dAMPKα to Ubiquitin-Gal4. Third instar larval 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 (Mirose et al., 2007) using the 488-nm argon stock 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 AMPKα2 were amplified by PCR using Pfu DNA Polymerase (Stratagene, La Jolla, CA) from rat AMPKα2 (Sigma, St. Louis, MO; plasmid 15951) and inserted into pEGFP-C1 (Clontech Laboratories, Palo Alto, CA). Mouse AMPKα wild-type and truncated versions were generated using PCR amplification from mouse cDNA and inserted into pEGFP-C1. The SV40-NLS (nuclear localization signal; PKKRRKVC), AMPKα2, and AMPKα1 C-terminal tail tags were cloned into the C-terminus of the pEGFP-C1:GFP construct. For expressing the SV40NLS and the AMPKα2 C-terminal tag together, the SV40-NLS coding sequence was inserted into the N-terminal forward primer for amplification of the GFP-coding sequence. The SV40NLS-GFP ampiclon was then inserted into a pEGFP-C2 plasmid containing the AMPKα2 C-terminal tail-coding sequence at the C-terminus of that plasmid.

L564A and L550A substitutions in the AMPKα1 C-terminal tail sequence were introduced into the reverse primer sequence and cloned using site-directed mutagenesis. Hemagglutinin (HA)-tagged human AMPKα1 and rat AMPKβ1 constructs were gifts from Reuben Shaw (UCSD), and mCherry-AMPKα1 was produced by inserting human AMPKα1 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 AMPKα and the AMPKα1/α2 C-terminal tail sequence at the C-terminus of that plasmid.

To generate AMPK-CRM1-NS (nuclear export sequence) fusions, sequences encoding residues Met1 to Val14 (human Snurportin-1) or Ser5 to liles (human SMAD4) were inserted into an independent protein kinase vector and cloned using site-directed mutagenesis. Hemagglutinin (HA)-tagged human AMPKα1 and rat AMPKβ1 constructs were gifts from Reuben Shaw (UCSD), and mCherry-AMPKα1 was produced by inserting human AMPKα1 into a modified pEGFP-N1 vector with GFP replaced by Flag:mCherry (gift from Tom Maynard, UNC-Chapel Hill). Muc-tagged clones of wild-type AMPKα and the AMPKα1/α2 C-terminal tail sequence at the C-terminus of that plasmid.

AMPKα/b/γ Coimmunoprecipitation

HEK293 cells were transiently transfected with myc-tagged AMPKα2, HA-tagged AMPKα1, and FLA-tagged Cherry-AMPKα1 in 10-cm dish. 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 by following overnight incubation of 1 ml of each lysate in mg/ml using Bio-Rad DC protein assay (Richmond, CA). Immune complexes were formed by incubation of 100 µl of anti-AMPKα1 mouse mAb (hemagglutinin (HA)-tagged human AMPKα1 and rat AMPKβ1 constructs were gifts from Reuben Shaw (UCSD), and mCherry-AMPKα1 was produced by inserting human AMPKα1 into a modified pEGFP-N1 vector with GFP replaced by Flag:mCherry (gift from Tom Maynard, UNC-Chapel Hill). Muc-tagged clones of wild-type AMPKα and the AMPKα1/α2 C-terminal tail sequence at the C-terminus of that plasmid.

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 DRAQ5 (Biotium Limited, Shipped, Leicestershire, United Kingdom) to visualize nuclei. For live cell imaging, 24 h after transfection the cells were examined using confocal microscopy (Olympus FV1000) to visualize nuclei. Tiling images were also taken using the Olympus FV1000 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 sequenced to verify fidelity. For myc-tagged constructs, the coding regions were amplified from the above GFP-AMPKα2 plasmids and inserted into the pCMV-myc vector.

Immunohistochemistry

AMPKα localization in fly tissue was determined using standard dissection and immunostaining procedures (Medina et al., 2006). The primary antibody was anti-AMPKα (mouse; Abcam, 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.

AMPKα Immunoprecipitation

Drosophila protein lysates for immunoprecipitation were prepared by collecting equal numbers of male and female flies (50 total) of each genotype in a 1.5-ml tube. One milliliter lysis buffer (20 mM Tris-Cl pH 7.5, 130 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate 1 mM β-glycerophosphate, 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 µl of anti-AMPKα1 mouse mAb (hemagglutinin (HA)-tagged human AMPKα1 and rat AMPKβ1 constructs were gifts from Reuben Shaw (UCSD), and mCherry-AMPKα1 was produced by inserting human AMPKα1 into a modified pEGFP-N1 vector with GFP replaced by Flag:mCherry (gift from Tom Maynard, UNC-Chapel Hill). Muc-tagged clones of wild-type AMPKα and the AMPKα1/α2 C-terminal tail sequence at the C-terminus of that plasmid.

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 µg 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 µg/ml) for 1 h followed by treatment with LMB (10 ng/ml, Sigma) and cycloheximide (1 µg/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.
Figure 1. AMPKα contains a highly conserved carboxy-terminal tail required for function in vivo. (A) Schematic of AMPKα homology with blocks highlighting regions with >50% conservation among bilatera (rat AMPKα2 as template; calculated by ProtPhyER). 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 orthologues with conserved residues highlighted in blue and invariant residues in red (AMPKα2 for species with multiple AMPKα subunits). (C) Peripheral neurons visualized by Gal4 109(2)80-driven expression of UAS-actin::GFP in wild-type (WT) Drosophila 2nd instar larvae, AMPKα loss of function (AMPKα3) larvae, AMPKα3 expressing a WT AMPKα transgene (80>UAS-AMPKα), or AMPKα2 expressing a C-terminally truncated AMPKα transgene (80>UAS-AMPKαΔC). Dendritic swellings are highlighted with arrows. Bar, 10 μm.

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

RESULTS

The AMPKα Carboxy-Terminus Is Required for Function

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

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

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

To explore why a 22-amino acid carboxy-terminal deletion of AMPKα fails to rescue AMPKα null mutants, we examined the localization of the truncated protein both by antibody immunohistochemistry and visualization of fluorescently tagged fusion protein. Expression of untagged AMPKα in transgenic animals and subsequent detection of AMPKα by immunohistochemistry revealed very clear subcellular localization differences between full-length and truncated protein (AMPKαΔC; Figure 2A). AMPKαΔC 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 AMPKαΔC 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 AMPKα with and without the carboxy-terminus is most clearly demonstrated using a live transgenic animal simultaneously expressing both full-length and AMPKαΔC in the same cells (Figure 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 AMPKα and AMPKαΔC (Figure 2D).

The AMPKα Carboxy-Tail Functions as a LMB-sensitive NES

To elucidate whether the carboxy-terminus might act as a NES in AMPKα localization, we used a previously described mammalian cell assay to test for sequences that alter the subcellular localization of proteins (Frederick et al., 2008). We fused the AMPKα carboxy-tail in frame at the C-terminus of two tandem GFP molecules (Figure 3A). Two consecutively 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 AMPKα2 (2xGFP-a2 tail) leads to localization predominantly in the cytoplasm (Figure 3B).

Because a previous study demonstrated that endogenous (untagged) AMPK protein in HeLa cells enriches in the nucleus upon treatment with LMB (Kodiba et al., 2007), a
specific inhibitor of CRM1-mediated nuclear export (Kutay and Guttinger, 2005), we tested whether LMB specifically inhibits this AMPKα tail-dependent nuclear export. Indeed, LMB treatment does result in altered localization of the 2xGFP-α2 tail from predominantly cytoplasmic (Figure 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 AMPKα2 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-α2L1–A4; Figure 3D), which altered localization to both nuclei and cytoplasm similar to treatment of 2xGFP-α2 tail with LMB. Identical results were obtained using the carboxy tail of AMPKα1 (data not shown), which also contains the same conserved bulky hydrophobic residues (including leucines) at the same positions and suggests that any difference between AMPKα1 and AMPKα2 localization is not due to their tail sequences. Using a larger GFP-tagged construct containing full-length AMPKα with the dual leucine mutations (GFP-AMPKαL1–A4) 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 AMPKα2–2xGFP was even sufficient to overcome nuclear targeting via the SV40 NLS (SV40-NLS α2 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 AMPKα 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. AMPKα 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-cyttoplasmic shuttling heat-stress protein, HsfA2, also vary from the canonical consensus containing four or five bulky hydrophobic residues at more flexible spacings (Heerklotz et al., 2001). According to these more flexible criteria, AMPKα proteins in animals may also match the CRM1 consensus sequence.

AMPKα carboxy-termini contain other conserved residues, including a conserved cysteine and threonine (Figure 1B), suggesting that these residues may be modified in vivo to alter either AMPK activity or localization. In Drosophila, mutating the carboxy tail Cys573 to serine rescued both the neuronal phenotype and lethality of AMPKα null mutants (data not shown), indicating that this cysteine is not essential. Further, in mammalian cells, mutating Thr536 to either a phosphomimetic (aspartate) residue or to an alanine failed to affect the localization of the 2xGFP-α2 tail protein compared 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 2. AMPKα lacking the carboxy-terminus localizes predominantly to nuclei in vivo and in vitro. (A) Indirect immunofluorescence of AMPKα (red) staining with anti-AMPKα antibody on transgenic Droso phila 3rd instar larvae expressing either full-length wild-type AMPKα or the carboxy-truncated AMPKα (AMPKαAC). All transgenic proteins are expressed using the Gal4-UAS system, driven by Ubiquitin-Gal4 (A and B). Nuclei were stained with ToPro-3 (blue). (B) Live animal images of larvae expressing amino-terminal mCherry-tagged wild-type (mCherry-AMPKα) or truncated (mCherry-AMPKαAC) AMPKα, GFP-tagged wild-type α (GFP-AMPKα) alone or in combination with mCherry-tagged truncated α (mCherry-AMPKαA+C+GFP-AMPKα). Bar, 10 μm. (C) Co-expression of amino-terminal fluorescently tagged full-length AMPKα2 (mCherry-AMPKα2, red) and truncated (GFP-AMPKα2C, green) in the same cotransfected mammalian cells (HEK293). (D) Scoring of transfected cells for subcellular localization of GFP-tagged full-length (GFP-AMPKα) or truncated (GFP-AMPKαAC) AMPKα as primarily nuclear (N), both nuclear and cytoplasmic (NC), or primarily cytoplasmic (C). Proper scoring for each group was confirmed by quantification of nuclear and cytoplasmic fluorescence for >50 cells in each group; N-NC fluorescence ratios were >2.0, 0.7–2.0, and <0.7, for N, NC, and C, respectively. (E) Wild-type GFP-AMPKα control cells demonstrating roughly equal fluorescence in cytoplasm and nucleus. Nuclei were stained with DRAQ5 (blue). Bar, 10 μm.
AMPK Cytoplasmic Localization Can Be Restored Using Other Defined NESs

Having identified sequences required for NES function at the carboxy-terminus of AMPKα, we wondered whether other well-documented NESs would function in lieu of the AMPKα carboxy-tail sequence. First, we further refined the putative AMPKα NES to within the carboxy-terminal 14 amino acids based on sequence alignment with other previously characterized CRM1-dependent NESs (Figure 3C) and confirmed the functional consequences of truncating only the final 14 amino acids (AMPKα/AMPKβ/AMPKγ C538), because this shorter truncation also localizes to the nucleus (Figure 4A). We then chose two well-characterized NESs to replace the putative AMPKα NES; there is a crystal structure of snurportin-1 (SNUPN) bound to CRM1 (Dong et al., 2009), whereas PKIA contains a distinct but also well-characterized NES (Fornerod et al., 1997). Both NESs served to restore the AMPKα/AMPKβ/AMPKγ C538 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 AMPKα chimeric constructs retain significant affinity for β and γ by communoprecipitation (Figure 4B), despite having no sequence identity with the AMPKα tail, indicating that these localization changes are not due to disruption of the AMPK heterotrimer.

As further confirmation that AMPK α/β/γ binding is not responsible for these α-tail-mediated changes in localization, we found that the construct which most strongly abolishes AMPKα cytoplasmic localization (AMPKα/AMPKβ/AMPKγ L,L,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 AMPKα Tail Is Not Required for Binding β/γ Subunits**

Although the carboxy tail of AMPKα appears to act as a NES, previous immunoprecipitation experiments with transfected cells mapped the β-binding site to amino acids 313–473 of AMPKα (Iseli et al., 2005), indicating that the carboxy tail of AMPKα is not required for association with the β/γ subunits. The AMPK heterotrimer crystal structures also indicate that the γ subunit has minimal contact...
with the carboxy tail of AMPKα/SNF1 (Amodeo et al., 2007; Xiao et al., 2007). Using coimmunoprecipitation experiments, we also confirmed that the carboxy tail is not required for AMPKα/β/γ association in transfected cells, as β and γ both still associate with AMPKαC (Supplementary Figure 2). However, the tail may increase complex association, affinity and/or stability (Iseli et al., 2005 and Supplementary Figure 2).

**AMPKα Nuclear Entry Is Dependent on Ran**

As the AMPK trimer complex is too large to passively diffuse into the nucleus, we sought to clarify the mechanism of its active transport. 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., 2003). 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 AMPKα isoforms contain similar sequences (TPS in α1 and TPT in α2), mutation of these residues to alanine or phosphomimetic glutamic acid residues failed to affect the localization of the truncated form of AMPKα (data not shown).

To further distinguish between the possible import pathways responsible for AMPK translocation, we examined the effect of a Ran mutant (RanQ69L) that halts Ran-dependent nuclear import and export by blocking its GTP hydrolysis (Bischoff et al., 1994). Transiently transfecting cells with this Ran mutant along with GFP-tagged AMPKα, we see that AMPKα is restricted to the cytoplasm (2nd row, Figure 5), as is its upstream activator LKB1 (Dorfman and Macara, 2008; 4th row, Figure 5). Even the carboxy-terminal AMPKα truncation (AMPKαC), which is normally strongly nuclear, localizes similarly to full-length AMPKα in the presence of RanQ69L (3rd row, Figure 5), indicating that AMPKα 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). AMPKα2 but not AMPKα1 has been proposed to contain a K-K/R-x-K/R NLS within the kinase domain that is activated by leptin in C2C12 cells (Suzuki et al., 2007), allowing differential localization between AMPKα1 and AMPKα2. Although it is not known whether HEK cells respond to leptin, we did not observe any difference in localization between AMPKα1 and AMPKα2 with or without mutations in this putative kinase domain NLS (data not shown), indicating that this leptin-stimulated nuclear translocation does not function in HEK cells. Along with our findings using RanQ69L and the nuclear localization of the truncated AMPKα in several cell types in Drosophila, these results ultimately suggest that AMPK contains another Ran-dependent NLS, either elsewhere in α1, or in the β or γ subunits that is basally active.

We also found that the phosphorylation state of the truncated AMPKα does not affect its nuclear translocation, as HEK cells transfected with C-terminally truncated AMPKα constructs containing T172D and T172A mutations in the activation loop localized similarly to truncated AMPKα (data not shown).
Heat Shock Increases Nuclear AMPKα In Vivo
Numerous in vitro studies have suggested changing cellular environments and conditions may change AMPKα localization, including alkaline pH, heat shock and oxidative stress, and leptin stimulation (Hong and Carlson, 2007; Kodiha et al., 2007; Suzuki et al., 2007). We wondered whether these stressors that affect AMPKα localization in vitro, might also affect AMPKα localization in vivo. Indeed, using live transgenic animal (larvae) imaging, we found that heat-shock induced nuclear enrichment of AMPKα in vivo (Figure 6). Although both GFP- and mCherry-tagged AMPKα 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). AMPKα 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 AMPKα localization as they had in vitro (data not shown).

The Truncated Nuclear AMPKα Isoform Shows Reduced Phosphorylation In Vivo
Although there are clear differences in AMPKα localization dependent on the carboxy-terminal putative NES, we wondered what downstream consequences might be elicited. For example, LKB1, an upstream activator of AMPKα, requires cytoplasmic localization for activation (Baas et al., 2003; Boudeau et al., 2003a). To determine whether or not the differentially localized AMPKα, with or without the putative NES, might also affect the phosphorylation of the invariant Thr172 (Thr184 in Drosophila) that is required for AMPKα activity (Lizcano et al., 2004), we measured the phosphorylation levels of Thr184 in transgenic Drosophila animals expressing either wild-type or truncated AMPKα by Western blot. Quantification of phospho-AMPKα (pAMPKα) in either total lysates (Figure 7A) or immunoprecipitated AMPKα, normalized to total AMPKα 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 AMPKα 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 AMPKα activation loop must be phospho-
Western blot of total lysates from transgenic and increased in the cytoplasmic form in human cells in vitro. (A) Importantly, decreased phosphorylation of predominantly AMPK/Thr2004/Thr2003/Thr183 (pAMPK) and phosphorylated for AMPK activity (Lizcano et al., 2004). Indeed, although both endogenous untagged and wild-type mCherry-tagged AMPK displayed dramatic increases in phospho-AMPK upon heat shock, the version missing the NES showed no change in phospho-AMPK (Figure 7C). Importantly, decreased phosphorylation of predominantly nuclear localized AMPKα appears to be highly conserved as cytoplasmic localization of AMPKα isoforms in human embryonic kidney (HEK) cells show increased phospho-AMPKα compared with the nuclear-enriched AMPKα isoform (Figure 7D). The importance of phospho-AMPKα regulation as an indicator of AMPK activity is demonstrated by the tight regulation between introduced transgenic/exogenous phospho-AMPKα and resulting in decreased endogenous phospho-AMPKα 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 (Nézu et al., 1999; Boudeau et al., 2003b; Xie et al., 2009). Although the mechanism of LKB1 localization is complex, it involves other proteins, including the STRADs (STE-related adapter) that form a complex with LKB1, promoting nuclear export and inhibiting nuclear import (Boudeau et al., 2003a; Dorfman and Macara, 2008). Although LKB1 is an upstream activator of AMPKα (Lizcano et al., 2004) and both proteins are kinases, LKB1 can function without other subunits bound, 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 PGC1α and PPARα/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 AMPKα2 in skeletal muscle (McGee et al., 2003), where AMPK is known to activate PGC1α and subsequent gene transcription (Jager et al., 2007). Another more recent study has demonstrated that AMPKα1 in the nucleus fluctuates in a circadian manner, regulating the circadian clock by inducing degradation of cryptochrome 1 (Lamia et al., 2009). Clearly, mechanisms that regulate AMPK subcellular localization are widely utilized to modulate its access to downstream substrates.

The nuclear pore complex (NPC) plays a key role as a molecular sieve to help compartmentalize proteins between nucleus and cytoplasm. Indeed, many nucleocytoplasmic shuttling proteins contain signals to direct them in and/or out through the NPC (Yasuhara et al., 2009). An AMPK trimer would far exceed the generally accepted nuclear pore diffusional 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 α/β/γ subunits (e.g., *Drosophila*), where localization models based on different genetically encoded isoforms are not applicable.

One previous model for nuclear AMPK localization proposes that AMPKα2, but not AMPKα1, contains an NLS in the kinase domain that becomes functional only upon addition of leptin (Suzuki *et al.*, 2007). Because not all organisms encode leptin and we did not observe any localization differences for AMPKα2 when mutating key residues in the proposed leptin-stimulated NLS in transfected HEK293 cells (data not shown), regulation of AMPK localization is likely cell type-dependent. This can also be seen in the differential localization between AMPKα1 and AMPKα2 isoforms in insulinoma cells (Salt *et al.*, 1998), in contrast to HEK cells, where α1 and α2 localize similarly (data not shown). These effects are also seen with the β subunit, because only AMPKβ1 enriches in the nucleus upon mutation of two phosphorylation sites in HEK cells (Warden *et al.*, 2001), whereas AMPK complexes containing β2 preferentially localize in the nucleus in C2C12 cells under leptin treatment (Suzuki *et al.*, 2007). Distinct SNF1 β subunits are also thought to promote differential subcellular localization in yeast (Vincent *et al.*, 2001). Altogether, these results suggest that cells differentially regulate AMPK localization, and thus activity, through multiple pathways, depending on their unique metabolic requirements and hormonal responses.

The findings herein identify amino acids at the carboxyterminus of AMPKα that modulate its nuclear export (Figures 1 and 3) that are nearly universal, with these sequences found across phyla (Figures 1 and 3), closely matching the consensus sequence for the leucine-rich CRM1-dependent NESs (la Cour *et al.*, 2004). Further, we identify a stress treatment in vivo, heat shock, which causes nuclear translocation of AMPKα, which requires the NES for increased phopho-AMPKα 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 AMPKα localizes to the nucleus in vitro in HEK cells and in vivo in *Drosophila* under unstressed conditions, this suggests that AMPKα is basally imported to the nucleus and that regulation of AMPK localization in response to stress would predominantly be affected through modulation of the export pathway. Adding further complexity, localization of the AMPK complex and partitioning of specific subunits may also be both cell type- and context-dependent. For instance, in multicellular organisms certain tissues (e.g., fat) provide energy to other tissues/organs (e.g., muscle) at their own expense. In these cases, AMPK activation likely leads to different physiologically 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 AMPKα. As the AMPKα carboxy-tail folds into a pocket formed by the α and β subunits after a long flexible loop (Amodeo *et al.*, 2007; Xiao *et al.*, 2007), altering the strength of these interactions could change its accessibility to CRM1, thus activating or inhibiting nuclear export. Although there are conserved residues that could be posttranslationally modified in the AMPKα carboxy-tail adjacent to the putative NES, we have so far been unable to identify residues flanking the NES that change the subcellular localization of AMPKα in vitro or are required for genetic rescue in vivo, as described earlier. One tantalizing possibility is that the potential phosphoserine mutations in β1 increase nuclear localization of AMPK by enhancing β interactions with the AMPKα tail, thus blocking nuclear export.

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

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