Diverse Cytopathologies in Mitochondrial Disease Are Caused by AMP-activated Protein Kinase Signaling[□]

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The complex cytopathology of mitochondrial diseases is usually attributed to insufficient ATP. AMP-activated protein kinase (AMPK) is a highly sensitive cellular energy sensor that is stimulated by ATP-depleting stresses. By antisense-inhibiting chaperonin 60 expression, we produced mitochondrially diseased strains with gene dose-dependent defects in phototaxis, growth, and multicellular morphogenesis. Mitochondrial disease was phenocopied in a gene dose-dependent manner by overexpressing a constitutively active AMPK α subunit (AMPK α T). The aberrant phenotypes in mitochondrially diseased strains were suppressed completely by antisense-inhibiting AMPK α expression. Phagocytosis and macropinocytosis, although energy consuming, were unaffected by mitochondrial disease and AMPK α expression levels. Consistent with the role of AMPK in energy homeostasis, mitochondrial “mass” and ATP levels were reduced by AMPK α antisense inhibition and increased by AMPK α T overexpression, but they were near normal in mitochondrially diseased cells. We also found that 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside, a pharmacological AMPK activator in mammalian cells, mimics mitochondrial disease in impairing *Dictyostelium* phototaxis and that AMPK α antisense-inhibited cells were resistant to this effect. The results show that diverse cytopathologies in *Dictyostelium* mitochondrial disease are caused by chronic AMPK signaling not by insufficient ATP.

INTRODUCTION

Mitochondrial diseases are a diverse group of degenerative disorders caused by mutations in either mitochondrial genes or nuclear genes encoding mitochondrial proteins (James and Murphy, 2002; Rossignol *et al.*, 2003; Maassen *et al.*, 2004; McKenzie *et al.*, 2004). Affecting one in ~4000 people, they vary in severity, age of onset, and the symptoms they cause, but they present mostly as forms of various neuromuscular disorders, heart disease, or diabetes. Although the precise nature of many of the disease-causing mutations is known, the relationship between genotype and phenotype in mitochondrial disease is complex and poorly understood. Individuals with the same genotype can exhibit very different symptoms, whereas different mutations can produce very similar clinical outcomes in different patients. This has been attributed to differences in the severity of the underlying genetic defect between individuals and to tissue-specific differences in the proportion of defective mitochondria, ATP demands, age-related accumulation of mitochondrial mutations, and the expression and function of specific isoforms of mitochondrial proteins. Despite these complexities it is accepted that mitochondrial disease pathology results primarily from a reduced capacity of the mitochondria to pro-

duce energy in the form of ATP (James and Murphy, 2002; Rossignol *et al.*, 2003; Maassen *et al.*, 2004; McKenzie *et al.*, 2004). It has therefore been assumed that the associated cytopathology is caused by ATP supplies being insufficient to support the affected cellular activities. By using the *Dictyostelium* model for mitochondrial disease, we have been able to study the underlying mechanisms of mitochondrial disease cytopathology without the superimposed complexities of metazoan developmental biology. This has revealed that diverse mitochondrial disease phenotypes arise, not because of insufficient ATP, but because of chronic activation of an energy-sensing alarm system that shuts down or interferes with some, but not all, cellular functions. That cellular alarm is AMP-activated protein kinase (AMPK).

AMPK is a ubiquitous, highly conserved protein kinase that maintains cellular energy homeostasis in healthy cells and in a variety of pathological situations, most notably diabetes, ischemic reperfusion injury, and cancer (Hardie and Hawley, 2001; Hardie, 2004; Kahn *et al.*, 2005; Hardie and Sakamoto, 2006). AMPK functions *in vivo* as a heterotrimer with a catalytic α and a regulatory γ subunit that are assembled into the holoenzyme on a scaffold provided by the β subunit. It is activated very sensitively by a reduction in ATP and concomitant increase in AMP levels resulting from stresses such as strenuous exercise, ischemia or glucose deprivation. The activated kinase phosphorylates target proteins and initiates downstream signaling pathways that shift metabolism from anabolic to catabolic pathways, stimulating glucose uptake and fatty acid oxidation, enhancing the energy-producing capacity of the cell through mitochondrial proliferation and inhibiting energy-consuming processes such as cell cycle progression and growth (Hardie and Hawley,

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MATERIALS AND METHODS

Chaperonin 60 antisense (pPROF128) and sense control (pPROF127) constructs were described previously (Kotsifas *et al.*, 2002). The AMPK α sense (control) and antisense constructs express a 1173-base pair AMPK α sense (pPROF361) or antisense (pPROF362) RNA corresponding to base pairs 364-1536 in the *snfA* sequence (Figure 1b) in DictyBase (DictyBase accession no. DDB0215396). The construct (pPROF392) for overexpression of a constitutively active, truncated AMPK α (Figure 1b) was created by subcloning the corresponding cDNA in the sense orientation into pA15GFP.

As described previously, all experiments were conducted with *D. discoideum* parental strain AX2 and transformants derived from it (Wilczynska *et al.*, 1997; Kotsifas *et al.*, 2002). Each transformant strain (names beginning with HPF) carried multiple copies of the following constructs: 1) the chaperonin 60 (*hspA*) antisense inhibition construct HPF405-416 (Kotsifas *et al.*, 2002) and HPF601-612, or its corresponding sense RNA control construct HPF417-418 (Kotsifas *et al.*, 2002); 2) the AMPK α (*snfA*) antisense inhibition construct (Figure 1c) HPF455-465 or its corresponding sense RNA control construct HPF466-468; 3) the AMPK α T overexpression construct (Figure 1b) HPF432-445; and 4) the *hspA* and *snfA* antisense and sense constructs in one of the four possible combinations: HPF501-512, *hspA/snfA* double antisense; HPF551-553, *hspA/snfA* double sense; HPF581-586, *hspA* antisense/*snfA* sense; and HPF576-580, *hspA* sense/*snfA* antisense. In no case did the presence of either the *snfA* sense construct or the *hspA* sense construct have any effect on any of the phenotypes investigated.

DNA and RNA Techniques

The 2.6-kb gene *snfA* (EMBL/GenBank accession no. AF118151) encoding the AMPK α subunit was amplified and cloned in pZErO-2 (Invitrogen, Carlsbad, CA) from AX2 genomic DNA with primers PAMKF1 (5'-gcgccttagattc-

Sequence analyses, alignments, and database searches were conducted using Web-based software through DICTYBase (<http://www.dictybase.org>), ExpASY (<http://www.expasy.org>), and the Australian Genome Research Facility (<http://www.agrf.org.au>).

Quantitative PCR. Expression of AMPK α mRNA was quantitated by real-time RT-PCR by using the iQ5 real-time PCR detection system and the iScript one-step RT-PCR Supermix (Bio-Rad, Hercules, CA) with SYBR Green I for amplicon detection. The 215-base pair target amplicon, shown in Figure 1d, was not present in the antisense RNA expression constructs or their corresponding sense RNA controls, so that the assay specifically measured the levels of the endogenous AMPK α mRNA. The 50- μ l reaction mixture contained 1 $\times$ iScript one-step RT-PCR Supermix with SYBR Green I (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 3 mM MgCl₂, 0.2 mM each dNTP, iTaQ antibody-mediated hot start DNA polymerase at 50 U/ml, 500 nM SYBR Green I, and 10–50 nM fluorescein), iScript Moloney murine leukemia virus reverse transcriptase (1 μ l of 50 $\times$ formulation), gene-specific primers (200 nM each PAAF1 and PAAR1), and purified total RNA template (1 pg–1 μ g). cDNA synthesis was performed at 42°C for 30 min. The experimental plate well factor was collected at 95°C for 90 s, and the PCR cycling and detection used 45 cycles of denaturation at 95°C for 1 min, annealing and extension at 55°C for 1 min. Expression levels for antisense RNA-expressing strains were measured relative to those of AX2 control cells.

Generation of Anti-peptide Antibody. Anti-peptide antibodies were raised against a mixture of two AMPK α peptide sequences: KILNRTKIKLNKMKDEKIRR (residues 60–78) in the catalytic domain and GSYDDDEVYSPNLVSPITTPIMS (residues 352–373) in the autoinhibitory region of the *D. discoideum* AMPK α subunit. Peptide synthesis and coupling was carried out by Mimotopes (Clayton, Victoria, Australia), and antibodies were raised in a rabbit by the Institute of Medical and Veterinary Science (Adelaide, South Australia, Australia).

Western Blotting. Each track of 10% polyacrylamide gels was loaded with 10 μ g of protein for SDS-polyacrylamide gel electrophoresis by using the Mini PROTEAN II apparatus (Bio-Rad). The proteins were electroblotted onto polyvinylidene difluoride membranes (GE Healthcare) by using the Mini Trans-Blot Electrophoretic transfer cell. The AMPK α primary antibody (1:500) and anti-rabbit immunoglobulin G horseradish peroxidase conjugate (1:2500; Promega, Madison, WI) as secondary antibody were used and the AMPK α subunit protein was detected using the 3-amino-9-ethylcarbazole staining kit (Sigma-Aldrich, St. Louis, MO).

ATP assays were conducted using the luciferase-based ATP determination kit (ENLITEN; Promega) by using cells grown axenically in HL-5 medium.

Figure 1. Genetic manipulation of levels of expression of the catalytic α subunit of AMPK and its truncated form AMPK α T. (a) Map of the *Dictyostelium* AMPK α polypeptide showing positions of the main functional features. Numbers indicate amino acid positions. The amino acid sequence is conserved except for a short N-terminal stretch and an asparagine-rich region in the C-terminal half of the molecule. Such asparagine-rich domains are very common in *Dictyostelium* proteins, but their functions are unknown. The β -subunit binding and autoinhibitory regions are required for assembly of the α subunit into the heterotrimeric holoenzyme and its regulation by AMP/ATP-binding to the γ subunit. The inset contains a Northern blot showing a developmental time course for native AMPK α mRNA expression. The probe was a genomic DNA fragment extending from position 2228 to 2642 base pairs in the genomic DNA sequence, numbering from the translational start codon. (b) The truncated form of AMPK α used in overexpression studies. The truncated form was created by introducing a single base deletion (A₁₁₂₀) during RT-PCR to produce a cDNA with a reading frame shift at that position and a premature stop codon 18 nucleotides downstream. In addition, our cDNA contains the following base substitutions: T₇C introduced with the 5' primer as well as T₁₁₃₄G and AAA₁₁₂₉CCC introduced with the 3' primer downstream of the frameshift. These additional changes arose because of sequence differences between GenBank record AF118151 and the since completed *Dictyostelium* genome sequence. S₃P and VLPRGQ₃₇₉ indicate amino acid substitutions, the latter resulting from the introduced frame shift at residue 374 that created a stop at codon 380. Numbers indicate amino acid positions. (c) The genomic DNA fragment used for antisense inhibition studies. The arrow indicates the direction of transcription of the fragment in the antisense RNA construct. The red section indicates the cDNA and the black bars indicate the position and size of introns in the fragment. Numbers represent nucleotide positions in the genomic sequence. (d) The amplicon used to measure antisense inhibition of expression of the native AMPK α mRNA by using real-time PCR. The sequences to which the primers anneal are not present in the AMPK α antisense construct. (e) Expression of the native AMPK α mRNA relative to the wild-type AX2 control in cells carrying the indicated number of copies of the AMPK α antisense RNA construct. Negative numbers are assigned to the copy numbers of the antisense inhibition construct, because it exerts a negative effect on expression of the native AMPK α mRNA. The Southern blot in the inset made use of a chromogenic substrate to show hybridization to the 8.25-kb antisense RNA construct as a function of the number of copies per genome. (f) Overexpression of the truncated AMPK α subunit (AMPK α T) mRNA as a function of the number of copies of the overexpression construct per genome. Expression levels relative to expression in the transformant with the highest copy number were measured in quantitative Northern blots. Insets contain separate Southern, Northern, and Western blots using chromogenic substrates to show the AMPK α T construct DNA, mRNA, and polypeptide levels at the indicated copy numbers. The antibody was not sufficiently sensitive to detect the native AMPK α subunit in Western blots, suggesting that the level of expression of AMPK α T is significantly higher than that of the endogenous full-length protein. This is consistent with Northern blots and quantitative RT-PCR results that showed that the levels of AMPK α T mRNA were 1 to 2 orders of magnitude higher than the mRNA for the native protein (data not shown).

Background luminescences measured before the assay were subtracted, and ATP concentrations were determined from a standard curve constructed using 10-fold serial dilutions of the ATP standard (1×10^{-7} – 1×10^{-11} M) in assay buffer.

Phenotypic Assays

Phototaxis and Thermotaxis. Semiquantitative phototaxis and thermotaxis assays were conducted as described previously (Fisher et al., 1981; Fisher and

Annesley, 2006) by using a small quantity of amoebae scraped from the edges of *Dictyostelium* colonies on *K. aerogenes* lawns. After incubation, slugs and slime trails were transferred to clear polyvinyl chloride (PVC) discs. The discs were stained for 5 min with Coomassie blue (Fisher et al., 1981), and then they were rinsed gently in running water. Start and end points of the stained trails were digitized, stored as a series of x, y coordinates by using a Summagraphics 120 digitizing tablet connected to a SUN workstation (SUN Microsystems, Santa Clara, CA), and analyzed using directional statistics based on the von Mises or circular normal distribution (Fisher and Annesley, 2006).

Growth on Bacterial Lawns. Two hundred and fifty microliters of *E. coli* B2 cells from an overnight Luria Broth culture were harvested, resuspended in sterile saline, spread uniformly onto each normal agar (NA: 20 g/l Bacto agar [Difco, Detroit, MI]; 1 g/l Bacto peptone [Oxoid, Basingstoke, Hampshire, England], 1.1 g/l anhydrous glucose, 1.9972 g/l KH_2PO_4 , and 0.356 g/l $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, pH 6.0) plate and allowed to dry. A 10- μl droplet of a suspension containing 1×10^6 *Dictyostelium* amoebae/ml was applied onto the center of the NA plate, allowed to dry in a laminar flow hood, and incubated at 21°C. The rate of expansion (diameter in millimeters) of the resulting *D. discoideum* plaque was measured at intervals of 8 or 12 h over a period of 5–7 d. The recorded values were analyzed by linear regression by using the “R” environment for statistical computing and graphics (<http://www.R-project.org>) to determine the growth rate from the growth curve. The 95% confidence intervals for the time taken for the plaque to expand 5 mm were determined from confidence intervals for the slope of the growth curve.

Growth in Axenic Medium. Cells from an exponentially growing *Dictyostelium* culture in HL-5 medium were inoculated into fresh HL-5 medium at an initial density of $\sim 1 \times 10^4$ cells/ml and incubated at 21°C with shaking at 150 rpm on an orbital shaker. The cell densities were determined using a hemocytometer at 8- or 12-h intervals over a period of 5–7 d and recorded. The cell densities were then analyzed by log-linear regression using the R programming environment computer software to determine the generation time from the exponential growth curve. The 95% confidence intervals for the generation time were calculated from confidence intervals for the slope of the log-linear portion of the growth curve.

Phagocytosis

Bacterial uptake by *Dictyostelium* strains was determined using as prey an *E. coli* strain expressing a fluorescent protein termed DsRed (Maselli *et al.*, 2002). DsRed-expressing *E. coli* (DsRed-Ec) cells were prepared at a density of $2\text{--}4 \times 10^{10}$ bacteria/ml (equivalent to $\text{OD}_{600} = 10\text{--}20$) in 20 mM Sorenson's buffer (2.353 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 17.65 mM KH_2PO_4 , pH 6.3). The fluorescence signal per million bacteria was determined from the density and fluorescence of the bacterial culture used in a given experiment. The relationship between OD_{600} and the density of the bacterial suspension was determined in a separate calibration curve.

Dictyostelium amoebae were harvested, washed, resuspended at $1.3\text{--}23 \times 10^6$ cells/ml and starved in Sorenson's buffer at 21°C for 30 min with shaking at 150 rpm. For the assay, 1 ml of the DsRed-Ec suspension added to 1 ml of starving *Dictyostelium* cells. At each time point in the assay (0 and 30 min), 0.5 ml of amoebae were washed free of uningested bacteria by differential centrifugation in the presence of 5 mM sodium azide (Maselli *et al.*, 2002), and their fluorescence was measured in a Modulus fluorometer (Turner BioSystems, Sunnyvale, CA) by using a specially constructed module designed for DsRed (530-nm excitation and 580-nm emission). Measurements were performed in duplicate at each time point. The hourly rate of consumption of bacteria by a single amoeba was calculated from the increase in fluorescence over 30 min, the fluorescence signal per million bacteria and the amoebal density.

Pinocytosis

Pinocytosis assays (Klein and Satre, 1986) were performed with fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich; average mol. mass, 70 kDa; working concentration, 2 mg/ml in HL-5 growth medium). Axenically growing *Dictyostelium* cells were harvested, resuspended in HL-5 at $2.5\text{--}25 \times 10^6$ cells/ml, and shaken at 150 rpm for 20 min at 21°C. FITC-dextran was added to the cells, and at each time point (0 and 60 or 70 min) 200- μl aliquots were transferred to 3 ml of ice-cold phosphate buffer (2 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and 15 mM KH_2PO_4 , pH 6.0). The cells were harvested, washed twice with ice-cold phosphate buffer, and lysed by addition of 2 ml of 0.25% (vol/vol) Triton X-100 in 100 mM Na_2HPO_4 , pH 9.2. The fluorescence of the lysate was measured in duplicate at each time point in a Modulus fluorometer (Turner BioSystems) using the Green Module. The hourly rate of uptake of medium was calculated from the cell density, the increase in fluorescence over 60 or 70 min, and a separate calibration curve relating the fluorescence signal to the volume of fluorescent medium.

Mitochondrial “Mass”

MitoTracker Green fluorescence was used to estimate mitochondrial mass (Pendergrass *et al.*, 2004). Axenically growing vegetative amoebae were harvested, washed once in Lo-Flo HL-5 (Liu *et al.*, 2002) (3.85 g/l glucose, 1.78 g/l proteose peptone, 0.45 g/l yeast extract, 0.485 g/l KH_2PO_4 , and 1.2 g/l $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$; filter sterile), incubated in Lo-Flo medium for 2 h, and then divided into two aliquots. One aliquot was resuspended in Lo-Flo HL-5 containing 200 nM MitoTracker Green FM (Invitrogen), whereas the other aliquot was resuspended in only Lo-Flo HL-5 as an unstained control. Both aliquots were incubated for an hour in the dark and then unbound MitoTracker Green was removed by washing the cells three times in Lo-Flo HL-5, with 10-min shaking on an orbital shaker (150 rpm) between washes. Finally, the cells were resuspended in Lo-Flo HL-5, and fluorescence was measured in duplicate in a Modulus fluorometer (Turner BioSystems) using the Blue

Module. The MitoTracker Green fluorescence per million cells was calculated after subtraction of the background fluorescence in the unstained cells.

Fluorescence Microscopy. Fluorescence microscopy was based on our previously reported methods (Gilson *et al.*, 2003). Vegetative amoebae were grown to log phase in HL-5 medium on sterile coverslips in six-well plates (Nalge Nunc, Naperville, IL), washed gently in Lo-Flo HL-5, and stained with 200 nM MitoTracker Red CMX-Ros (Invitrogen) in Lo-Flo HL-5 for 1 h in the dark. Unbound MitoTracker Red was removed by washing the cells three to four times in Lo-Flo HL-5 over 2 h. After two washes in phosphate buffer (12 mM Na_2HPO_4 and 12 mM NaH_2PO_4 , pH 6.5), the cells were fixed and flattened at the same time by placing the coverslips upside down on a layer of 1% agarose in phosphate buffer containing 3.7% paraformaldehyde for 30 min. The fixed cells on the coverslips were washed four times (5 min each) in phosphate-buffered saline and mounted for microscopy.

Morphology

Fruiting body morphology after multicellular development was scored as described previously (Kotsifas *et al.*, 2002) after culturing transformants on *K. aerogenes* lawns on SM agar plates with or without 0.5% (wt/vol) activated charcoal. The SM agar plates were prepared by spreading 0.1 ml of a dense *K. aerogenes* suspension in sterile saline onto the surface of the plates and allowing the inoculum to dry in the laminar flow hood before streak inoculating amoebae of the transformants onto the lawns. The plates were incubated at 21°C for 4–6 d to allow growth and multicellular development.

Statistical Techniques

Analysis of Directional Data. The directions of travel of individual slugs were analyzed using directional statistics based on the circular normal (von Mises) distribution as described previously (Fisher *et al.*, 1981; Fisher and Annesley, 2006). The accuracy of phototaxis is the concentration parameter (κ) of the circular normal (von Mises) distribution, which measures how concentrated individual directions are around the mean direction μ (toward the light source). The κ values range from 0 for no preferred direction of migration (all directions equally probable) to ∞ for perfect orientation (all directions exactly toward the light).

Regression and Correlation Analysis. Regression analysis was performed, and the coefficient of variation (R^2) was calculated using standard linear regression models. R^2 equals the square of the Pearson product-moment r , which was used where appropriate to determine the significance probability for some correlations – the probability of the observed results occurring under the null hypothesis that there is no correlation. The significance of all correlations was also tested by calculating the nonparametric Kendall rank r and in all cases the outcomes ($p > 0.1$ or $p < 0.01$) were the same as those yielded by the Pearson coefficient. In some cases, Kolmogorov–Smirnov, Kruskal–Wallace, and Student's t two-sample tests were also performed.

Three-Dimensional Surface Regression Plots. Three-dimensional scatter plots and surface regressions were created using a local adaptation of the “scatter3d” function in the R environment for statistical computing and graphics (<http://www.R-project.org>). Quadratic (phototaxis and growth rate data) or planar (other data) surfaces of best fit were plotted for quantitative phenotypic data relating the phenotypes (vertical axis) to both the chaperonin 60 and AMPK α expression indices (horizontal axes). The results are presented as rotating animations in Supplemental Material.

RESULTS

We previously reported that mitochondrial disease can be created and studied in *Dictyostelium* in two ways: targeted disruption of mitochondrial genes (Wilczynska *et al.*, 1997), or antisense inhibition of nuclear genes encoding mitochondrial proteins (e.g., chaperonin 60) essential for mitochondrial respiratory functions (Kotsifas *et al.*, 2002). Regardless of which strategy was used, the phenotypic consequences of mitochondrial dysfunction in *Dictyostelium* were defects in phototaxis, thermotaxis, growth, and multicellular development. In view of the diverse regulatory roles played by AMPK in response to ATP-depleting stressors, we decided to test whether AMPK activation and signaling were responsible for these diverse phenotypic outcomes of mitochondrial disease. If this were so, then increasing cellular AMPK activities should cause the same defects as mitochondrial dysfunction, whereas depressing AMPK levels in mitochondrially diseased cells should ameliorate these defects.

Dictyostelium AMPK α Is Similar to the α Subunits of AMPK in Other Eukaryotes and Is Expressed throughout Development

Searches of the predicted *Dictyostelium* proteome (Eichinger *et al.*, 2005; Chisholm *et al.*, 2006) revealed a single isoform of each of the α , β , and γ subunits of the AMPK heterotrimer (DictyBase accession nos. DDB0215396, DDB0204006, and DDB0217034, respectively). The α subunit gene, named *snfA* after the *Saccharomyces cerevisiae* orthologue, contains four introns and encodes a polypeptide of 727 amino acids. The predicted protein sequence was confirmed by sequencing full-length cDNAs (data not shown). The cDNA encodes a protein that is highly similar to AMPK α subunits from other eukaryotes except for a short N-terminal stretch of 27 amino acids and a 241-residue asparagine-rich domain located 98 residues upstream of the C terminus, both of which are not conserved in other organisms (Figure 1a). Aside from this asparagine-rich domain, all of the features of AMPK α subunits in other organisms were found to be present in the *Dictyostelium* protein in the expected location. These include a kinase domain near the N terminus (70% identical and 84% similar to the human $\alpha 1$ isoform), a phosphorylatable threonine (T₁₈₈) corresponding to the regulatory T₁₇₂ of the human AMPK $\alpha 2$ isoform, ATP-binding and kinase catalytic site signatures, and a C-terminal region with recognizable, albeit lower similarity to the autoinhibitory and β subunit-binding domain of other α subunits (28% identical and 47% similar to the equivalent region of human AMPK $\alpha 1$). In the active form of the AMPK heterotrimer, there is one molecule of AMP bound to each of two Bateman domains in the γ subunit (Scott *et al.*, 2004) and the regulatory threonine on the α subunit is phosphorylated (Weekes *et al.*, 1994; Hardie *et al.*, 2003). Although the major upstream activating kinase in mammalian cells, LKB1 (Hawley *et al.*, 2003; Hong *et al.*, 2003; Shaw *et al.*, 2004), is constitutively active, phosphorylation of T₁₇₂ is normally autoinhibited in the heterotrimer, and this inhibition must be relieved by AMP binding to the γ subunit. ATP acts as a competitive inhibitor of AMPK at the AMP-binding sites on the γ subunit. *Dictyostelium* also possesses an LKB1 homologue (DictyBase accession no. DDB02290349).

For AMPK to be responsible for the deranged phenotypes associated with mitochondrial dysfunction in *Dictyostelium*, it must be present in wild-type cells at all stages of the life cycle. A Northern blot confirmed that the α subunit mRNA is present throughout development and suggested that the levels may increase during the first 5 h of starvation-induced differentiation (Figure 1a, inset).

Generation of Clones with Increased or Decreased Levels of AMPK α

To study the role of AMPK in mitochondrial disease in vivo in *Dictyostelium*, we chose a molecular genetic approach. To facilitate this, we amplified and cloned a cDNA encoding a truncated form (AMPK α T) of the *Dictyostelium* AMPK α subunit (Figure 1b). The encoded protein contains single amino acid changes at positions 3 and 374 through 379 at which point the polypeptide terminates prematurely. Although the S₃P substitution near the N terminus is not expected to affect the function of the protein, the six C-terminal substitutions and truncation of the protein mean that AMPK α T will not bind to the β subunit and will not be subject to the normal autoinhibitory mode of regulation of this enzyme. Truncating the human AMPK α isoform 1 in this region has been shown to have both of these effects and to thereby produce an active kinase that is phosphorylated

constitutively by upstream kinases (Crute *et al.*, 1998; Iseli *et al.*, 2005). Ectopic overexpression of AMPK α T will therefore increase the total AMPK activity in the cell.

The AMPK α T cDNA was subcloned into the *Dictyostelium* expression vector pA15GFP to create an overexpression construct. We also created antisense inhibition (and sense control) constructs by using a portion of the AMPK α gene (Figure 1c). Multiple, independent, stable transformants of the wild-type *Dictyostelium* strain AX2 were isolated and retained in each case.

To relate the phenotypes of these transformants to their genotypes, it was necessary to verify that the level of expression of AMPK α or AMPK α T was correlated in the expected manner with the number of copies of the plasmids with which they had been stably transformed. Quantitative RT-PCR of the amplicon in Figure 1d showed that the reduction of the native AMPK α mRNA level was correlated with the copy number of the antisense RNA-expression construct (Figure 1e). Conversely, in quantitative Northern blots the steady-state level of AMPK α T mRNA was tightly correlated with the copy number of the AMPK α T expression construct (Figure 1f). Western blots using an antibody directed against two specific AMPK α peptides confirmed that these cells overexpressed (in a copy number-dependent manner), a novel 42-kDa protein (AMPK α T) not present in wild-type cells (inset). Both constructs clearly affect expression in the expected copy number-dependent manner. Accordingly in further studies, we used the copy number of the corresponding constructs as an AMPK α expression index. To simplify analysis and presentation of the data, we assigned negative values to the copy numbers for antisense constructs and positive values to the copy numbers for overexpression constructs.

Overexpression of the AMPK α Catalytic Domain Phenocopies Mitochondrial Disease, whereas AMPK α Antisense Inhibition Suppresses Mitochondrial Disease Phenotypes

Because mitochondrially diseased strains of *Dictyostelium* exhibit impaired phototactic orientation, slow growth in liquid medium and defective multicellular morphogenesis (Wilczynska *et al.*, 1997; Kotsifas *et al.*, 2002; Chida *et al.*, 2004), we investigated whether AMPK α T overexpression caused similar defects and whether AMPK α antisense inhibition suppressed those defects (in chaperonin 60 antisense-inhibited cells). We also investigated whether the impaired growth was due to slower uptake of food by phagocytosis or macropinocytosis.

Phototaxis

In the multicellular migratory phase of its life cycle, the so-called slug, *Dictyostelium* exhibits extraordinarily sensitive and accurate orientation responses to light (phototaxis) and temperature gradients (thermotaxis). Although they are not well understood, the photo- and thermosensory transduction pathways in *Dictyostelium* are known to involve a variety of typically eukaryotic signaling molecules (Fisher, 1997, 2001; Bandala-Sanchez *et al.*, 2006). If any of them were downstream targets of AMPK signaling, then AMPK activation would cross-talk and interfere with phototaxis and thermotaxis. Chronic AMPK activation would then account for the impaired phototaxis and thermotaxis observed in mitochondrial disease.

Figure 2 shows that slugs became increasingly disoriented in phototaxis as the copy number of either a chaperonin 60 antisense construct (Figure 2a) or an AMPK α T expression construct (Figure 2b) increased. AMPK α T overexpression

Figure 2. Effect of chaperonin 60 and AMPK α expression levels on *Dictyostelium* slug phototaxis. Each circle represents a different clonal cell line (strain) carrying the indicated number of copies of either the chaperonin 60 antisense construct, the AMPK α antisense construct, or the AMPK α T overexpression construct. Lines are fitted only to the data represented by the circles. Each square represents a different strain carrying different numbers of copies of both the chaperonin 60 antisense construct and the AMPK α antisense construct. Data for these strains therefore occurs in both panels, in each of which it is plotted according to the copy number relevant to that panel only. The accuracy of phototaxis is the concentration parameter (κ) of the circular normal (von Mises) distribution, which measures how concentrated individual directions are around the direction toward the light source. κ ranges from 0 in the case of no preferred direction of migration (all directions equally probable) to ∞ in the case of perfect orientation (all directions exactly toward the light). Vertical bars are 90% confidence intervals. Negative values indicate the copy numbers of antisense inhibition constructs, whereas positive values indicate copy numbers of overexpression constructs. Copy numbers of zero include both the wild-type strain (AX2) and control strains carrying sense construct controls, but no copies of the relevant antisense or overexpression construct. (a) Phototaxis by mitochondrially diseased (chaperonin 60 antisense inhibited) *Dictyostelium* slugs formed and migrating on charcoal agar at 21°C. (b) Phototaxis by slugs of *Dictyostelium* strains with different levels of expression of the AMPK α subunit (antisense inhibition, negative copy numbers) or a truncated form of the α subunit containing the catalytic domain (AMPK α T overexpression, positive copy numbers). An animated three-dimensional scatter plot combining data from both panels a and b is contained in the Supplemental Material video file AMPK_photo_spin.mov.

thus phenocopies in a dose-dependent manner the phototaxis defect caused by mitochondrial dysfunction. AMPK α antisense inhibition in an otherwise wild-type genetic background had little effect on slug phototaxis causing, if any-

thing, a slight improvement in orientation. However mitochondrially diseased strains that would otherwise exhibit impaired phototaxis, behaved normally if AMPK α expression was also inhibited in the same cells (Figure 2).

Phototaxis and thermotaxis share the same downstream signaling pathways (Fisher, 1997, 2001), and mitochondrial disease impairs both (Wilczynska *et al.*, 1997; Kotsifas *et al.*, 2002). In other experiments (data not shown) we also found that AMPK α T overexpression impairs thermotaxis and that AMPK α antisense inhibition rescues the thermotaxis defect caused by chaperonin 60 inhibition. We conclude that in mitochondrial disease in *Dictyostelium* the defects in photosensory and thermosensory signal transduction are a result of AMPK α signaling. This pathway is likely to be conserved in higher eukaryotes, because AMPK was reported recently to inhibit motility and chemotactic signal transduction in mammalian monocytes (Kanellis *et al.*, 2006). In *Dictyostelium* phototaxis, the downstream target proteins could include RasD and extracellular signal-regulated kinase (ERK)2, which were recently reported to be part of a photosensory signaling complex (Bandala-Sanchez *et al.*, 2006). In mammalian cell lines, AMPK has been reported to inhibit upstream elements of the ERK1/2 signaling pathways (Sprenkle *et al.*, 1997; Kim *et al.*, 2001; Nagata *et al.*, 2004).

Growth Rates

Although AMPK signaling was responsible for defective phototaxis and thermotaxis in *Dictyostelium* mitochondrial disease, the other phenotypes could have been caused by a different mechanism. For example, the dramatically reduced cell growth and division rates reported previously in *Dictyostelium* mitochondrial disease (Wilczynska *et al.*, 1997; Kotsifas *et al.*, 2002) could have been caused by limitations in the energy available for growth. If this were the case, AMPK α T overexpression would not phenocopy and AMPK α antisense inhibition would not rescue the growth defects caused by mitochondrial dysfunction. Instead, the homeostatic function of AMPK in restoring the cellular energy status to normal would, if anything, produce the reverse outcomes.

Figure 3 shows that overexpression of the catalytic domain of AMPK and mitochondrial disease (antisense inhibition of chaperonin 60) dramatically impaired *Dictyostelium* growth both on plates on a bacterial food source (Figure 3, a and b) and in shaken culture in a nutrient broth (Figure 3, c and d). In otherwise healthy cells, AMPK α antisense inhibition actually enhanced growth slightly; the generation times in liquid decreased from ~9 to ~7 h, whereas growth on plates also accelerated (Figure 3, b and d). Most remarkably, the impaired growth of mitochondrially diseased cells (chaperonin 60 antisense inhibition) was completely restored to normal by AMPK α antisense inhibition (Figure 3, a and c). This dramatic suppression of the mitochondrial disease phenotype occurred both for growth on plates and in liquid medium. It shows that the cause of the impaired growth in *Dictyostelium* mitochondrial disease is chronic AMPK activation not insufficient energy.

Phagocytosis and Macropinocytosis Rates

Growth and division of cells rely on their continued ability to take up nutrients and in *Dictyostelium*, this is achieved by phagocytosis during growth on bacterial lawns and by macropinocytosis during growth in liquid medium. The two types of endocytosis depend upon signaling pathways containing both shared and distinct elements (Cardelli, 2001; Maniak, 2003) that could be downstream targets of AMPK signaling. It was thus possible that the impaired growth of mitochondrially diseased cells was due to impaired signal

Figure 3. Effect of chaperonin 60 and AMPK α expression levels on *Dictyostelium* growth. Symbols used are as in Figure 2. Vertical bars are 95% confidence intervals. Negative values indicate the copy numbers of antisense inhibition constructs, whereas positive values indicate copy numbers of the overexpression construct. Copy numbers of zero include both the wild type strain (AX2) and control strains carrying sense construct controls, but no copies of the relevant antisense or overexpression construct. (a and b) Time taken for a growing *Dictyostelium* colony (plaque) to expand 5 mm during growth at 21°C on an *E. coli* B2 lawn on SM agar. The growth time was calculated from the slope of the line measured by linear regression analysis of plaque diameter versus time during 5–7 d of growth. An animated three-dimensional scatter plot combining data from both a and b is contained in the Supplemental Material video file AMPK_plate_growth_spin.mov. (c and d) Generation time for *Dictyostelium* cells growing in HL-5 liquid medium at 21°C, shaken at 150 rpm. Generation times were calculated from growth curve slopes using log-linear regression analysis of cell counts during the exponential phase of growth. An animated three-dimensional scatter plot combining data from both c and d is contained in the Supplemental Material video file AMPK_HL-5_growth_spin.mov.

transduction for phagocytosis, macropinocytosis, or both. We therefore assayed in our strains the rate of bacterial uptake in phagocytosis and the rate of fluid uptake in macropinocytosis. Figure 4 shows that neither phagocytosis nor macropinocytosis were significantly affected by the levels of expression of either chaperonin 60 or AMPK α during 30- or 60-min assays, respectively. Thus, the dramatically slower growth that AMPK activity elicits in mitochondrial disease does not result from impaired ingestion of extracellular nutrient sources but from effects on the pathways that control cell growth and proliferation.

In other organisms, activated AMPK also inhibits cell growth and proliferation (Sprenkle *et al.*, 1997; Kim *et al.*, 2001; Nagata *et al.*, 2004; Igata *et al.*, 2005), and, in coupled p53-dependent pathways, additionally induces apoptosis in a variety of mammalian cell types (Campas *et al.*, 2003; Kefas *et al.*, 2003; Kobayashi *et al.*, 2004; Shaw *et al.*, 2004). Although *Dictyostelium* lacks both caspase-mediated apoptosis and p53, it does possess components of the target of rapamycin pathway, a central integrator of signals regulating cell growth and a major means by which AMPK inhibits cell growth in metazoa (Feng *et al.*, 2005; Inoki *et al.*, 2005). This conserved signaling pathway therefore provides a possible general mechanism for AMPK-mediated inhibition of cell proliferation in mitochondrial disease.

Multicellular Development

Development in *Dictyostelium* is initiated by starvation-induced differentiation followed by chemotactic aggregation, further differentiation into multiple cell types, and complex morphogenetic movements to form a fruiting body—a drop-let of spores (sorus) atop a stalk and basal disk. Multicellu-

larity evolved independently in the amoebozoia (*Dictyostelium*) and metazoa, so that some of the associated intracellular signaling molecules are different, whereas others have been conserved and recruited for use in regulation of cell type choice and differentiation in both lineages (Williams, 2006; Strmecki *et al.*, 2005). The developmental signaling pathways thus provide a mixture of potential downstream targets of AMPK signaling in mitochondrially compromised *Dictyostelium* cells, some unique and others conserved.

Mitochondrial disease in *Dictyostelium* has been shown to result in dysmorphogenesis so that as the disease becomes more severe, fewer fruiting bodies form, and those that do are morphologically aberrant with short thick stalks (Kotsifas *et al.*, 2002) resulting from misregulation of cell type choice in favor of the stalk differentiation pathway (Chida *et al.*, 2004). We found that a similar phenotype results from overexpression of AMPK α T in an otherwise wild-type background (Figure 5, d and f versus b). Conversely, antisense inhibition of AMPK α expression restored normal development in mitochondrially diseased cells in which chaperonin 60 expression was inhibited (Figure 5, a and c versus b). Consistent with this data, prestalk gene expression is enhanced in *Dictyostelium* cells that accumulate AMP because of inactivation of the AMP deaminase (Chae *et al.*, 2002). Thus, the abnormal multicellular development associated with mitochondrial disease in *Dictyostelium* is also mediated by AMPK signaling.

In the wild-type genetic background, antisense inhibition of AMPK α expression also caused a developmental abnormality in that cells growing on bacterial lawns failed to initiate development in areas where the bacteria had been

Figure 4. Effect of chaperonin 60 and AMPK α expression levels on *Dictyostelium* phagocytosis and pinocytosis rates. Symbols are as in Figure 2. Negative values indicate the copy numbers of antisense inhibition constructs, whereas positive values indicate copy numbers of overexpression construct. Copy numbers of zero refer to the wild-type parental strain AX2. (a and b) Rates of phagocytosis by *Dictyostelium* cells engulfing *E. coli* cells expressing the fluorescent red protein Ds-Red. Rates were measured from duplicate fluorescence measurements immediately and 30 min after addition of fluorescent bacteria to the amoebal suspension. An animated three-dimensional scatter plot combining data from both a and b is contained in the Supplemental Material video file AMPK_phago_spin.mov. (c and d) Rates of macropinocytosis by *Dictyostelium* cells in HL-5 medium containing 2 mg/ml FITC-dextran. The uptake of fluorescent medium was measured in duplicate immediately and 60 or 70 min after addition of FITC-dextran to the amoebal suspension. An animated three-dimensional scatter plot combining data from both c and d is contained in the Supplemental Material video file AMPK_pino_spin.mov.

consumed (Figure 5e). This result shows that in otherwise healthy cells, AMPK signaling is required for the initiation of development by starvation. The simplest explanation is that during normal development, starvation results in temporary ATP depletion and AMPK activation, which signals a halt to cell proliferation and entry into the pathways for early differentiation. The aggregation defect caused by AMPK α antisense inhibition was not observed in the mitochondrially diseased background of cells in which chaperonin 60 expression was also antisense inhibited. This makes sense if, in these cells, the mitochondrial dysfunction were to cause more severe ATP depletion than usual at the onset of development. The lower levels of AMPK expression in these cells would then be offset by more complete activation of the AMPK that is present so that development is normal.

AMPK Stimulates Mitochondrial Biogenesis and ATP Production

In mammalian cells, particularly in muscle tissues, AMPK activity leads to mitochondrial proliferation (Bergeron *et al.*, 2001; Zong *et al.*, 2002). This is part of the response to strenuous physical training in athletes and is a component of roles of AMPK in energy homeostasis in healthy cells. However, mitochondrial proliferation is sometimes also a feature

of mitochondrial diseases (Campos *et al.*, 1997; Graham *et al.*, 1997; Agostino *et al.*, 2003). We therefore examined whether mitochondrial dysfunction and AMPK activation might cause this same cytopathology in *Dictyostelium*. Figure 6b shows that overexpression of the AMPK α catalytic domain resulted in a stronger MitoTracker Green fluorescence signal per cell, whereas AMPK α antisense inhibition reduced the fluorescence signal. However, the fluorescence signal was unaltered in the mitochondrially diseased cells whether or not AMPK α expression was also antisense inhibited (Figure 6a). Fluorescence microscopy of cells stained with MitoTracker Red confirmed that mitochondrial biogenesis in *Dictyostelium* is stimulated by AMPK as in mammalian cells. We found no evidence for the proliferation of mitochondria in mitochondrially diseased *Dictyostelium* cells, presumably because in these cells any reduction in mitochondrial biogenesis caused by chaperonin 60 undersupply is counterbalanced by the effects of enhanced AMPK activity. Because mitochondrial biogenesis was stimulated in AMPK α T-overexpressing cells and reduced in AMPK antisense-inhibited cells, we expected that ATP levels would be altered in a similar manner. Figure 6, c and d, shows that this is so. ATP levels were greater in AMPK α T-overexpressing cells, were reduced in AMPK α antisense-inhibited cells, and were not significantly altered in mitochondrially diseased cells.

Figure 5. Effect of chaperonin 60 and AMPK α expression levels on multicellular morphogenesis in *Dictyostelium*. Photographs were taken from above (main panels) or from the side (insets) of fruiting bodies formed during growth at 21°C on a bacterial lawn (*K. aerogenes*). Apart from b, the parental wild-type strain (AX2), the strains contained (a) antisense inhibition constructs for both chaperonin 60 (main panel, 73 copies; inset, 56 copies) and AMPK α (main panel, 109 copies; inset, 98 copies) or (c) the chaperonin 60 antisense construct only (main panel, 48 copies; inset, top row, 67 copies; inset, other rows, 74 copies) or (e) the AMPK α antisense inhibition construct (143 copies) only or (d and f) the AMPK α T overexpression construct only (30 copies and 148 copies, respectively). Mitochondrial disease (chaperonin 60 antisense inhibition) and AMPK α T overexpression both caused the formation of fruiting bodies with thick, short stalks (red arrows in c, d, and f). Fruiting body morphology was normal for a mitochondrially diseased strain (chaperonin 60 antisense inhibition) in which AMPK α expression was antisense inhibited (cyan arrows in a). In otherwise healthy cells, AMPK α antisense inhibition resulted in formation of fewer, smaller fruiting bodies that were morphologically normal (green arrows in e). For comparative purposes, the strains were selected to show the phenotypes at moderate copy numbers of the various constructs. The aberrant phenotypes in c, d, e, and f were more severe at higher copy numbers.

AICAR, a Pharmacological Activator of AMPK in Mammalian Cells, Impairs Phototaxis in the Wild-Type but Not in AMPK α Antisense-inhibited Cells

Treatment with 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) is commonly used to activate AMPK in vitro and in vivo (Corton *et al.*, 1995; Hardie and Hawley, 2001). AICAR is taken up by cells by an adenosine transporter and converted intracellularly by adenosine kinase to AICA-ribotide (ZMP) (Nakamaru *et al.*, 2005), an analogue of AMP that activates AMPK. The *Dictyostelium* genome encodes homologues of these proteins (Ent family transporters; DictyBase accession nos. DDB0185520, DDB0205638, and DDB0205639; adenosine kinase, DictyBase accession no. DDB0230174) as well as the AMPK kinase LKB1 that is the primary mediator of AICAR activation (Hawley *et al.*, 2003). The presence of these genetic components makes it feasible, but does not prove, that AICAR treatment activates AMPK in *Dictyostelium* as it does in mammalian cells (Sugden *et al.*, 1999).

As a further test of whether AMPK activation could cause the defects seen in mitochondrial diseases, we examined the effect of AICAR on *Dictyostelium* slug phototaxis. Wild-type *Dictyostelium* cells were allowed to develop to the slug stage in the presence of AICAR and then to migrate in the presence of the drug. These slugs became increasingly disoriented as the AICAR concentrations increased (Figure 7a). These effects of long-term (>48 h) AICAR exposure could have been mediated directly or indirectly by some other adenosine- or AMP-binding protein. We therefore tested whether AICAR would still impair phototaxis in a strain in

which the expression of the α subunit of AMPK had been antisense inhibited. Figure 7b shows that the antisense-inhibited strain was resistant to the effects of AICAR on phototaxis. Although this result does not prove that AICAR impairs phototaxis by activating AMPK, it does indicate that AMPK is required for the AICAR effects and is consistent with a role for AMPK in mitochondrial disease.

DISCUSSION

The complex pathology of mitochondrial diseases is usually assumed to result from ATP depletion to levels that are insufficient to support normal cellular activities. Our results show that diverse cytopathologies in the *Dictyostelium* mitochondrial disease model are due not to insufficiency of the ATP supply but to chronic activation of the cellular energy sensor AMPK. Overexpression of the catalytic domain of AMPK phenocopies mitochondrial disease, whereas antisense inhibition of expression of the AMPK α subunit suppresses all of the aberrant disease-associated phenotypes. Mitochondrial disease emerges thus as an AMPK-mediated signaling disorder rather than an energy insufficiency per se.

There is every indication that this novel insight into the nature of mitochondrial disease will be generally applicable. During preparation of this article, it was reported that cell cycle progression in the developing *Drosophila* eye is halted specifically by AMPK signaling in response to the ATP-depleting effects of a mitochondrial mutation (Mandal *et al.*, 2005). Mitochondrial electron transport uncouplers such as dinitrophenol have been shown, like other cellular stressors, to activate AMPK. Much of the cytopathology of mitochondrial disorders in humans (James and Murphy, 2002; Rossignol *et al.*, 2003; Maassen *et al.*, 2004; McKenzie *et al.*, 2004)—mitochondrial proliferation and ragged red muscle fibers, cellular hypertrophy, and induction of apoptosis—is known also to occur in response to AMPK activation (Bergeron *et al.*, 2001; Hardie and Hawley, 2001; Zong *et al.*, 2002; Hardie, 2004; Kahn *et al.*, 2005; Hardie and Sakamoto, 2006). Finally, symptoms that are strongly reminiscent of mitochondrial disease are seen in a rare human genetic disorder, AICA-ribosiduria, in which an inactive AICAR transformylase causes the AMPK activator ZMP to accumulate in cells (Marie *et al.*, 2004). In addition to dysmorphic features, the affected patient, a female infant, suffered from severe neurological dysfunction, epilepsy, and congenital blindness.

Figure 8 shows an explanatory model for the interactions between mitochondrial function, AMPK, and the various energy-consuming cellular activities investigated in this work (flame-shaded section). Mitochondrial dysfunction was created by antisense inhibition of chaperonin 60 expression as described previously (Kotsifas *et al.*, 2002). The model suggests that this results in a reduction in mitochondrial biogenesis and ATP-generating capacity. Rossignol *et al.* (2003) discussed multiple layers of biochemical “thresholds” that determine the degree to which ATP-generating capacity is actually compromised as a result of genetic defects affecting the mitochondria. When these thresholds are exceeded AMPK would become chronically activated in a homeostatic response that returns mitochondrial mass and ATP levels to near normal but chronically inhibits some of the major energy-consuming activities of the cell (e.g., growth and cell cycle progression, multicellular morphogenesis, and photo- and thermosensory signal transduction). These major symptoms of mitochondrial disease in *Dictyostelium* were both phenocopied by overexpression of AMPK α T in otherwise healthy cells, and they were relieved

Figure 6. Effect of chaperonin 60 and AMPK α expression levels on mitochondrial mass and ATP levels in *Dictyostelium*. Symbols are as in Figure 2. Negative values indicate the copy numbers of antisense inhibition constructs, whereas positive values indicate copy numbers of the overexpression construct. Copy numbers of zero refer to the wild-type parental strain AX2. (a and b) Mitochondrial mass as measured by fluorescence with the mitochondrion-specific dye MitoTracker Green after subtraction of autofluorescence from unstained cells from the same suspension. The insets in b show MitoTracker Red fluorescence microscopy of typical cells from wild-type AX2 and from representative AMPK α antisense-inhibited strains (with and without chaperonin 60 antisense inhibition) and a representative AMPK α T-overexpressing strain. Compared with wild-type cells, the MitoTracker Red fluorescence seems brighter and the mitochondria more numerous in the case of AMPK α T overexpression, but fainter and the mitochondria less numerous in the AMPK α antisense-inhibited cells. The cells that are antisense-inhibited for both AMPK α and chaperonin 60 do not seem to differ from the wild-type cells in the brightness of the MitoTracker Red fluorescence or in the numbers of mitochondria. An animated three-dimensional scatter plot combining data from both a and b is contained in the Supplemental Material video file AMPK_mtmass_spin.mov. (c and d) ATP levels in *Dictyostelium* cells as measured using luciferase-based luminescence in a Modulus fluorometer (Turner BioSystems) with the luminescence module. An animated three-dimensional scatter plot combining data from both c and d is contained in the Supplemental Material video file AMPK_ATP_spin.mov. The significance of the correlations in b and d was verified both by using the nonparametric Kendall rank r and by two-sample tests (t test with unequal variances, Kolmogorov-Smirnov test and Kruskal-Wallis test) of the difference between the overexpression strains and the antisense-inhibited strains.

by antisense inhibition of AMPK α expression in mitochondrially diseased cells.

Mitochondrial proliferation and significantly reduced ATP levels are not always evident in mitochondrially dis-

Figure 7. Impairment of phototaxis by the pharmacological AMPK activator AICAR. *Dictyostelium* slugs of the parental strain (AX2; a) or an AMPK α antisense-inhibited transformant (143 copies of the antisense construct; b) were allowed to form and migrate toward a lateral light source on water agar supplemented with the indicated concentrations of AICAR. Slug trails were blotted onto PVC discs stained with Coomassie blue protein stain, digitized, and plotted from a common origin. The light source was to the right of the Figure. In the presence of AICAR, the wild-type slugs were disoriented so that their trails wound about much more during phototaxis. This did not occur with the AMPK α antisense-inhibited strain. The inset shows a separate experiment with wild-type cells in which the dose response to AICAR was clearer.

Knowing that AMPK activity is responsible for diverse cytopathologies in mitochondrial disease should open new possibilities for successful treatment of the symptoms with pharmacological inhibitors of AMPK and other elements of the downstream signaling pathways. Naturally, such treatments would not cure the disease nor would they ameliorate symptoms once ATP supplies really did become insufficient to support normal cellular activities. However, in the *Dicystostelium* model at least, that point seems not to be reached—AMPK α antisense inhibition in mitochondrially diseased cells always restored the wild-type phenotypes completely. It seems likely that in mitochondrially diseased mammalian cells as well, diverse cellular functions would be impaired and apoptosis induced by AMPK before ATP supplies actually become insufficient.

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REFERENCES

- Agostino, A. *et al.* (2003). Constitutive knockout of Surf1 is associated with high embryonic lethality, mitochondrial disease and cytochrome c oxidase deficiency in mice. *Hum. Mol. Genet.* 12, 399–413.
- Bandala-Sanchez, E., Annesley, S. J., and Fisher, P. R. (2006). A phototaxis signalling complex in *Dictyostelium discoideum*. *Eur. J. Cell Biol.* 85, 1099–1106.

- Barth, C., Fraser, D. J., and Fisher, P. R. (1998a). A rapid, small scale method for characterization of plasmid insertions in the *Dictyostelium* genome. *Nucleic Acids Res.* 26, 3317–3318.
- Barth, C., Fraser, D. J., and Fisher, P. R. (1998b). Coincidental replication is responsible for tandem multimer formation during plasmid integration into the *Dictyostelium* genome. *Plasmid* 39, 141–153.
- Bergeron, R., Ren, J. M., Cadman, K. S., Moore, I. K., Perret, P., Pypaert, M., Young, L. H., Semenkovich, C. F., and Shulman, G. I. (2001). Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis. *Am. J. Physiol. Endocrinol. Metab.* 281, E1340–E1346.
- Campas, C., Lopez, J. M., Santidrian, A. F., Barragan, M., Bellosillo, B., Colomer, D., and Gil, J. (2003). Acadesine activates AMPK and induces apoptosis in B-cell chronic lymphocytic leukemia cells but not in T lymphocytes. *Blood* 101, 3674–3680.
- Campos, Y., Martín, M. A., Rubio, J. C., Gutiérrez del Olmo, M. C., Cabello, A., and Arenas, J. (1997). Bilateral striatal necrosis and MELAS associated with a new T3308C mutation in the mitochondrial ND1 gene. *Biochem. Biophys. Res. Commun.* 238, 323–325.
- Cardelli, J. (2001). Phagocytosis and macropinocytosis in *Dictyostelium*: phosphoinositide-based processes, biochemically distinct. *Traffic* 2, 311–320.
- Chae, S.-C., Fuller, D., and Loomis, W. F. (2002). Altered cell-type proportioning in *Dictyostelium* lacking adenosine monophosphate deaminase. *Dev. Biol.* 241, 183–194.
- Chida, J., Yamaguchi, H., Amagai, A., and Maeda, Y. (2004). The necessity of mitochondrial genome DNA for normal development of *Dictyostelium* cells. *J. Cell Sci.* 117, 3141–3152.
- Chisholm, R. L., Gaudet, P., Just, E. M., Pilcher, K. E., Fey, P., Merchant, S. N., and Kibbe, W. A. (2006). DictyBase, the model organism database for *Dictyostelium discoideum*. *Nucleic Acids Res.* 34, D423–D427.
- Corton, J. M., Gillespie, J. G., Hawley, S. A., and Hardie, D. G. (1995). 5-aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur. J. Biochem.* 229, 558–565.
- Crute, B. E., Seefeld, K., Gamble, J., Kemp, B. E., and Witters, L. A. (1998). Functional domains of the $\alpha 1$ catalytic subunit of the AMP-activated protein kinase. *J. Biol. Chem.* 273, 35347–35354.
- Eichinger, L. *et al.* (2005). The genome of the social amoeba *Dictyostelium discoideum*. *Nature* 435, 43–57.
- Feng, Z., Zhang, H., Levine, A. J., and Jin, S. (2005). The coordinate regulation of the p53 and mTOR pathways in cells. *Proc. Natl. Acad. Sci. USA.* 102, 8204–8209.
- Fisher, P. R. (1997). Genetics of phototaxis in a model eukaryote, *Dictyostelium discoideum*. *Bioessays* 19, 397–408.
- Fisher, P. R. (2001). Genetic analysis of phototaxis in *Dictyostelium*. In: *Photomovement. ESP Comprehensive Series in Photosciences*, Vol. 1, ed. D.-P. Häder and M. Lebert, Amsterdam, The Netherlands: Elsevier Science, 519–559.
- Fisher, P. R., and Annesley, S. J. (2006). Slug phototaxis, thermotaxis and spontaneous turning behaviour. In: *Dictyostelium discoideum* Protocols. *Methods in Molecular Biology*, Vol. 346, ed. L. Eichinger and F. Rivero, Totowa, NJ: Humana Press, 137–170.
- Fisher, P. R., Smith, E., and Williams, K. L. (1981). An extracellular chemical signal controlling phototactic behaviour by *D. discoideum* slugs. *Cell* 23, 799–807.
- Gilson, P. R., Yu, X.-C., Hereld, D., Barth, C., Savage, A., Kiefel, B. R., Lay, S., Fisher, P. R., Margolin, W., and Beech, P. L. (2003). Two *Dictyostelium* orthologs of the prokaryotic cell division protein FtsZ localize to mitochondria and are required for the maintenance of normal mitochondrial morphology. *Eukaryot. Cell* 2, 1315–1326.
- Graham, B. H., Waymire, K. G., Cottrell, B., Trounce, I. A., MacGregor, G. R., and Wallace, D. C. (1997). A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. *Nat. Genet.* 16, 226–234.
- Hardie, D. G. (2004). The AMP-activated protein kinase pathway—new players upstream and downstream. *J. Cell Sci.* 117, 5479–5487.
- Hardie, D. G., and Hawley, S. A. (2001). AMP-activated protein kinase: the energy charge hypothesis revisited. *Bioessays* 23, 1112–1119.
- Hardie, D. G., and Sakamoto, K. (2006). AMPK: a key sensor of fuel and energy status in skeletal muscle. *Physiology* 21, 48–60.
- Hardie, D. G., Scott, J. W., Pan, D. A., and Hudson, E. R. (2003). Management of cellular energy by the AMP-activated protein kinase system. *FEBS Lett.* 546, 113–120.
- Hawley, S. A., Boudeau, J., Reid, J. L., Mustard, K. J., Udd, L., Mäkelä, T. P., Alessi, D. R., and Hardie, D. G. (2003). Complexes between the LKB1 tumor suppressor, STRAD α/β and MO25 α/β are upstream kinases in the AMP-activated protein kinase cascade. *J. Biol.* 2, 28.
- Hemminki, A. *et al.* (1998). A serine/threonine kinase gene defective in Peutz-Jeghers syndrome. *Nature* 391, 184–187.
- Hong, S.-P., Leiper, F. C., Woods, A., Carling, D., and Carlson, M. (2003). Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proc. Natl. Acad. Sci. USA.* 100, 8839–8843.
- Igata, M. *et al.* (2005). Adenosine monophosphate-activated protein kinase suppresses vascular smooth muscle cell proliferation through the inhibition of cell cycle progression. *Circ. Res.* 97, 837–844.
- Inoki, K., Ouyang, H., Li, Y., and Guan, K.-L. (2005). Signaling by Target of Rapamycin proteins in cell growth control. *Microbiol. Mol. Biol. Rev.* 69, 79–100.
- Iseli, T. J., Walter, M., van Denderen, B.J.W., Katsis, F., Witters, L. A., Kemp, B. E., Michell, B. J., and Stapleton, D. (2005). AMP-activated protein kinase β subunit tethers α and γ subunits via its C-terminal sequence (186–270). *J. Biol. Chem.* 280, 13395–13400.
- James, A. M., and Murphy, M. P. (2002). How mitochondrial damage affects cell function. *J. Biomed. Sci.* 9, 475–487.
- Jenne, D. E., Reimann, H., Nezu, J., Friedel, W., Loff, S., Jeschke, R., Muller, O., Back, W., and Zimmer, M. (1998). Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase. *Nat. Genet.* 18, 38–43.
- Kahn, B. B., Alquier, T., Carling, D., and Hardie, D. G. (2005). AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* 1, 15–25.
- Kanellis, J., Kandane, R. K., Etemadmoghadam, D., Fraser, S. A., Mount, P. F., Leviodiotis, V., Kemp, B. E., and Power, D. A. (2006). Activators of the energy sensing kinase AMPK inhibit random cell movement and chemotaxis in U937 cells. *Immunol. Cell Biol.* 84, 6–12.
- Kefas, B. A., Cai, Y., Ling, Z., Heimberg, H., Hue, L., Pipeleers, D., and van de Castele, M. (2003). AMP-activated protein kinase can induce apoptosis of insulin-producing MIN6 cells through stimulation of c-Jun-N-terminal kinase. *J. Mol. Endocrinol.* 30, 151–161.
- Kim, J., Yoon, M. Y., Choi, S. L., Kang, I., Kim, S.-S., Kim, Y.-S., Choi, Y.-K., and Ha, J. (2001). Effects of stimulation of AMP-activated protein kinase on insulin-like growth factor 1- and epidermal growth factor-dependent extracellular signal-regulated kinase pathway. *J. Biol. Chem.* 276, 19102–19110.
- Klein, G., and Satre, M. (1986). Kinetics of fluid-phase pinocytosis in *Dictyostelium discoideum* amoebae. *Biochem. Biophys. Res. Commun.* 138, 1146–1152.
- Kobayashi, H., Ouchi, N., Kihara, S., Walsh, K., Kumada, M., Abe, Y., Funahashi, T., and Matsuzawa, Y. (2004). Selective suppression of endothelial cell apoptosis by the high molecular weight form of adiponectin. *Circ. Res.* 94, e27–e31.
- Kotsifas, M., Barth, C., Lay, S. T., de Lozanne, A., and Fisher, P. R. (2002). Chaperonin 60 and mitochondrial disease in *Dictyostelium*. *J. Muscle Res. Cell Motil.* 23, 839–852.
- Kurth-Kraczek, E. J., Hirshman, M. F., Goodyear, L. J., and Winder, W. W. (1999). 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* 48, 1667–1671.
- Liu, T., Mirschberger, C., Chooback, L., Arana, Q., Dal Sacco, Z., MacWilliams, H., and Clarke, M. (2002). Altered expression of the 100 kDa subunit of the *Dictyostelium* vacuolar proton pump impairs enzyme assembly, endocytic function and cytosolic pH regulation. *J. Cell Sci.* 115, 1907–1918.
- Maassen, J. A., 't Hart, L. M., van Essen, E., Heine, R. J., Nijpels, G., Tafrech, R.S.J., Raap, A. K., Janssen, G.M.C., and Lemkes, H.H.P.J. (2004). Mitochondrial diabetes. Molecular mechanisms and clinical presentation. *Diabetes* 53, S103–S109.
- Mandal, S., Guptan, P., Owusu-Ansah, E., and Banerjee, U. (2005). Mitochondrial regulation of cell cycle progression during development as revealed by the *tenured* mutation in *Drosophila*. *Developmental Cell* 9, 843–854.
- Maniak, M. (2003). Fusion and fission events in the endocytic pathway of *Dictyostelium*. *Traffic* 4, 1–5.
- Marie, S., Heron, B., Bitoun, P., Timmerman, T., van den Berghe, G., and Vincent, M.-F. (2004). AICA-ribosiduria: a novel, neurologically devastating inborn error of purine biosynthesis caused by mutation of ATIC. *Am. J. Hum. Genet.* 74, 1276–1281.
- Maselli, A., Laevsky, G., and Knecht, D. A. (2002). Kinetics of binding, uptake and degradation of live fluorescent (DsRed) bacteria by *Dictyostelium discoideum*. *Microbiology* 148, 413–420.

- McKenzie, M., Liolitsa, D., and Hanna, M. G. (2004). Mitochondrial disease: mutations and mechanisms. *Neurochem. Res.* 29, 589–600.
- Nagata, D., Takeda, R., Sata, M., Satonaka, H., Suzuki, E., Nagano, T., and Hirata, Y. (2004). AMP-activated protein kinase inhibits angiotensin II-stimulated vascular smooth muscle cell proliferation. *Circulation* 110, 444–451.
- Nakamaru, K. *et al.* (2005). AICAR, an activator of AMP-activated protein kinase, down-regulates the insulin receptor expression in HepG2 cells. *Biochem. Biophys. Res. Commun.* 328, 449–454.
- Pendergrass, W., Wolf, N., and Poot, M. (2004). Efficacy of MitoTracker Green™ and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. *Cytometry* 61A, 162–169.
- Rosignol, R., Faustin, B., Rocher, C., Malgat, M., and Mazat, J.-P., and Letellier, T. (2003). Mitochondrial threshold effects. *Biochem. J.* 370, 751–762.
- Russell, R. R., Bergeron, R., Shulman, G. I., and Young, L. H. (1999). Translocation of myocardial GLUT4 and increased glucose uptake through activation of AMPK by AICAR. *Am. J. Physiol.* 277, H643–H649.
- Scott, J. W., Hawley, S. A., Green, K. A., Anis, M., Stewart, G., Scullion, G. A., Norman, D. G., and Hardie, D. G. (2004). CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J. Clin. Invest.* 113, 274–284.
- Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Cantley, L. C. (2004). The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc. Natl. Acad. Sci. USA* 101, 3329–3335.
- Sprenkle, A. B., Davies, S. P., Carling, D., Hardie, D. G., and Sturgill, T. W. (1997). Identification of Raf-1 Ser⁶²¹ kinase activity from NIH 3T3 cells as AMP-activated protein kinase. *FEBS Lett.* 403, 254–258.
- Strmecki, L., Greene, D. M., and Pears, C. J. (2005). Developmental decisions in *Dictyostelium discoideum*. *Dev. Biol.* 284, 25–36.
- Sugden, C., Crawford, R. M., Halford, N. G., and Hardie, D. G. (1999). Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5'-AMP. *Plant J.* 19, 433–439.
- Tarnopolsky, M. A., and Parise, G. (1999). Direct measurement of high-energy phosphate compounds in patients with neuromuscular disease. *Muscle Nerve* 22, 1228–1233.
- Weekes, J., Hawley, S. A., Corton, J., Shugar, D., and Hardie, D. G. (1994). Activation of rat liver AMP-activated protein kinase by kinase kinase in a purified, reconstituted system. Effects of AMP and AMP analogues. *Eur. J. Biochem.* 219, 751–757.
- Wilczynska, Z., Barth, C., and Fisher, P. R. (1997). Mitochondrial mutations impair signal transduction in *Dictyostelium discoideum* slugs. *Biochem. Biophys. Res. Commun.* 234, 39–43.
- Williams, J. G. (2006). Transcriptional regulation of *Dictyostelium* pattern formation. *EMBO Rep.* 7, 694–698.
- Witters, L. A., and Kemp, B. E. (1992). Insulin activation of acetyl-CoA carboxylase accompanied by inhibition of the 5'-AMP-activated protein kinase. *J. Biol. Chem.* 267, 2864–2867.
- Woods, A., Azzout-Marniche, D., Foretz, M., Stein, S. C., Lemarchand, P., Ferre, P., Foufelle, F., and Carling, D. (2000). Characterisation of the role of AMP-activated protein kinase in the regulation of glucose-activated gene expression using constitutively active and dominant negative forms of the kinase. *Mol. Cell. Biol.* 20, 6704–6711.
- Zong, H., Ren, J. M., Young, L. H., Pypaert, M., Mu, J., Birnbaum, M. J., and Shulman, G. I. (2002). AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc. Natl. Acad. Sci. USA* 99, 15983–15987.