AMP directly inhibits NDPK through a phosphoserine switch to maintain cellular homeostasis

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ABSTRACT AMP-activated protein kinase (AMPK) is a key energy sensor that regulates metabolism to maintain cellular energy balance. AMPK activation has also been proposed to mimic benefits of caloric restriction and exercise. Therefore, identifying downstream AMPK targets could elucidate new mechanisms for maintaining cellular energy homeostasis. We identified the phosphotransferase nucleoside diphosphate kinase (NDPK), which maintains pools of nucleotides, as a direct AMPK target through the use of two-dimensional differential in-gel electrophoresis. Furthermore, we mapped the AMPK/NDPK phosphorylation site (serine 120) as a functionally potent enzymatic “off switch” both in vivo and in vitro. Because ATP is usually the most abundant cellular nucleotide, NDPK would normally consume ATP, whereas AMPK would inhibit NDPK to conserve energy. It is intriguing that serine 120 is mutated in advanced neuroblastoma, which suggests a mechanism by which NDPK in neuroblastoma can no longer be inhibited by AMPK-mediated phosphorylation. This novel placement of AMPK upstream and directly regulating NDPK activity has widespread implications for cellular energy/nucleotide balance, and we demonstrate in vivo that increased NDPK activity leads to susceptibility to energy deprivation–induced death.

INTRODUCTION

AMP-activated protein kinase (AMPK) functions as a cellular energy sensor activated by hypoxia, low glucose, and other stressors that lower ATP levels and raise AMP levels (Hardie et al., 2006; Shaw, 2006). In response to AMP/ATP ratio–altering events, activated AMPK turns on ATP-generating pathways and inhibits ATP-consuming pathways, thereby restoring the AMP/ATP ratio (Williams and Brenman, 2008). AMPK was first discovered as a protein whose activity inhibited preparations of acetyl-CoA carboxylase (ACC1), a regulator of cellular fatty acid synthesis (Winder et al., 1997). AMPK is a heterotrimeric protein with a 63-kDa catalytic α subunit and two regulatory β and γ subunits (38 and 36 kDa, respectively), each of which is encoded by distinct genes (α1, α2; β1, β2; γ1, γ2, γ3; Davies et al., 1994; Mitchellhill et al., 1994; Gao et al., 1996; Stapleton et al., 1996; Nielsen et al., 2003), and AMPK is implicated in a number of signaling pathways (Hardie, 2004, 2007; Shaw, 2009).

Upstream activation of AMPK is mediated by the tumor suppressor liver kinase B1 (LKB1; Shaw et al., 2004) and Ca2+/calmodulin–dependent kinase β (Hawley et al., 2005; Hurley et al., 2005). Although LKB1 has clear roles in metabolism, LKB1 is also known as Par-4 in Caenorhabditis elegans, a key regulator of cell polarity (Watts et al., 2000; Chartier et al., 2011). Nonetheless, all known AMPK upstream kinases phosphorylate AMPKα threonine 172 (Thr-172) in the “activation loop” of the catalytic α subunit (both α1 and α2 isoforms), and this phosphorylation event causes >100-fold increase in kinase activity (Hawley et al., 1996). Conversely, dephosphorylation of Thr-172 by phosphatases can turn AMPK activity off (Sanders et al., 2007; Rubenstein et al., 2008). In general, mammalian AMPK activity stimulates processes involved in ATP-producing, catabolic pathways (e.g., increasing the glucose transporter GLUT4 and mitochondrial biogenesis) and inhibits ATP-consuming anabolic metabolic pathways (e.g., increasing fatty acid synthesis and mitochondrial biogenesis).

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Abbreviations used: AMPK, AMP-activated protein kinase; hGFAP-Cre, human glial fibrillary acidic protein-Cre; LKB1, liver kinase B1; NDPK, nucleoside diphosphate kinase; ACC1, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; 2D-DIGE, two-dimensional differential in-gel electrophoresis. © 2012 Onyenwoke et al. This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).

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pathways (e.g., gluconeogenesis, lipogenesis, and protein synthesis; Hardie and Hawley, 2001; Barnes et al., 2002; Holmes and Dohm, 2004; Hardie, 2007). The best-defined direct target of AMPK is the fatty acid synthesis and rate-limiting enzyme ACC, which AMPK phosphorylates and inhibits to subsequently lower malonyl-CoA levels and increase fatty acid uptake into mitochondria (Merrill et al., 1997; Winder et al., 1997; Hardie and Hawley, 2001).

Beyond lipid synthesis, AMPK can also switch off protein synthesis by using two different pathways. These pathways include activation of elongation factor-2 kinase, which causes inhibition of the elongation step of translation (Winder et al., 1997; Horman et al., 2002), and inhibition of the target-of-raphamycin (TOR) pathway, which stimulates the initiation step of protein synthesis by the phosphorylation of multiple targets (Proud, 2004). TOR is directly activated by an upstream signaling pathway involving the TSC1–TSC2 (tuberous sclerosis complex) heterodimer. AMPK directly phosphorylates TSC2 and thereby activates the TSC (Inoki et al., 2003). There is also evidence suggesting that AMPK might directly target and inhibit TOR (Cheng et al., 2004). More recent studies identified MRLC, raptor, a clock-related gene, and ULK1 as direct targets of AMPK (Lee et al., 2007; Gwinn et al., 2008; Lamia et al., 2009; Egan et al., 2011).

In this study, we searched for potential new targets of AMPK activity. From these efforts, we identified nucleoside diphosphokinase (NDPK) as a potential downstream target of AMPK. NDPK is a ubiquitous enzyme that catalyzes the transfer of the γ-phosphate group from a nucleoside or deoxyribonucleoside triphosphate (NTP or dNTP) to a nucleoside or deoxyribonucleoside diphosphate (NDP or dNDP, respectively) involving a high-energy phosphoenzyme intermediate (Rosengard et al., 1989; Engel et al., 1995). Functionally, NDPK maintains pools of nucleoside and deoxyribonucleoside triphosphates for processes central to energy utilization, for example, DNA synthesis and translation, using diphosphate substrates (Engel et al., 1995). In addition, the Drosophila homologue of NDPK is required in vivo during normal development for guided cell migration (Rosengard et al., 1989; Randazzo et al., 1991; Dammai et al., 2003; Nallamothu et al., 2008). Here we suggest mechanisms by which AMPK normally inhibits NDPK activity through phosphorylation of a highly conserved serine within NDPK.

RESULTS

Two-dimensional differential in-gel electrophoresis analyses identify NDPK as a phosphoprotein

The overall goal of our study was the identification of potential new targets of AMPK—keys for understanding energy homeostasis and metabolic disease that might be mediated by AMPK signaling.

We performed two-dimensional differential in-gel electrophoresis (2D-DIGE) using cytosolic brain lysates derived from wild-type (WT) and AMPKα1/2 double-knockout (KO) mice (Williams et al., 2011) devoid of all AMPK catalytic activity to identify proteins altered in abundance or posttranslational modification between the WT and KO. One particular protein spot showed a potential shift in migration when comparing WT- and KO-derived lysates but a similar total abundance (Figure 1A).

Given that AMPKα functions as a kinase and the WT spot migrated higher on the gel, this spot might contain a modified protein that was no longer phosphorylated in the KO and thus would migrate differently. A second 2D-DIGE experiment was performed with only WT lysate. However, the WT sample was divided into two samples; the first sample was treated with phosphatase, and the second sample was untreated. A qualitatively similar result to the WT/KO 2D-DIGE comparison was observed (Figure 1B).

After performing the 2D-DIGE experiments, all four adjacent spots of the gel corresponding to the WT or KO spots were excised and identified. All protein spots were confirmed to be nucleoside diphosphate kinase A (NDPK-A; EC 2.7.4.6), also known as Nm23 (nonmetastatic 23). Western blotting indicated that there was no significant difference in NDPK protein expression levels in the total WT versus KO lysate fractions (Figure 1C, Load, and Supplemental Figure S1A). However, a difference was noted when phosphoprotein enrichment was performed prior to Western blot analysis for NDPK, which indicated more phospho-NDPK exists in WT compared with KO brain lysate (Figure 1C, Eluate, and Supplemental Figure S1A).

Drosophila NDPK activity is inhibited by AMPK function, and loss of NDPK function can compensate for loss of AMPKα function

Although there are multiple genes that encode for NDPK activity in mammals (Boissan et al., 2009), there is only a single NDPK gene in Drosophila with previously characterized genetic loss-of-function mutations available for studies. We therefore used Drosophila genetics and a well-established biochemical NDPK activity assay (Timmons et al., 1995; Krishnan et al., 2001) to study NDPK activity in Drosophila. Using two genetically defined null alleles, we demonstrated gene-dosage responsiveness for NDPK activity (Figure 2A). We subsequently confirmed that the decreased NDPK biochemical activity was roughly correlated with the NDPK protein levels detected in various genetic NDPK mutant genotypes by Western blot (Figure 2B and Supplemental Figure S1B).

We next investigated the seemingly inverse relationship between NDPK and AMPK activities by reducing AMPKα function through the use of a transgenic RNA interference (RNAi)–based expression system that phenocopies genetic loss of AMPKα function and only allows Drosophila development to reach the late pupal/pharate adult stage without producing eclosing adults (Johnson et al., 2010). When larvae with decreased AMPKα function, through RNAi (Johnson et al., 2010; Supplemental Figure S2, A and B), were assayed for NDPK activity, a significant increase was observed (Figure 2C).
null AMPKα loss-of-function allele 1 rescue (% expected)
<table>
<thead>
<tr>
<th>Transgene or loss-of-function mutation</th>
<th>AMPKα loss-of-function allele 1 rescue (number rescued/total scored)</th>
<th>AMPKα loss-of-function allele 2 rescue (number rescued/total scored)</th>
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<tbody>
<tr>
<td>UAS-S6k (DN)</td>
<td>Yes (11)</td>
<td>Yes (14/130)</td>
</tr>
<tr>
<td></td>
<td>No (0/68)</td>
<td>No (23/122)</td>
</tr>
<tr>
<td>UAS-TOR (DN)</td>
<td>Yes (13)</td>
<td>No (0/63)</td>
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<td></td>
<td>No (0/52)</td>
<td>No (0/77)</td>
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<tr>
<td>NDPK1</td>
<td>Yes (26)</td>
<td>Yes (7/91)</td>
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<td></td>
<td>No (0/52)</td>
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<td>NDPK2</td>
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<td></td>
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<td></td>
<td>No (0/77)</td>
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**TABLE 1:** AMPKα RNAi and AMPKα loss-of-function rescue by NDPK.

Although these results are suggestive that AMPK might inhibit NDPK activity, because no prior report described the regulation of NDPK activity by phosphorylation, it was unclear whether loss of NDPK would enhance or inhibit AMPK genetic function. Initially, we used the transgenic RNAi-based expression system to reduce AMPKα function and found that introducing a single NDPK loss-of-function mutation for either NDPK allele (Figure 2B and Supplemental Figure S1B) rescued otherwise lethal AMPKα knockout animals to viability/eclosion (Table 1). In addition, heterozygous NDPK loss-of-function alleles were able to rescue previously published (Mirouse et al., 2007) null AMPKα lethal loss-of-function alleles to viability as well, an effect otherwise only seen by dominant-negative S6 kinase, which is known to antagonize AMPK function (Montagne et al., 1999). In support of these results, increased NDPK expression did not rescue AMPKα knockout or loss-of-function animals but instead made the transgenic animals susceptible to energetic stress by accelerating starvation-induced death in a defined starvation paradigm (Johnson et al., 2010; Figure 3).

**AMPK directly inhibits NDPK activity through phosphorylation**

The preceding results suggested that AMPK and NDPK genetically antagonize each other. Combination of these genetic results with the biochemical identification of decreased NDPK phosphorylation in AMPKα mutant brain suggests a potential model by which AMPK directly phosphorylates NDPK to inhibit NDPK function. The relationship between NDPK and AMPK was subsequently investigated in vitro using AMPK purified from a cell expression system (Dyck et al., 1996), which was subsequently added to NDPK protein. When these protein complexes were incubated together before performing NDPK assays, NDPK activity was significantly decreased and substantially further decreased upon additional AMPK activation (Figure 4A and Supplemental Figure S1C) with cobalt chloride (Lee et al., 2003; Supplemental Figure S3; CoCl₂ treatment was the most effective AMPK activator of those tested), which demonstrates AMPK activity inhibits NDPK activity in vitro (Figure 4B).

After establishing that AMPK can directly phosphorylate NDPK in vitro, we identified potential NDPK phosphorylation sites in vivo. The protein spot corresponding to NDPK from WT mouse brain was excised for phosphopeptide mapping (Yale Mass Spectrometry...
established gold standard substrate for AMPK activity (Davies
Supplemental Figure S6) versus the SAMS peptide, a specific, well-
tide, with all serines except S120 mutated to alanine; Figure 5A and
into a modified version of the NDPK phosphopeptide (the NDPK
kinase assays were performed to monitor the incorporation of
phorylated to inhibit NDPK activity (Figure 5B).

Indeed, adding the AMPK complex to S125A decreased NDPK ac-
tivities to measure AMPK-mediated inhibition of NDPK activity.

We therefore added purified AMPK to purified activated phos-
phoprotein—either WT or with the serine-to-alanine muta-
ments of either serine (S120 or S125) to alanine in NDPK had no significant
effect on NDPK activity. Similarly, mutating S125 to the phospho-
mimetic amino acid glutamate also had no effect on NDPK activity
(Figure 5B). However, mutating S120 to glutamate (E) resulted in
insoluble protein under native protein purification conditions. In ad-
dition, when solubilized under denaturing/renaturation conditions,
the S120E mutant protein was inactive even when WT NDPK that
underwent the same treatment maintained significant activity (Sup-
portmental Figure S5). We therefore added purified AMPK to puri-
fied NDPK protein—either WT or with the serine-to-alanine muta-
tions to measure AMPK-mediated inhibition of NDPK activity. Indeed,
adding the AMPK complex to S125A decreased NDPK ac-
tivity; however, adding AMPK to S120A had no inhibitory effect on
NDPK activity, suggesting that S120 is a residue that can be phos-
phorylated to inhibit NDPK activity (Figure 5B).

To determine whether AMPK could phosphorylate NDPK, in vitro
kinase assays were performed to monitor the incorporation of $^{32P}$
into a modified version of the NDPK phosphopeptide (the NDPK-
tide, with all serines except S120 mutated to alanine; Figure 5A and
Supplemental Figure S6) versus the SAMS peptide, a specific, well-
established gold standard substrate for AMPK activity (Davies et al.,
1989). The kinase assays revealed very similar calculated specific
activities of wild-type and each NDPK mutant with (dark-colored
columns) and without (light-colored columns) the addition of activated
AMPK. The decrease in activity for the S120A mutant protein was not
statistically significant ($p = 0.36, n = 3$). $^{*} p < 0.05, n = 3$. Data are
shown as mean ± SEM.

**DISCUSSION**

Elaborating targets for the cellular energy sensor AMPK is key for
understanding the roles this molecule plays in energy homeostasis
and metabolic disease. Through the use of proteomic techniques,
we were able to identify proteins that are potentially regulated and/
or phosphorylated by AMPK and further prioritize these proteins for
study based on genetic evaluation in Drosophila. On the basis of
these criteria and studies, the protein NDPK was identified as a
good candidate for additional study.

Phosphopeptide mapping identified a peptide (Figure 5A, un-
derlined in red) that contained a phosphorylated serine residue. Mu-
tagenesis studies performed on the two conserved serine residues
indicated that S120 is the critical residue for NDPK regulation
(Figure 5B), which has been a speculated but, up to this point, not
experimentally validated mechanism for NDPK regulation
(Venerando et al., 2011). These results correlate well with previous
studies indicating that NDPK is phosphorylated at serine residue(s)
AMPK directly phosphorylates NDPK

MATERIALS AND METHODS

Materials

All chemicals were of an analytical grade and, unless otherwise noted, from Sigma-Chemical (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ).
Lawn, NJ). [γ-32P]ATP (specific activity 3000 Ci/mmol) was from PerkinElmer (Boston, MA). The SAMS (HMRSMASGLHLVKKRR) and NDPK (RNHIHGDAVAKAKRR) peptides were synthesized by Abgent (San Diego, CA) and the University of North Carolina Microprotein Sequencing and Peptide Synthesis Facility (Chapel Hill, NC), respectively. The hemagglutinin (HA)-tagged human AMPKα1 and rat AMPKβ1 constructs were gifts from R. Shaw. Glutathione S-transferase (GST)-tagged AMPKα1 was a gift from L. Witters. The antibodies used were as follows: anti-NDPK (C-20, sc-343; Santa Cruz Biotechnology, Santa Cruz, CA), anti–phospho-AMPK (α2, C-1) (Cell Signaling, Beverly, MA), and anti–α-tubulin (clone B-5-1-2; Sigma-Aldrich). Anti-dNDPK (reactive to protein of *Drosophila* origin, dNDPK) was a gift from T. Hsu.

Transgenic animals and brain sample/lysis preparation

Three-month-old male mice were killed to obtain brain tissue (kindly provided by T. Williams, University of North Carolina). The genotypes used were as follows: wild type, human glial fibrillary acidic protein-Cre (hGFAP-Cre) mice (K. McCarthy, University of North Carolina), and conditional AMPKα1/2 knockout mice (Williams et al., 2011) to produce AMPKα1/2-αβ/β-hGFAP-Cre mice. The animals were handled under protocols approved by the Institutional Animal Care and Use Committee (Institutional Animal Care and Use Committee ID 09-149.0) of the University of North Carolina–Chapel Hill and in accordance with National Institutes of Health guidelines.

For the preparation of brain lysate, a whole mouse brain (375–425 mg) was processed in 10 ml of ice-cold lysis buffer A (50 mM Tris-HCl, pH 7.5, protease [P2714; Sigma-Aldrich] and phosphatase [P5726; Sigma-Aldrich] inhibitor cocktails [note: for the preparation of the phosphatase-treated WT brain lysate for 2D-DIGE, the phosphatase inhibitor cocktail was omitted for 2.5 U/ml alkaline phosphatase (final concentration); P6774; Sigma] and the sample was incubated for 1 h with the addition of 1 mM MgCl2 (37°C) before the high-speed centrifugation step), and benzonase nuclease) using a TissueMiser homogenizer (Fisher Scientific). The lysate was centrifuged at 1000 × g for 20 min (4°C). The supernatant was then centrifuged for a second time. The resultant clarified supernatant was centrifuged at 100,000 × g to produce the cytosol. The pellet was discarded, and the supernatant (cytosol) was directly used for Western blotting and then for 2D-DIGE analyses after it was cleaned using a 2D Clean-Up Kit (GE Healthcare, Piscataway, NJ).

2D-DIGE protocols and protein identifications

The 2D-DIGE experiments were performed by the University of North Carolina Systems-Proteomics Center using previously described methodologies (Osorio et al., 2007). Protein spot identification was performed by the Yale Mass Spectrometry and Proteomics Resource Core (New Haven, CT) using peptide mass fingerprinting tandem mass spectrometry data, as previously described (Osorio et al., 2007; Pinaud et al., 2008).

**Purification of phosphoproteins by immobilized metal affinity chromatography**

Phosphoproteins were purified from mouse brain lysates using a PhosphoProtein Kit (Qiagen, Valencia, CA), as described by the manufacturer. The brain lysates were prepared as described; however, 0.25% 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulphonate was included in the buffer and throughout the purification process, as recommended by the manufacturer's protocol.

**Plasmid construction**

A pET21b (Novagen, Gibbstown, NJ) construct containing the cDNA for wild-type NDP kinase A, inserted in the BamHI–Ndel site, was obtained from M.-L. Lacombe. The NDPK gene was PCR amplified from this construct using the primer pair F, 5′-AAAGATCCGGCCAACGTGTAGGTCACCTTC-3′, and R, 5′-AAAGATCCGGCCAACGTGTAGGTCACCTTC-3′, digested with BamHI and SalI, and ligated into a pET28b (Novagen) vector cut with the same restriction enzymes for expression as a histidine (His)-tagged protein.

Site-directed mutagenesis of NDP kinase

NDPK was carried out using bridging PCR. The primer pairs for the construction of each NDPK variant are as follows: S120E (F, 5′-ATTACATGGGAGATCTTGGGAAGTGC-3′; R, 5′-TCCACAGAATCTCCTGCTGATATAATGCTTCCTG-3′), S125E (F, 5′-TTCTGTGAGGAGGAGGAGAAGGATCCCGG-3′, R, 5′-CTTCTTCTGGCTTCTCCACAAATCGACTGCC-3′), S120A (F, 5′-ATTACATGCGGCTAGTCTGGGAAGTGC-3′; R, 5′-TCCACAGAATCTCCTGCTGATATAATGCTTCCTG-3′), and S125A (F, 5′-TTCTGTGGAGGAGGAGGAGAAGGATCCCGG-3′, R, 5′-CTTCTTCTGGCTTCTCCACAAATCGACTGCC-3′).

**Purification of NDP kinase**

Full-length His-tagged wild-type and mutant proteins were expressed in high yields using E. coli BL21-Gold (DE3) pLysS competent cells (Agilent Technologies, Palo Alto, CA) transformed with the appropriate construct using heat shock, as described by the...
manufacturer, and purified using batch/gravity-flow column purification with Talon IMAC resin (Clontech, Mountain View, CA) under native conditions (denaturing conditions, i.e., 4 M urea, were used throughout the purification process for the S120E mutant) following the manufacturer's instructions.

Cell culture and AMPKα/β/γ coimmunoprecipitation

For mammalian cell expression of AMPK, the AMPK subunits were used as previously described (Dyck et al., 1996). HEK293 cells were cultured in complete DMEM (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37°C in 5% CO₂. For the transient expression of AMPK protein, the cells were plated 24 h before the experiments in 15-cm dishes and then transfected with the three plasmids using Lipofectamine 2000 (1 μg DNA per 2 μl; Invitrogen, Carlsbad, CA) following the manufacturer's protocols. Note: For some kinase assays, GST-AMPKα1 was replaced with WT or kinase-dead (KD) myc-AMPK (in a pCMV-myc vector; Clontech; Kazgan et al., 2010).

After 24 h, fresh medium containing CoCl₂ (200 μM) was added to the cells for 1 h in the incubator to activate AMPK, as previously described (Lee et al., 2003). Cells were then harvested, lysed in 0.5 ml of lysis buffer A plus 1.0% Triton X-100 with shaking for 1 h (4°C), and centrifuged at 16,000 × g for 10 min (4°C). GST- and myc-tagged AMPK were purified from the supernatants using GST pull-down, using glutathione Sepharose 4B (Amersham, GE Healthcare), and immunoprecipitation performed, using anti-c-Myc antibody (9E10; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at a 1:10 dilution for 1 mg/ml lysate and A/G agarose (Pierce Protein Research Products, Rockford, IL), respectively, according to the manufacturer's instructions and as previously described (Kazgan et al., 2010). Washed, bead-adsorbed GST-AMPK was used for NDPK assays, as previously described (Dyck et al., 1996), and both GST- and myc-tagged AMPK were used for kinase assays.

NDPK assays

A well-known procedure to assay NDP kinase activities was used (Timmons et al., 1993; Krishnan et al., 2001). In brief, 10 μl of diluted enzyme (lysate or purified NDPK; see further discussion) was added to a 990-ml reaction mixture containing 100 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 100 mM KCl, 0.4 mM NADH, 6 mM ATP, 0.7 mM TDP, 4 mM phosphoenolpyruvate (PEP), and 10 U of pyruvate kinase and lactate dehydrogenase each. The absorbance of NADH at 340 nm was then recorded. A unit of activity is defined as the amount of enzyme (lysate or purified NDPK; see further discussion) required to convert 1 μmol of NADH to NAD⁺ in 1 min.

For NDPK assays with flies or fly larvae, 20 adult flies or 40–50 fly larvae were homogenized in 50 μl of ice-cold buffer (100 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 100 mM KCl). The lysates were centrifuged at 10,000 × g for 10 min (4°C). The clarified supernatant was then diluted 1:10 for inclusion in the described assay (0.5–2 μg of protein used). Purified NDPK was purchased from Sigma-Aldrich (N2635) and also produced as purified His-tagged versions and used at amounts of 10–50 and 50–200 ng, respectively, in NDPK assays. For the inhibition assays including AMPK, 3 μg of bead-adsorbed GST-AMPK was incubated with purified NDPK or purified His-tagged NDPK at room temperature for 2 h before executing the NDPK assays.

Kinase assays

Kinase assays were performed according to previously described methods (Davies et al., 1989). Briefly, AMPK activity assays with GST-AMPK were performed at room temperature (25°C) in a 25-μl reaction mixture containing 3–12 μg of protein in kinase buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.0, 75 mM NaCl, 5 mM sodium acetate, 5 mM magnesium chloride, 1 mM dithiothreitol, 0.8% glycerol, 0.1 mM EDTA, 200 μM AMP and ATP, and 2 μCi of [γ-32P]ATP) with or without the SAMS or NDPK peptide. After a 30-min incubation period, the reaction mixtures were counted in a scintillation counter. Kinase assays with myc-tagged KD-AMPK and myc-tagged WT-AMPK were performed in the same manner as described earlier, but 0.5 μg of protein was added to the reaction mixture. AMPK activity is expressed as picomoles of 32P incorporation into the peptide per minute per microgram of protein.

Western blottting

Fly protein lysates for immunoblotting were prepared by collecting equal numbers of male and female flies (50 total) of each genotype in a 1.5-ml microfuge tube. One milliliter of lysis buffer A was added to each sample. Flies were then ground to homogeneity, incubated for 1 h with shaking (4°C), and centrifuged at 16,000 × g for 10 min (4°C). Supernatants were collected, and protein concentrations were determined using the Bio-Rad DC protein assay (Richmond, CA). Note: The preparation of brain lysate samples was described earlier.

Proteins (50 μg) were then boiled in loading buffer and subjected to SDS-PAGE (Invitrogen), followed by Western analyses using 1:1000 dilutions of all primary antibodies, with the exception of anti-α tubulin (1:16,000). Secondary antibodies (IRDye infrared antibodies; LI-COR Biosciences, Lincoln, NE) were used at a dilution of 1:2000. Scanning, analyzing, and quantification of blots were performed via the Odyssey Infrared Imaging System (LI-COR Biosciences). Three or more independent experiments were performed for all immunoblotting data.

Fly stocks, crosses, rescue experiments, and lifespan measurements

Drosophila melanogaster strains obtained from the Bloomington Stock Center (Bloomington, IN) included the following: Act-GAL4, tub-GAL4, awdRNAI (referred to as NDPK₁ in this study), awdMS659 (NDPK₂), UAS-AMPKαRNAI, the SNF1A¹ and SNF1A² mutants, UAS-SNF1A, UAS-S6K-KQ, UAS-Tor.TED, and UAS-GFP. UAS-SNF4 and UAS-NDPK were gifts from D. Kretzschmar and T. Hsu, respectively. All flies were maintained at 25°C in yeast-cornmeal vials, and all crosses were also performed in cornmeal-yeast vials.

For the AMPKα RNAI rescue experiments, males carrying a transgene or loss-of-function mutation on the second or third chromosome were mated to virgin females carrying a GAL4 (either tub-GAL4 or Act-GAL4, respectively). From these crosses, male progeny carrying the transgene or loss-of-function mutation and the GAL4 were mated to virgin females carrying UAS-AMPKαRNAI. The progeny from this second cross were then scored for rescue, that is, viable adult flies in spite of AMPKα RNAI knockdown.

For the AMPKα loss-of-function rescue experiments, males carrying a transgene or loss-of-function mutation on the second or third chromosome were mated in parallel to virgin females from both the SNF1A¹ and SNF1A² loss-of-function lines. The male progeny from both of these crosses were scored for rescue, that is, viable adult flies in spite of carrying the lethal loss-of-function mutation/phenotypical, non–bar-eyed males.

Measurements of lifespan have been widely used in Drosophila as a metric of stress sensitivity (Johnson et al., 2010). Thirty 3- to 5-d-old male flies were starved in empty food vials that contained pieces of filter paper saturated with deionized H₂O. We assessed the percentage survival of at least three replicate vials three times daily.
Statistical analyses
Comparisons were made using the unpaired Student’s t test with p < 0.05 considered significant. Values are presented as the mean ± the SE of the mean (SEM) and are represented as error bars. Indirect immunofluorescent detection of a secondary antibody (LI-COR Biosciences) was scanned and standardized to an internal standard to calculate and quantify arbitrary units using the Odyssey Infrared Imaging System, and a representative Western blot is shown in each figure.

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AMPK directly phosphorylates NDPK