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The Energy Sensor AMP-activated Protein Kinase Directly Regulates the Mammalian FOXO3 Transcription Factor*[S]

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The maintenance of homeostasis throughout an organism's life span requires constant adaptation to changes in energy levels. The AMP-activated protein kinase (AMPK) plays a critical role in the cellular responses to low energy levels by switching off energy-consuming pathways and switching on energy-producing pathways. However, the transcriptional mechanisms by which AMPK acts to adjust cellular energy levels are not entirely characterized. Here, we find that AMPK directly regulates mammalian FOXO3, a member of the FOXO family of Forkhead transcription factors known to promote resistance to oxidative stress, tumor suppression, and longevity. We show that AMPK phosphorylates human FOXO3 at six previously unidentified regulatory sites. Phosphorylation by AMPK leads to the activation of FOXO3 transcriptional activity without affecting FOXO3 subcellular localization. Using a genome-wide microarray analysis, we identify a set of target genes that are regulated by FOXO3 when phosphorylated at these six regulatory sites in mammalian cells. The regulation of FOXO3 by AMPK may play a crucial role in fine tuning gene expression programs that control energy balance and stress resistance in cells throughout life.

The maintenance of cellular energy levels in response to changes in nutrient availability, exercise, or stress stimuli is pivotal for organismal homeostasis throughout life. Disruption of this balance is associated with a number of pathologies, including diabetes and cancer. The AMP-activated protein kinase (AMPK)[3] plays a crucial role in translating changes in energy levels into adaptive cellular responses (1, 2). AMPK is a heterotrimeric protein kinase composed of three subunits: a catalytic subunit (α), a scaffolding subunit (β), and an AMP-sensing subunit (γ). AMPK is activated by stimuli that increase the AMP/ATP ratio in cells. Excess AMP activates AMPK by inhibiting the dephosphorylation of the α catalytic subunit (3) and by inducing a change in conformation in the AMPK heterotrimeric complex that promotes the phosphorylation and activation of the α catalytic subunit by the AMPK-activating protein kinases, LKB1 and calmodulin-dependent protein kinase kinase (4–8).

AMPK controls cell metabolism and growth in response to low energy levels by phosphorylating a variety of substrates in cells, including acetyl-CoA carboxylase (ACC), tuberous sclerosis complex 2, and $p27^{KIP1}$ (9–11). AMPK also regulates gene expression by phosphorylating co-activators, such as transducer of regulated CREB, thyroid hormone receptor interactor 6, and the transcription factor p53 (12–16). The LKB1-AMPK pathway plays a pivotal role in tumor suppression (17, 18), in diabetes prevention (19), and in longevity (20). Thus, identifying novel AMPK substrates is important to understand how the LKB1-AMPK pathway mediates its effects in the organism.

FOXO transcription factors are good candidates to be regulated by AMPK. The FOXO family of Forkhead transcription factors (FOXO1, FOXO3, FOXO4, and FOXO6 in mammals) plays an important role in the regulation of organismal glucose metabolism, tumor suppression, and longevity (21–26). In mammalian cells, FOXO family members up-regulate target genes involved in glucose metabolism, cell cycle arrest, stress resistance, and even cell death (27–32). FOXO transcription factors integrate cellular signals emanating from insulin, growth factors, cytokines, and oxidative stress via a combination of post-translational modifications, including phosphorylation, acetylation, and ubiquitination (30, 33–37). In response to insulin and growth factors, AKT phosphorylates FOXO proteins, thereby sequestering them in the cytoplasm and inhibiting their function (30, 34). Stimuli that activate AMPK have been reported to inhibit FOXO1-dependent transcription (38). However, whether FOXO family members are direct substrates of AMPK and how they mediate the effects of AMPK on gene expression is not known.

Here, we show that the energy-sensing AMPK directly phosphorylates human FOXO3 at six previously unidentified residues *in vitro*. At least three of these sites are phosphorylated in mammalian cells in response to stimuli that activate AMPK. AMPK is necessary and sufficient for the phosphorylation of FOXO3 at these sites in cells. We show that mutating the

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[3] The abbreviations used are: AMPK, adenosine monophosphate-activated protein kinase; ACC, acetyl-CoA carboxylase; 2DG, 2-deoxyglucose; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; GST, glutathione *S*-transferase; DN, dominant negative; MEF, mouse embryo fibroblast; LC, liquid chromatography; MS/MS, mass spectrometry; PI3K, phosphatidylinositol 3-kinase; RT, reverse transcription.

AMPK phosphorylation sites of FOXO3 impairs FOXO3-dependent transcription but does not affect FOXO3 localization. A genome-wide microarray analysis indicates that mutation of AMPK phosphorylation sites in FOXO3 specifically impairs the expression of a subset of target genes, including oxidative stress resistance and energy metabolism genes. We identify a connection between the AMPK pathway and FOXO3 that may play an important role in the maintenance of cellular homeostasis.

EXPERIMENTAL PROCEDURES

Constructs—GST-FOXO3 bacterial expression constructs, FLAG-FOXO3 mammalian expression constructs, and retroviral constructs were described previously (30, 36). FOXO3 mutants (T179A, S399A, S413A. S439A, S555A, S588A, and S626A) in the pGEX-4T3, the pECE, and the NH_2 vectors were generated by site-directed mutagenesis (Stratagene), using the following sets of primers: T179A-F (CGGACAAACGGCTAGCTCTGTCCCAGATC), T179A-R (GATCTGGGACAGAGCTAGCCGTTTGTCCG), S399A-F (CACGCTCCCGCCGGCCCAGCCATCGCCC), S399A-R (GGGCGATGGCTGGGCCGGCGGGAGCGTG), S413A-F (CATGCAGCGGAGCTCAGCTTTCCCGTATACC), S413A-R (GGTATACGGGAAAGCTGAGCTCCGCTGCATG), S439A-F (GGTGTTCGGACCTTCAGCTCTGAATTCCCTACGCCAGTCTCC), S439A-R (GGAGACTGGCGTAGGGAATTCAGAGCTGAAGGTCCGAACACC), S555A-F (CCTTGTCGAATTCTGTCGCCAACATGGGCTTGAG), S555A-R (CTCAAGCCCATGTTGGCGACAGAATTCGACAAGG), S588A-F (CCTCTCGGACTCTCTCGCAGGATCCTCCTTGTACTC), S588A-R (GAGTACAAGGAGGATCCTGCGAGAGAGTCCGAGAGG); S626A-F (GGAATGTGACATGGAGGCCATTATCCGTAG), S626A-R (CTACGGATAATGGCCTCCATGTCACATTCC).

GST-SIRT1, GST-FOXO1, GST-FOXO4, and GST-FOXO6 bacterial expression constructs were created by subcloning the human cDNA (SIRT1, FOXO1, and FOXO4) or mouse cDNA (FOXO6) into the pGEX-4T3 vector in frame with GST.

The mutations and flanking regions were verified by sequencing. Human AMPK β1 and γ1 expression plasmids were obtained from Origene. Human AMPKα2 WT cDNA (Origene) was subcloned into the pECE mammalian expression vector in frame with the FLAG epitope. CA AMPK α2 was generated by truncating AMPK α2 cDNA at the position corresponding to amino acid 310, as previously described (39). Dominant negative (DN) AMPK α2 K45R mutant was obtained by site-directed mutagenesis using the following primers: K45R-F (CCATAAAGTGGCAGTTAGAATCTTAAATAGAC) and K45R-R (GTCTATTTAAGATTCTAACTGCCACTTTATGG).

Antibodies and Reagents—The antibodies to β-actin and FLAG epitope were obtained from Novus Biologicals and Sigma, respectively. The antibodies to phospho-Ser^{413} and total FOXO3 were described previously (36). The antibodies to the C-terminal region of FOXO3 were generated by injection of a fusion protein between GST and amino acids 497–601 of mouse Foxo3 into rabbits, and the antibodies were purified by affinity (Quality Controlled Biochemicals). Phosphopeptides of the sequences p-T179 (CESSPDKRLpTLSQI), p-S399 (CLDNITLPPpSQPSP), p-S555 (CSRALSNSVpSNMGL), p-S588 (CMQTLSDSLpSGSSL), and p-S626 (CGSLECDMEpSIIRS) were coupled to KLH and injected into rabbits, and the phosphoantibodies were purified by affinity (Quality Controlled Biochemicals for P-S399, P-555, and P-S588; Covance for P-179, P-588, and P-S626). Unless otherwise stated, chemicals were purchased from Sigma. Compound C was a generous gift from Dr. Gaochao Zhou (40).

AMPK in Vitro Kinase Assay—Purified AMPK (Upstate Biotechnology) was incubated with various substrates (1 μg) in the kinase reaction buffer (HEPES, pH 7.0 (15 mM), dithiothreitol (450 μM), $MgCl_2$ (18.75 mM), β-glycerophosphate (6.25 mM), EGTA (1.25 mM), and ATP (125 μM)) and 12.5 μCi of radiolabeled ATP, with or without 150 μM AMP, at 30 °C for 15 min. Phosphorylation was detected by incorporation of radiolabeled [γ-^{32}P]ATP. Quantifications were performed using the Amersham Biosciences Storm PhosphorImager and ImageQuant 5.2 software.

Immunoblotting—The 293T human epithelial kidney cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (50 units/ml penicillin and 265 μg/ml streptomycin). 293T cells were seeded in 6-well plastic dishes at a density of 7×10^5 cells/well. They were transfected by the calcium phosphate technique with 5 μg of the constructs of interest. Forty-eight hours after transfection, cells were incubated in serum-free Dulbecco's modified Eagle's medium for 3 h and stimulated with various AMPK activators. Extracts were obtained by lysing the cells in lysis buffer (Tris-HCl, pH 8.0 (50 mM), NaCl (100 mM), EGTA (2 mM), NaF (10 mM), β-glycerophosphate (40 mM), Triton X-100 (0.4%), aprotinin (10 μg/ml), and phenylmethylsulfonyl fluoride (1 mM)). Protein extracts were resolved on SDS-PAGE (10%) and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies, and the primary antibody was visualized using horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies and ECL (Amersham Biosciences).

Immunoprecipitation—293T cells were seeded in a 10-cm dish at 3.5×10^6 cells/dish. 24 h later, protein extracts from four dishes were pooled and incubated with the C-terminal antibody to FOXO3 chemically coupled to agarose beads using the Profound™ co-immunoprecipitation kit (Pierce). The immune complexes were washed three times in the wash buffer (Pierce), and the proteins were eluted from the beads using the elution buffer (Pierce).

Immunocytochemistry—293T cells were seeded in 12-well plastic dishes on coverslips at a density of 3.5×10^5 cells/well. Cells were transfected by the calcium phosphate technique with 2.5 μg of the constructs of interest. Forty-eight hours after transfection, cells were fixed in 4% formaldehyde and permeabilized with 0.4% Triton X-100. Coverslips were incubated with the FLAG antibody (1:2000; Sigma) and the Alexa Fluoro 488 goat anti-mouse antibody. Coverslips were mounted in Aquamount and examined under epifluorescent illumination. FOXO3 subcellular localization was examined in 300 cells in a blinded manner.

Tandem Mass Spectrometry—A Coomassie-stained band corresponding to GST-FOXO3 phosphorylated by AMPK was excised from an SDS-polyacrylamide gel, divided in half,

reduced with dithiothreitol, alkylated with iodoacetamide, and digested with either trypsin or chymotrypsin. Peptide mixtures were separated by microcapillary reverse-phase chromatography and analyzed online in a hybrid linear ion trap-Orbitrap (LTQ-Orbitrap; Thermo Electron) mass spectrometer. Mass spectra were data base-searched using the SEQUEST algorithm. All peptide matches were initially filtered based on enzyme specificity, mass measurement error, Xcorr and ΔCorr′ scores and further manually validated for peptide identification and site localization.

Luciferase Reporter Assay—WT mouse embryonic fibroblasts (MEFs), prepared from embryonic day 13.5 embryos from CD1 WT mice, were seeded at 35,000 cells/well in 24-well plates and were transfected with 200 ng of FOXO3 plasmids together with 200 ng of a luciferase reporter construct driven by six tandem repeats of DAF-16/FOXO binding elements (6 × DBE-Luciferase) (41) and 200 ng of a *Renilla* luciferase reporter construct driven by the thymidine kinase promoter (TK-Renilla) using Fugene (Roche Applied Science). 48 h after transfection, cells were stimulated with appropriate stimuli, and luciferase and *Renilla* activities were measured using the STOP and GLOW kit (Promega) using the manufacturer's protocol.

Electrophoretic Mobility Shift Assay—The forward (AAATAACACACACGTGTGCTGGTAAACAAGCGCGCCAGCC) and reverse (GGCTGGCGCGCTTGTTTACCAGCACACGTGTGTGTTATTT) oligonucleotides corresponding to the FOXO canonical binding site (DBE) were annealed and labeled with polynucleotide kinase in the presence of [^{32}P]ATP for 30 min. The double-stranded probe was purified from a 15% native gel. 16,000 cpm of labeled probes were incubated for 20 min at room temperature with 100–500 ng of recombinant protein and 6 μg of salmon sperm DNA in electrophoretic mobility shift assay binding buffer (Tris-HCl, pH 7.5 (50 mM), KCl (250 mM), dithiothreitol (5 mM), $MgCl_2$ (25 mM), glycerol (50%), and Nonidet P-40 (0.25%)). The reactions were then resolved by electrophoresis on 4% native acrylamide gels. Gels were dried and autoradiographed.

Retroviral Infection—Foxo3$^{-/-}$ primary MEFs were prepared from embryonic day 13.5 embryos from *Foxo3*$^{-/-}$ mice (FVB/N background). For retroviral infection, 293T cells were co-transfected with the retroviral constructs of interest together with plasmids encoding the Gag, Pol, and Env proteins of the MLV retrovirus. 20 h after transfection, 0.45-μm filtered conditioned medium from infected 293T cells was added onto *Foxo3*$^{-/-}$ MEF cells in the presence of 8 μg/ml Polybrene (hexadimethrine bromide; Sigma). Three successive rounds of infections were performed at 12-h intervals, and the infected cells were selected for their resistance to G418 (Invitrogen) for 1 week. Cells were seeded in 6-well plates at 3×10^5 cells/well and stimulated as described. RNA was extracted using Trizol following the manufacturer's protocol (Invitrogen).

Quantitative RT-PCR—The expression of mouse *Gadd45a*, *Aldh3a*1, and *Mt*1 were determined by reverse transcription of total RNA, followed by quantitative PCR analysis. One μg of total RNA was reverse transcribed with random hexamers using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real time PCR was performed on a Bio-Rad iCycler using iQ SYBR green (Bio-Rad) with the following primers: Aldh3a1 F, AGACATCAAGCGGTGGAGTGA; Aldh3a1 R, CGAAGCTTTTCTTGCCATGG; Mt1 F, ATCTCGGAATGGACCCCAACT; Mt1 R, TATTTACACGTGGTGGCAGCG; Gadd45a F, AGCAAGGCTCGGAGTCAGC; Gadd45a R, ACGTTGAGCAGCTTGGCAG; β-actin F, TGTTACCAACTGGGACGACA; β-actin R, TCTCAGCTGTGGTGGTGAAG; Rab39b F, GGAAAACGCATCAAGCTCCA; Rab39b R, AAGGACCTGCGGTTGGTAATG; Ucp2 F, CCCAGCCTACAGATGTGGTAA; Ucp2 R, GAGGTTGGCTTTCAGGAGAGT; Pgc1a F, AGCGCCGTGTGATTTACGTT; Pgc1a R, CCGCAGATTTACGGTGCATT; Slc40a1 F, TTCCGCACTTTCCGAGATG; Slc40a1 R, AGTCAAAGCCCAGGACTGTCA; Atp6v0d2 F, CAAGCCTTTGTTTGACGCTGT; Atp6v0d2 R, TGCCTGTTGAATGCCAGCA.

The experiments were conducted in triplicate, and the results were expressed as $2^{(-(\text{Gene of interest number of cycles} - \text{actin number of cycles}))}$. Control PCRs were also performed on total RNA that had not been reverse-transcribed to test for the presence of genomic DNA in the RNA preparation. The specificity of the products of the quantitative RT-PCR reactions was verified on 2% agarose gels.

Genome-wide Microarray Analysis—Foxo3$^{-/-}$ MEFs expressing either WT FOXO3 or the 6A mutant constructs were left unstimulated, or duplicates were incubated in serum-free media in the presence of 2-deoxyglucose (2DG; 1 mM) for 8 h. RNA was extracted by Trizol extraction and was checked for integrity and quantity with the Agilent Bio-Analyzer QC. Microarray hybridization was performed at the Stanford PAN facility. Briefly, RNA was reverse transcribed to cDNA, followed by *in vitro* transcription and biotinylation. Biotinylated cRNAs were then fragmented, mixed with control cRNA fragments, and hybridized to Affymetrix oligonucleotide arrays (Mouse Genome 430 2.0 array). Microarray results were normalized across all probe sets, such that the mean signal of each experiment is normalized to the mean signal of the base line for all probe sets with GCOS (GeneChip Operating Software) from Affymetrix so that changes in gene expression could be compared across different samples. Normalized data were subsequently filtered using the following parameters. 1) A probe was removed if normalized values for that particular probe were missing in greater than 20% of the samples (to ensure that each probe was placed in the proper cluster). 2) A probe was removed if the S.D. value of the normalized values across individual conditions tested in the microarray (WT nonstimulated, 6A nonstimulated, WT stimulated by nutrient deprivation 1, WT stimulated by nutrient deprivation 2, 6A stimulated by nutrient deprivation 1, and 6A stimulated by nutrient deprivation 2) was less than 2.0 (to ensure that only genes whose expression changes significantly were compared). 3) A probe was removed if it did not display an absolute normalized value greater than 20 in at least four samples, as in Ref. 42 (to exclude probes with low values, which are known to be less reliable. Values ranged in expression from 0.1 to 36,786.4). Data were then log-transformed to ensure that negative-fold changes were given equal importance as positive-fold changes. Finally, a complete linkage

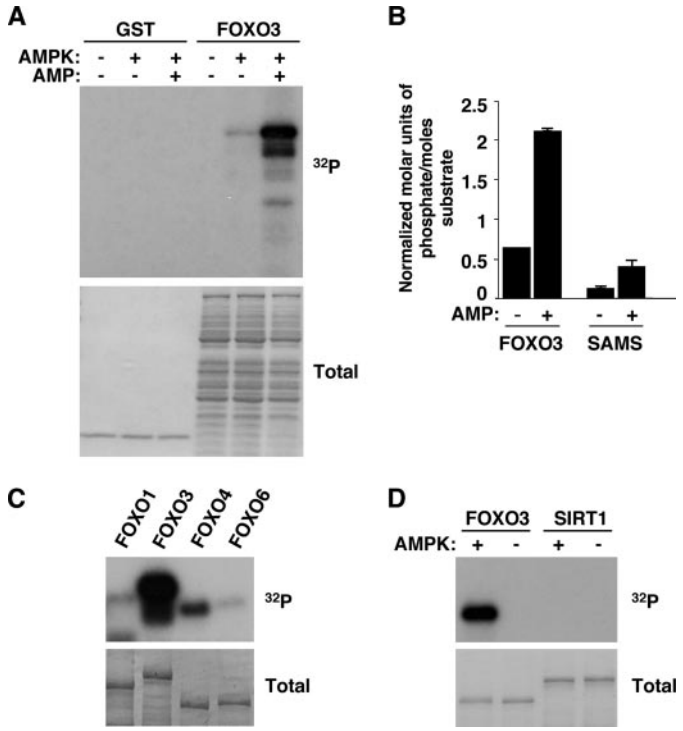

FIGURE 1. **AMPK directly phosphorylates mammalian FOXO transcription factors.** *A*, AMPK phosphorylates human FOXO3 in an AMP-dependent manner. AMPK was incubated with GST or GST-FOXO3 in the absence or presence of AMP. *B*, stoichiometry of phosphorylation of FOXO3 and the SAMS peptide by AMPK. The graph represents the mean and S.D. of normalized molar units of phosphate incorporated per mol of substrate of three independent experiments. *C*, AMPK phosphorylates FOXO3 more potently than the other FOXO family members. Purified human AMPK was incubated with GST-FOXO1, GST-FOXO3, GST-FOXO4, and GST-FOXO6 in the presence of [γ-^{32}P]ATP and AMP. *D*, AMPK phosphorylates GST-FOXO3 but not GST-SIRT1 *in vitro*.

hierarchical clustering was performed using Gene Cluster 3.0 (43). Clustering results were further analyzed with Java Treeview (44).

RESULTS

AMPK Directly Phosphorylates FOXO Transcription Factors in Vitro—To determine if AMPK directly phosphorylated FOXO transcription factors, we performed *in vitro* kinase assays using purified AMPK and bacterially expressed forms of GST-FOXO fusion proteins as substrates. We found that AMPK directly phosphorylated mammalian FOXO3 and that the phosphorylation of FOXO3 by AMPK was greatly enhanced by the presence of AMP (Fig. 1*A*). This result indicates that the purified kinase responsible for FOXO3 phosphorylation is AMP-dependent. The stoichiometry of phosphorylation of FOXO3 by AMPK was higher than that of SAMS, a 15-amino acid peptide containing a known AMPK phosphorylation site (45) (Fig. 1*B*), suggesting that FOXO3 is a good *in vitro* substrate of AMPK. AMPK also phosphorylated the other FOXO family members (FOXO1, FOXO4, and FOXO6) but displayed a strong preference toward FOXO3 (Fig. 1*C*). In contrast, AMPK did not phosphorylate GST alone (Fig. 1*A*), the small GTPase RAC1 (data not shown), or the NAD^+-dependent deacetylase SIRT1 (Fig. 1*D*), indicating that even *in vitro*, AMPK displays some specificity toward FOXO transcription factors. Together, these results show that AMPK potently phosphorylates FOXO3 *in vitro*.

AMPK Phosphorylates FOXO3 at Six Previously Unidentified Residues—We next sought to determine the residues of FOXO3 that were phosphorylated by AMPK. Scanning of FOXO3 protein sequence revealed the presence of six sites that conform to varying degrees to the consensus motif phosphorylated by AMPK (amphipathic helix; $\phi X\beta XX(S/T)XXX\phi$, where ϕ represents a hydrophobic residue and β represents a basic residue) (46–48): Thr^{179}, Ser^{399}, Ser^{413}, Ser^{439}, Ser^{555}, and Ser^{588} (Fig. 2*A*). A deletion analysis showed that the C-terminal domain of FOXO3 (amino acids 301–673) was more potently phosphorylated by AMPK than the N-terminal domain (amino acids 1–300) (Fig. 2*B*). This observation indicates that most of the AMPK phosphorylation sites are present in the C-terminal portion of FOXO3, which is the transactivation domain. We replaced the potential AMPK phosphorylation sites in FOXO3 with alanines, individually or in combination, and analyzed the phosphorylation of these FOXO3 mutants by AMPK. The T179A, S399A, S413A, S555A, and S588A single mutants were all slightly less phosphorylated by AMPK than WT FOXO3 (Fig. 2*C*) (data not shown), whereas the FOXO3 S439A mutant was phosphorylated by AMPK to the same extent as WT FOXO3 (data not shown). These results suggest that Ser^{439} was not modified by AMPK, at least under these conditions. A mutant of four of the C-terminal residues (S399A/S413A/S555A/S588A; 4SA) displayed a 60% reduction in phosphorylation by AMPK compared with WT FOXO3 (Fig. 2*C*), indicating that these four C-terminal sites contribute significantly to the phosphorylation of FOXO3 by AMPK.

To confirm our mutation analysis and potentially identify other residues of FOXO3 that are phosphorylated by AMPK, we analyzed the phosphopeptides of FOXO3 phosphorylated by AMPK using liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). This LC-MS/MS approach confirmed the phosphorylation of two residues, Ser^{413} and Ser^{555} (supplemental Table S1). LC-MS/MS also revealed that several other residues of FOXO3 were phosphorylated by AMPK *in vitro* (supplemental Table S1). In particular, Ser^{626}, although not a perfect AMPK phosphorylation site, is located in the highly phosphorylated C-terminal fragment of FOXO3. The replacement of Ser^{626} by an alanine reduced the phosphorylation of FOXO3 by AMPK, suggesting that this site is phosphorylated *in vitro* (data not shown). The replacement of Thr^{179}, Ser^{399}, Ser^{413}, Ser^{555}, Ser^{588}, and Ser^{626} by alanine (6A mutant) resulted in a significant reduction (84%) in phosphorylation compared with WT FOXO3 (Fig. 2*C*), indicating that these six phosphorylation sites are the main AMPK phosphorylation sites of FOXO3. However, the 6A mutant was still phosphorylated by AMPK to some extent, consistent with the finding that other sites in FOXO3 can be phosphorylated by AMPK (supplemental Table S1). Taken together, these results indicate that AMPK phosphorylates at least six residues of FOXO3 *in vitro* (Thr^{179}, Ser^{399}, Ser^{413}, Ser^{555}, Ser^{588}, and Ser^{626}).

FOXO3 Is Phosphorylated at Several AMPK Phosphorylation Sites in Cells in Response to Stimuli That Activate AMPK—To assess the phosphorylation of FOXO3 by AMPK within cells, we generated phosphospecific antibodies to each of the six residues of FOXO3 that are phosphorylated *in vitro* by AMPK. We

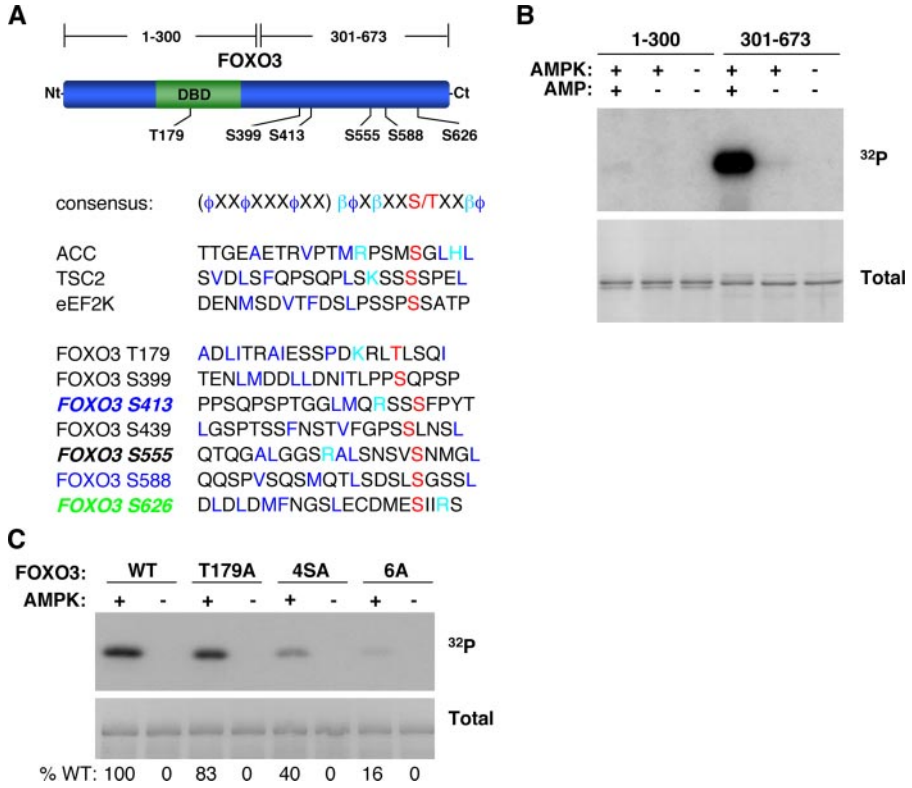

FIGURE 2. **Identification of AMPK phosphorylation sites in FOXO3.** *A*, location of the sites phosphorylated *in vitro* by AMPK in FOXO3. *DBD*, DNA binding domain. Alignments of the AMPK consensus phosphorylation motif with putative phosphorylation sites (in *red*) in human FOXO3 and sites in known substrates of AMPK. ϕ, hydrophobic (*dark blue*); β, basic (*light blue*). The sites indicated in *boldface* and *italic type* were identified as being phosphorylated *in vitro* by AMPK by LC-MS/MS. The site indicated in green was identified as being phosphorylated in cells by LC-MS/MS. The sites indicated in *blue* were found to be phosphorylated in cells using phosphospecific antibodies. *B*, AMPK phosphorylates the C-terminal domain of FOXO3. The N-terminal (residues 1–300) or C-terminal (residues 301–673) domains of FOXO3 were incubated with purified AMPK in the presence of AMP and [γ-^{32}P]ATP. *C*, FOXO3 T179A mutant, quadruple mutant (S399A/S413A/S555A/S588A) (4SA), and sextuple mutant (T179A/S399A/S413A/S555A/S588A/S626A) (6A) display a reduction in phosphorylation by AMPK compared with WT FOXO3 in an *in vitro* kinase assay. The experiments presented in this figure are representative of at least two independent experiments.

first tested the specificity of these phosphospecific antibodies by incubating FOXO3 WT or mutants in the absence or presence of AMPK *in vitro* and by analyzing the products of this reaction in Western blot experiments (Fig. 3*A*). We found that the phosphospecific antibodies to Ser^{413} and Ser^{588} recognized WT FOXO3 that was phosphorylated by AMPK but did not recognize the nonphosphorylated form of WT FOXO3 (Fig. 3*A*). The phosphospecific antibodies to Thr^{179}, Ser^{399}, Ser^{555}, and Ser^{626} displayed some recognition of the nonphosphorylated form of FOXO3 (data not shown), suggesting that the epitopes including these four sites are not highly antigenic or are masked by other modifications. Alternatively, Thr^{179}, Ser^{399}, Ser^{555}, and Ser^{626} may not be stoichiometrically phosphorylated by AMPK *in vitro*. Importantly, the phosphospecific antibodies to Ser^{413} and Ser^{588} recognized WT FOXO3 that was incubated with AMPK but did not recognize the forms of FOXO3 in which these residues were replaced by alanines even in the presence of active AMPK (Fig. 3*A*). Mutation of the other potential AMPK sites appeared to reduce the phosphorylation by AMPK at both Ser^{588} and Ser^{413}, suggesting that phosphorylation at these other sites might help recruit AMPK to FOXO3 to facilitate the phosphorylation at Ser^{588} and Ser^{413} (Fig. 3*A*). Taken together, these results indicate that the antibodies to phospho-Ser^{413} and -Ser^{588} are specific to the phosphorylated form of FOXO3, which allows us to test the phosphorylation of FOXO3 at these sites in cells.

To determine if AMPK phosphorylates FOXO3 in cells, we ectopically expressed the three AMPK subunits (α, β, and γ) in human 293T cells together with FLAG-tagged forms of FOXO3 (WT, FOXO3 single mutants, or 6A mutant) and incubated these cells with 2DG, a nonhydrolyzable form of glucose, which activates AMPK in cells (10, 49). FOXO3 phosphorylation in transfected cells was assessed by Western blotting using the phosphospecific antibodies that we generated. We found that 2DG induces the phosphorylation of Ser^{413} and Ser^{588} (Fig. 3*B*). The phosphorylation of FOXO3 at Ser^{413} and Ser^{588} was specific to these sites, because FOXO3 mutants at each residue were not recognized by the corresponding phosphospecific antibody but were still recognized by the other phosphospecific antibody (Fig. 3*B*). The LC-MS/MS analysis of FOXO3 immunoprecipitated from 2DG-stimulated cells with antibodies to the FLAG tag confirmed that Ser^{626} was phosphorylated in cells stimulated with 2DG. This mass spectrometry analysis also revealed the phosphorylation of other sites of FOXO3 (supplemental Table S2), raising the possibility that other sites of FOXO3 may be phosphorylated by AMPK or related protein kinases *in vivo*. LC-MS/MS was not sensitive enough to identify FOXO3 peptides with phospho-Ser^{413} or phospho-Ser^{588} in cells, which could be due to the characteristics of these peptides or the stoichiometry of phosphorylation at these sites *in vivo*. Together, these results indicate that FOXO3 is phosphorylated within cells at least at three of the six phosphorylation sites identified *in vitro* (Ser^{413}, Ser^{588}, and Ser^{626}).

To analyze if AMPK also induced the phosphorylation of endogenous FOXO3 within cells, we treated 293T cells with stimuli that activate AMPK, such as 2DG and phenformin. We found that these stimuli increased the phosphorylation of endogenous FOXO3 at Ser^{413} to a similar extent as they increased phosphorylation of ACC, a known AMPK substrate (Fig. 3*C*). Immunoprecipitation followed by Western blot also indicated that the phosphorylation of endogenous FOXO3 at Ser^{413} and Ser^{588}, was increased in response to 2DG stimulation (Fig. 3*D*). Thus, stimuli that activate AMPK in cells induce the phosphorylation of endogenous FOXO3 at least at Ser^{413} and Ser^{588}, suggesting that AMPK is responsible for the phosphorylation of these sites in mammalian cells.

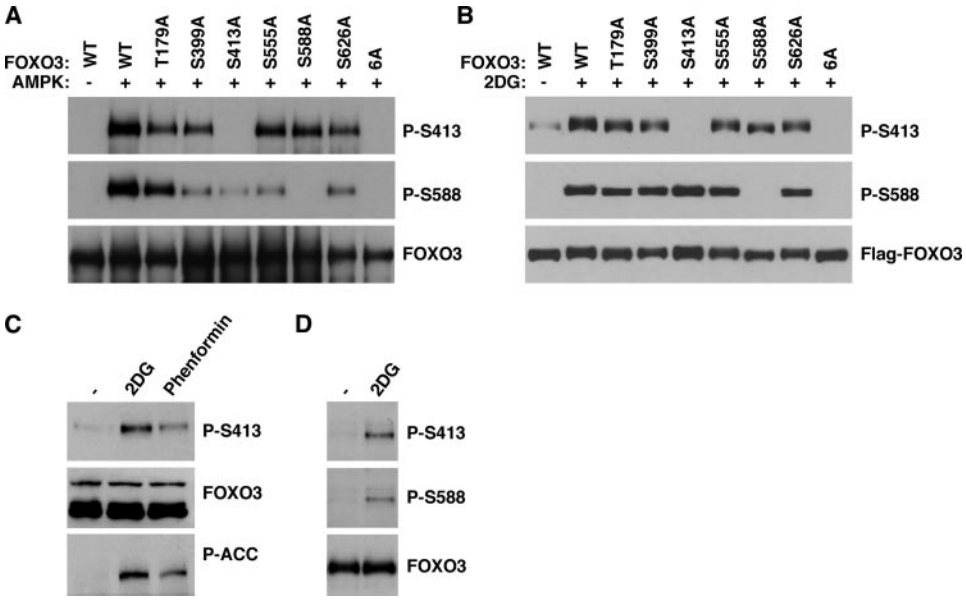

FIGURE 3. **Stimuli that activate AMPK lead to the phosphorylation of FOXO3 in cells.** *A*, specificity of phosphospecific antibodies to Ser^{413} and Ser^{588} of FOXO3. Purified FOXO3 WT or FOXO3 mutant at each phosphorylation site was incubated *in vitro* with AMPK in the presence of AMP and ATP and analyzed by Western blot using the phosphospecific antibodies to Ser^{413} and Ser^{588}. Total protein amounts were visualized by Western blot with an antibody to FOXO3. *B*, phosphospecific antibodies to AMPK phosphorylation sites in FOXO3 are specific in cells. 293T cells were co-transfected with constructs encoding AMPK subunits and FLAG-FOXO3 WT or FOXO3 phosphorylation mutants. Cells were deprived of growth factors for 2 h and then incubated in the absence (−) or presence (+) of 2DG (100 mM, 5min). Cell extracts were analyzed by Western blot with phosphospecific antibodies to FOXO3 (P-S413 and P-S588) and to FLAG (FOXO3). *C*, endogenous FOXO3 is phosphorylated in response to stimuli that activate AMPK. 293T cells were deprived of growth factors for 2 h and incubated with 2DG (100 mM, 5 min) or phenformin (10 mM, 1 h). Cell extracts were analyzed by Western blot with antibodies to P-S413, total FOXO3, or phospho-ACC. *D*, endogenous FOXO3 is phosphorylated at Ser^{413} and Ser^{588} in response to stimuli that activate AMPK. 293T cells were deprived of growth factors for 2 h and incubated with 2DG (100 mM, 5 min). Protein extracts were immunoprecipitated with antibodies to FOXO3 and analyzed by Western blot with antibodies to P-S413, P-S588, and total FOXO3. The experiments presented in this figure are representative of at least two independent experiments.

AMPK Is Necessary and Sufficient for FOXO3 Phosphorylation in Cells—To determine if AMPK activity was required for FOXO phosphorylation, we generated a catalytically inactive version of the α2 subunit of AMPK (K45R mutant) that acts as a dominant-negative (DN) form (50). We expressed WT AMPK α2 or DN AMPK α2 in combination with the β1 and γ1 subunits of AMPK together with WT FOXO3 in 293T cells. 2DG stimulation led to a potent increase in the phosphorylation of ectopically expressed FOXO3 at Ser^{588} and Ser^{413} in the presence of WT AMPK α2. In contrast, expressing DN AMPK α2 did not allow FOXO3 phosphorylation by 2DG (Fig. 4*A*), indicating that AMPK activity is required for the phosphorylation of ectopically expressed FOXO3 in cells.

To confirm the importance of AMPK activity in FOXO3 phosphorylation, we used Compound C, a chemical inhibitor of AMPK that does not significantly inhibit structurally related protein kinases, including protein kinase A, protein kinase C, or SYK (40). We found that treating cells with Compound C potently inhibited phosphorylation of ectopically expressed FOXO3 at Ser^{413} and Ser^{588} in response to 2DG (Fig. 4*B*). Compound C also inhibited the phosphorylation of endogenous FOXO3 at S413 (Fig. 4*C*). These results indicate that AMPK activity is required for FOXO3 phosphorylation at Ser^{413} and Ser^{588} in cells.

To explore if AMPK activation was sufficient to promote FOXO3 phosphorylation in cells, we generated a constitutively active version of AMPK by truncating the C-terminal inhibitory domain of the AMPK α2 subunit (51). The expression of this active version of AMPK elicited the phosphorylation of ectopically expressed FOXO3 at Ser^{413} and Ser^{588} even in the absence of stimuli, such as 2DG (Fig. 4*D*). Ser^{413}, but not Ser^{588}, was also highly phosphorylated when WT AMPK was ectopically expressed (Fig. 4*D*), suggesting that Ser^{413} may be more efficiently phosphorylated by AMPK than Ser^{588}. Together, these results indicate that active AMPK is sufficient to promote FOXO3 phosphorylation even in the absence of stimuli. To address if active AMPK induced phosphorylation of FOXO3 in cells at other sites, we expressed the constitutively active form of AMPK together with FOXO3 in 293T cells and immunoprecipitated FOXO3. LC-MS/MS analysis revealed that Ser^{626}, as well as other sites, was phosphorylated in cells expressing the constitutively active form of AMPK (supplemental Table S2). These experiments indicate that AMPK is necessary and sufficient to phosphorylate FOXO3 in cells at Ser^{413} and Ser^{588}. Ser^{626} is also likely to be phosphorylated *in vivo* in response to AMPK activation.

The Phosphorylation of FOXO3 by AMPK Enhances FOXO3-dependent Transcription—Having shown that AMPK phosphorylates FOXO3 at several previously unidentified phosphorylation sites in cells, we asked if phosphorylation of FOXO3 by AMPK affected FOXO3 function as a transcription factor (Fig. 5). To this end, we used the FOXO3 mutant that can no longer be phosphorylated by AMPK, because all six phosphorylation sites were replaced by alanine residues (6A mutant; see Fig. 2*C*). We used this 6A mutant rather than a mutant of the three residues of FOXO3 phosphorylated by AMPK in cells (Ser^{413}, Ser^{588}, and Ser^{626}), because we have not ruled out the possibility that the other three sites (Thr^{179}, Ser^{399}, and Ser^{555}) may also be phosphorylated by AMPK in cells. We tested the ability of WT or 6A FOXO3 to transactivate a minimal promoter containing six FOXO-responsive elements (6 × DBE) driving luciferase expression in MEFs (41) (Fig. 5*A* and supplemental Fig. S1). We found that complete nutrient deprivation (growth factor deprivation in the presence of 2DG) activates WT FOXO3 transactivation of the 6 × DBE-luciferase reporter to a larger extent than growth factor deprivation alone (Fig. 5*A*). The FOXO3 6A mutant was significantly impaired in its ability to transactivate the 6 × DBE-luciferase reporter in response to nutrient deprivation (Fig. 5*A* and supplemental Fig. S1). Given that five of the six AMPK phosphorylation sites of FOXO3 are located within

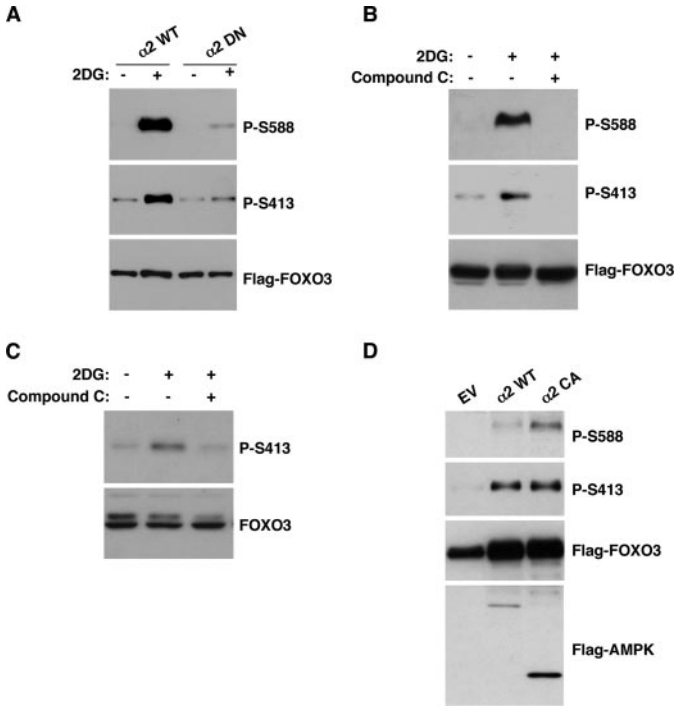

FIGURE 4. **AMPK is necessary and sufficient for FOXO3 phosphorylation in cells at Ser^{413} and Ser^{588}.** *A*, AMPK activity is important for FOXO3 phosphorylation. 293T cells were co-transfected with constructs encoding FLAG-FOXO3 together with vectors encoding WT α2 AMPK (α2 WT) or an inactive form of α2 AMPK (K45R mutant) (α2 DN) in the presence of the other AMPK isoforms (β1 and γ1). Cells were deprived of growth factors for 2 h and stimulated with 2DG (100 mM, 5 min), and protein extracts were analyzed by Western blot with phosphospecific antibodies to FOXO3 (P-S588 and P-S413) and FLAG (FOXO3). *B*, blocking AMPK activity with a chemical inhibitor prevents FOXO3 phosphorylation in cells. 293T cells were co-transfected with a construct encoding FLAG-FOXO3 and a construct encoding WT α2 AMPK. Cells were deprived of growth factors for 2 h and then incubated for 1 h with the AMPK inhibitor compound C (40 μM) prior to stimulation with 2DG (100 mM, 5 min), and cell extracts were analyzed by Western blot with antibodies to phosphospecific antibodies to FOXO3 (P-S588 and P-S413) and FLAG (FOXO3). *C*, blocking AMPK activity with a chemical inhibitor prevents endogenous FOXO3 phosphorylation in cells. 293T cells were deprived of growth factors for 2 h and then incubated for 1 h with the AMPK inhibitor compound C (40 μM) prior to stimulation with 2DG (100 mM, 5min), and cell extracts were analyzed by Western blot with phosphospecific antibodies to FOXO3 (P-S413) and antibodies to FOXO3. *D*, AMPK activity is sufficient to induce FOXO3 phosphorylation within cells. 293T cells were co-transfected with an empty vector construct (*EV*), constructs encoding WT α2 AMPK (*α2 WT*) or a version of AMPK rendered constitutively active by truncation of the inhibitory C-terminal domain (*α2 CA*) together with FLAG-FOXO3. Cells were deprived of growth factors for 2 h. Protein extracts were analyzed by Western blot with phosphospecific antibodies to FOXO3 (P-S588 and P-S413) and to FLAG (FOXO3 and AMPK). The experiments presented in this figure are representative of at least two independent experiments.

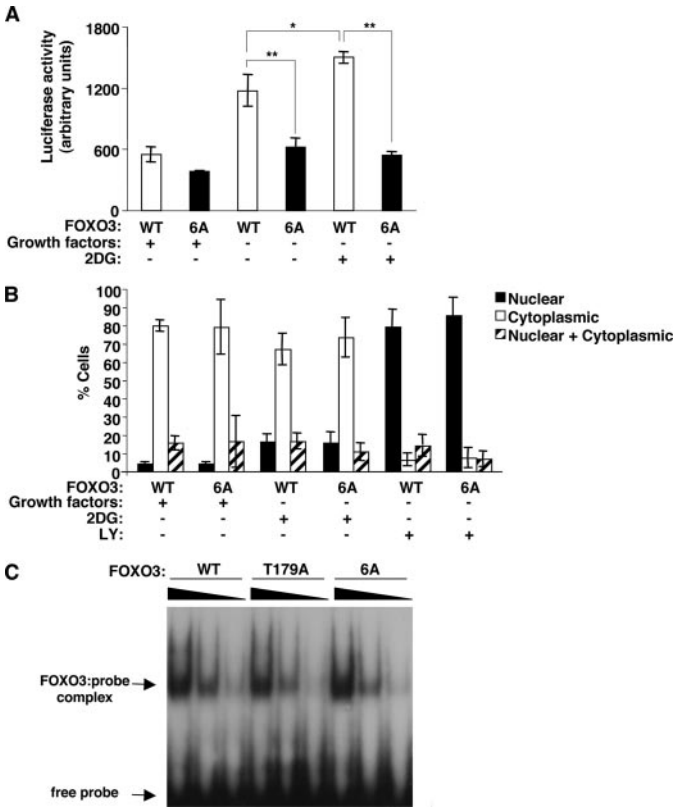

FIGURE 5. **AMPK phosphorylation of FOXO3 is required to enhance FOXO3-dependent transcription in response to nutrient deprivation.** *A*, mutation of six AMPK phosphorylation sites on FOXO3 decreases FOXO3-dependent transcription in response to nutrient deprivation. WT MEFs were co-transfected with constructs encoding either WT FOXO3 (*WT*) or the 6A mutant (T179A/S399A/S413A/S555A/S588A/S626A) together with a luciferase reporter construct driven by a FOXO-dependent promoter containing a repeat of six DBE (FOXO binding sites) and an internal *Renilla* luciferase reporter construct driven by the thymidine kinase promoter. Cells were either unstimulated, deprived of growth factors, or deprived of growth factors in the presence of 2DG (1 mM, 7 h), and luciferase and *Renilla* luciferase activities were measured. The data presented correspond to the mean and S.E. of five independent experiments conducted in triplicate (*, $p < 0.05$; **, $p < 0.01$ in a one-way analysis of variance statistical test). *B*, the mutation of the AMPK phosphorylation sites in FOXO3 does not affect FOXO3 subcellular localization. 293T cells were transfected with FLAG-FOXO3 WT or the 6A mutant (T179A/S399A/S413A/S555A/S588A/S626A). Cells were deprived of growth factors for 2 h and then stimulated with 2DG (10 mM, 15 min) or LY294002 (*LY*; 20 μM, 1 h), an inhibitor of PI3K that is known to induce FOXO3 relocalization to the nucleus. FLAG-FOXO3 localization was analyzed by immunofluorescence using an antibody to FLAG. The percentage of cells displaying FOXO3 in the nucleus, cytoplasm, or both compartments was counted. The graph represents the mean and S.D. of two independent experiments. *C*, the mutation of the AMPK phosphorylation sites in FOXO3 does not affect FOXO3 binding to DNA. The DNA binding activity of 500, 250, or 100 ng of purified WT FOXO3 (*WT*), T179A mutant of FOXO3 (T179A), or the 6A mutant (T179A/S399A/S413A/S555A/S588A/S626A) was analyzed by electrophoretic mobility shift assays using ^{32}P-labeled oligonucleotide probe containing a consensus FOXO binding sequence (DBE).

the C-terminal transcription activation domain, our results further suggest that phosphorylation at these sites normally activates FOXO3 transcriptional activity.

To determine if the mutation of the AMPK phosphorylation sites in FOXO3 impaired other functions of this transcription factor, we examined the subcellular localization of FOXO3 WT and 6A mutant. Immunofluorescence experiments indicate that the subcellular localization of the FOXO3 6A mutant was similar to that of WT FOXO3 in the absence or presence of 2DG (Fig. 5*B*). This result suggests that phosphorylation by AMPK does not affect the subcellular localization of FOXO3 under these conditions. In addition, the 6A mutant, similar to WT FOXO3, still translocates to the nucleus in response to LY294002 (*LY*), a potent inhibitor of the PI3K-AKT pathway (Fig. 5*B*). The observation that the 6A mutant retains its ability to translocate to the nucleus indicates that these mutations do not grossly alter the general conformation of FOXO3.

To determine if the mutation of the AMPK phosphorylation sites in FOXO3 impaired the ability of FOXO3 to bind DNA, we performed electrophoretic mobility shift assays using FOXO3 WT and T179A and 6A mutants. We found that purified FOXO3 WT, T179A, or 6A bound with similar affinity to a probe containing a canonical DBE (Fig. 5*C*). This finding indi-

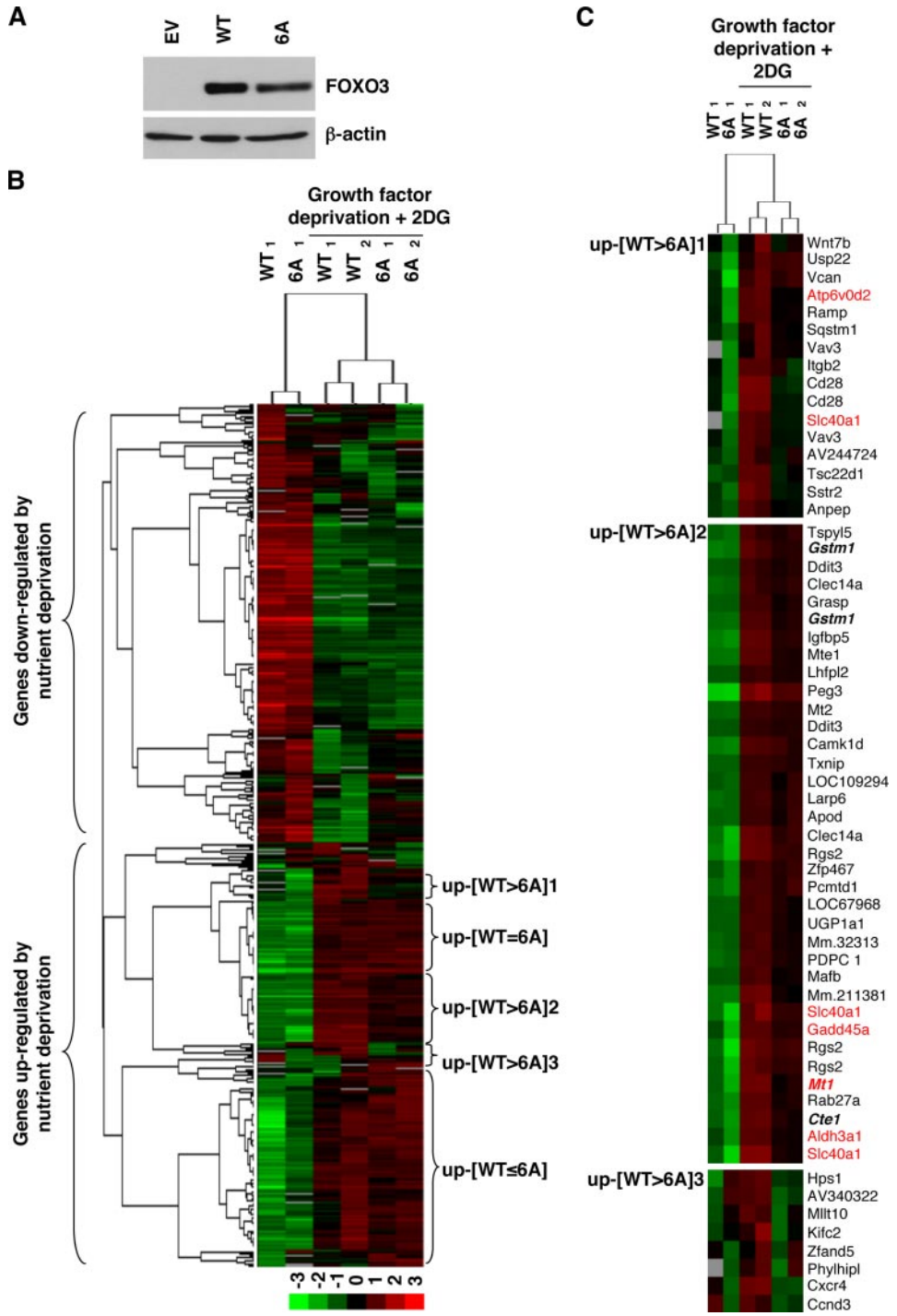

FIGURE 6. **Genome-wide microarray identifies specific targets affected by AMPK phosphorylation of FOXO3.** *A*, *Foxo3*$^{-/-}$ MEFs were infected with retroviruses encoding empty vector (*EV*), FOXO3 WT, or 6A, and the levels of FOXO3 were assessed by Western blotting with an antibody to FOXO3 and to β-actin. *B*, FOXO3-rescued *Foxo3*$^{-/-}$ MEFs were either left unstimulated or deprived of growth factors in the presence of 2DG (1 mM, 8 h). The genome-wide gene expression profiles in these samples were analyzed using Affymetrix Mouse Genome 420 arrays. The data presented correspond to one experiment conducted in duplicate (WT_1 or WT_2 and $6A_1$ or $6A_2$). *Red*, up-regulated gene expression; *green*, down-regulated gene expression; *black*, no change in gene expression; *gray*, probes that gave no reading. *C*, an unsupervised clustering analysis revealed three clusters containing genes that are more potently up-regulated in cells expressing FOXO3 WT than in cells expressing FOXO3 6A in response to nutrient deprivation (up-[WT > 6A]). Genes highlighted in *red* were verified by quantitative RT-PCR. Genes indicated in *boldface* and *italic type* were also identified in tissues from caloric restricted mice (52, 53).

these results suggest that AMPK phosphorylation of FOXO3 enhances FOXO3 transcriptional activity but do not exclude the possibility that AMPK phosphorylation also modulates FOXO3 recruitment to promoters *in vivo*.

The Mutation of the AMPK Phosphorylation Sites of FOXO3 Impairs the Regulation of a Subset of Endogenous Target Genes—We next examined whether AMPK phosphorylation of FOXO3 also affected transcription of endogenous FOXO3 target genes in MEFs. To eliminate the contribution of endogenous FOXO3, we generated primary MEFs from *Foxo3*$^{-/-}$ mice and reconstituted these *Foxo3*$^{-/-}$ MEFs with retroviruses expressing either WT FOXO3 or the 6A mutant. We verified that the expression of WT FOXO3 and 6A proteins was similar by Western blotting (Fig. 6*A*). To analyze the expression of FOXO3 target genes that are specifically affected by AMPK phosphorylation, we performed a genome-wide microarray analysis to compare mRNA expression in *Foxo3*$^{-/-}$ MEFs expressing WT FOXO3 or the 6A mutant in the absence or presence of stimuli that activate AMPK (growth factor deprivation and 2DG). An unsupervised hierarchical clustering analysis of the microarray data revealed that the duplicate samples for the 6A mutant ($6A_1$ and $6A_2$) clustered with each other rather than with the duplicate samples for the WT FOXO3 (WT_1 and WT_2), indicating that the 6A mutant elicited a different overall transcriptional response from WT FOXO3 (Fig. 6*B*). We focused on the three self-assembled clusters containing genes that were increased more potently by FOXO3 WT than by the 6A mutant (up-[WT > 6A]1 to -3) (Fig. 6, *B* and C, and supplemental Table S3), because the expression profile of genes in these three clusters was similar to the expression of the 6 × DBE-luciferase reporter gene in cells expressing FOXO3 WT or 6A in response to stimuli activating AMPK. Interestingly, the up-[WT > 6A]2 cluster contained *Gadd45a*, a known FOXO3 target involved in resist-

cates that the replacement of six phosphorylation sites by alanine residues does not disrupt in major ways the intrinsic DNA binding properties of the FOXO3 molecule. Taken together,

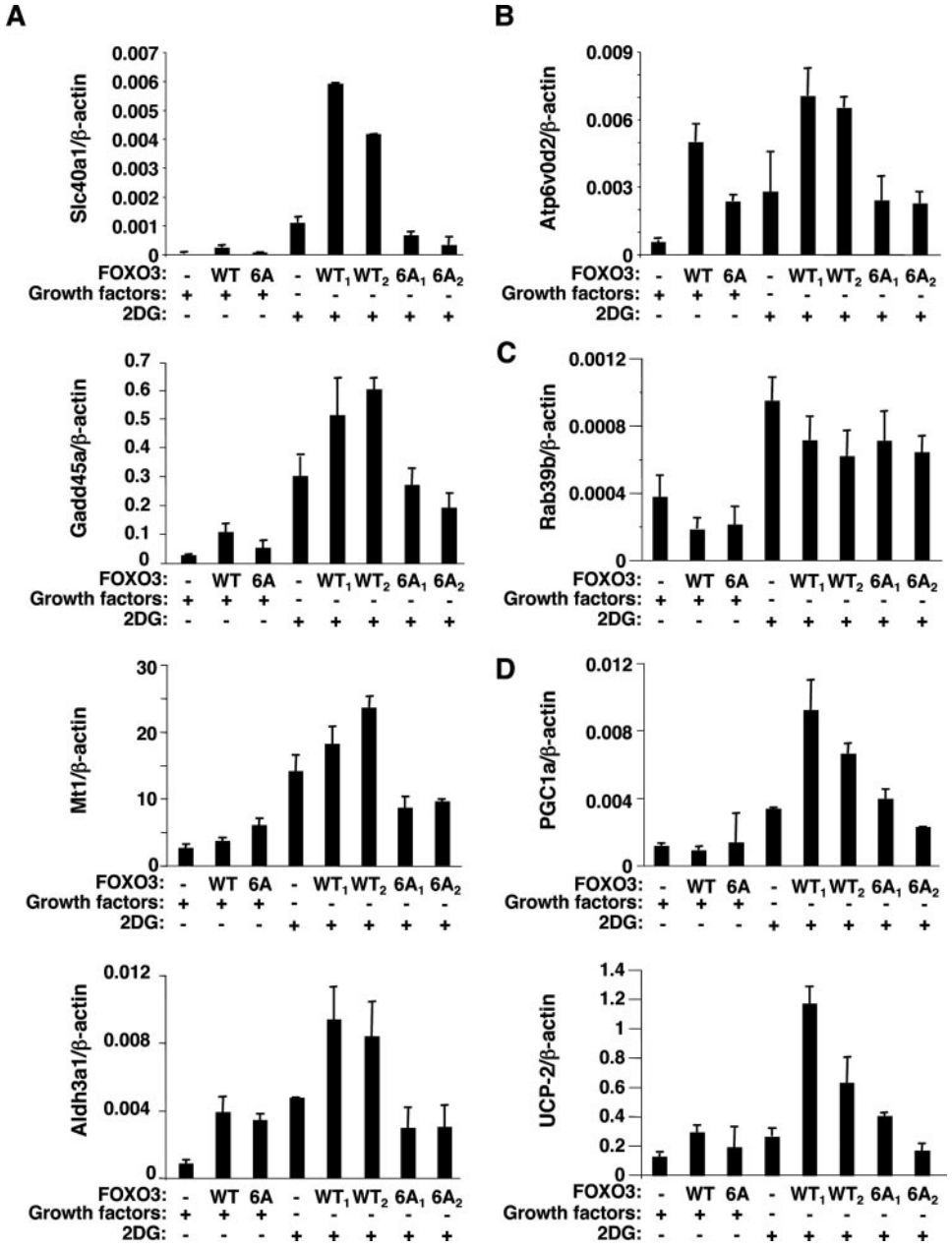

FIGURE 7. **AMPK phosphorylation of FOXO3 is required to enhance the expression of specific genes in response to nutrient deprivation.** *A*, four of the genes identified in cluster up-[WT > 6A]2 were verified using quantitative RT-PCR with specific primers. WT_1 and WT_2, duplicates of FOXO3 WT; $6A_1$ and $6A_2$, duplicates of FOXO3 6A. *Gadd45a* changes in gene expression were found in three independent experiments. *B*, one gene identified in cluster up-[WT > 6A]1 was verified using quantitative RT-PCR with specific primers. *C*, not all genes that are induced by nutrient deprivation are affected by the mutation of the six AMPK phosphorylation sites. *D*, other target genes involved in energy metabolism are affected by the mutation of the six AMPK phosphorylation sites in FOXO3. These genes were not clustered because they did not meet the stringent filtering criteria, but they followed a similar trend in gene expression.

ance to oxidative stress stimuli (29), as well as several genes identified in tissues from caloric restricted mice (Mt1, Gstm1, and Cte1) (52, 53) (Fig. 6*C* and supplemental Table S3).

Using quantitative real time PCR, we confirmed that genes in clusters up-[WT > 6A]1 and up-[WT > 6A]2, such as *Gadd45a*, metallothionein 1, aldehyde dehydrogenase 3A1, lysosomal ATPase, and ferroportin 1 (*Slc40a1*), were indeed induced to a greater extent by FOXO3 WT than by the 6A mutant (Fig. 7, *A* and *B*), thereby validating the microarray data. The finding that WT FOXO3 up-regulated *Gadd45a* more potently than the 6A mutant was confirmed in three independent MEF infections (data not shown). Quantitative RT-PCR also revealed that the 6A mutation did not affect the expression of *Rab39b*, a gene present in a cluster in which genes were induced equally well by FOXO3 WT or 6A (Fig. 7*C*). This observation indicates that the mutation of the six phosphorylation sites of FOXO3 does not affect all genes in the same manner. Furthermore, microarray and quantitative RT-PCR experiments indicated that *Foxo3*$^{-/-}$ MEFs expressing the FOXO3 6A mutant had a different gene expression profile than *Foxo3*$^{-/-}$ MEFs expressing empty vector (data not shown), confirming that the replacement of the six phosphorylation sites of FOXO3 by alanine does not completely disrupt the function of the FOXO3 molecule.

A GO-term analysis revealed that cluster up-[WT > 6A]1 was significantly enriched ($p < 0.01$) for genes involved in signal transduction activity. Interestingly, cluster up-[WT > 6A]2 was significantly enriched ($p < 0.05$) for genes involved in oxidative stress resistance (*e.g.* *Gadd45a*, glutathione *S*-transferase m1, DNA damage-induced transcript 3 (*Gadd153*), metallothionein I, and metallothionein II). In addition, several genes present in cluster up-[WT > 6A]2 showed a GO-term enrichment of energy metabolism genes ($p < 0.1$) using sources other than glucose (*e.g.* aldehyde dehydrogenase 3A1 and acetyl-CoA thioesterase 1). The other clusters (supplemental Tables S3 and S4) were not significantly enriched for a particular pathway in GO-term analysis. Consistent with the possibility that AMPK phosphorylation of FOXO3 controlled genes involved in energy metabolism, quantitative RT-PCR experiments confirmed that AMPK phosphorylation sites on FOXO3 were necessary for the up-regulation of other genes known to be involved in the control of energy metabolism and stress resistance, such as the peroxisome proliferator-activated receptor γ co-activator *Pgc1a* (54) and the uncoupling protein *Ucp2* (55) (Fig. 7*D*). These two genes were not clustered because of the high stringency of the filtering parameters. Taken together, these observations sug-

gest that the phosphorylation of FOXO3 at the AMPK sites is required for the up-regulation of genes implicated in energy metabolism and stress resistance to counteract the effects of nutrient deprivation. Thus, AMPK phosphorylation of FOXO3 may help maintain cellular homeostasis in response to nutrient deprivation.

DISCUSSION

FOXO3 Is Phosphorylated by AMPK at Six Novel Phosphorylation Sites—AMPK phosphorylates FOXO3 at residues that are different from the residues that were identified previously as being regulated by other protein kinases (AKT, SGK, CK1, DYRK1, JNK, IKK-β, and MST1) (30, 36, 56–62). The phosphorylation of the AMPK sites in FOXO3 is triggered by stimuli that decrease cellular energy levels. Thus, in addition to integrating information about insulin, growth factors, and oxidative stress, FOXO3 also senses energy levels.

The six phosphorylation sites in FOXO3 that we identified may also be targeted by other protein kinases. For example, other members of the large AMPK family, which comprises 13 other members (BRSK, SIK, MARK, etc.), may also phosphorylate FOXO3 at a subset of these six phosphorylation sites (7). Among the AMPK family, AMPK is unique in that it contains a γ subunit that senses AMP levels in cells (7). Thus, AMPK may phosphorylate FOXO3 in response to changes in energy levels, whereas the other members of the AMPK family may phosphorylate FOXO3 in response to other types of stimuli (*e.g.* oxidative stress).

Some of the sites that we identified in FOXO3 are partially conserved in other FOXO isoforms, but the conservation is not total. For example, the consensus motif surrounding Ser^{413} is present in FOXO3 and FOXO4 but not in FOXO1 and FOXO6. Conversely, the motif surrounding Ser^{588} is present in FOXO3 and FOXO1 but not in FOXO4 and FOXO6. This observation may explain why AMPK favors FOXO3 over other FOXO family members in *in vitro* phosphorylation assays (Fig. 1*C*). A subset of the AMPK phosphorylation sites in FOXO3 is also conserved in other species, including in the FOXO orthologue in *Caenorhabditis elegans*, DAF-16. We recently found that AMPK also phosphorylates DAF-16 in *C. elegans* (63), raising the possibility that the AMPK-FOXO connection is conserved throughout evolution.

Role of AMPK Phosphorylation on FOXO3 Function—Although AKT, JNK, and MST1 phosphorylation of FOXO3 mostly control FOXO subcellular localization (30, 58, 62), AMPK phosphorylation of FOXO3 appears to enhance the ability of these transcription factors to up-regulate the expression of specific target genes. The mechanism by which phosphorylation by AMPK affects FOXO3-dependent transcription is likely to be complex. Phosphorylation of FOXO3 at the AMPK sites may modulate FOXO3 transcriptional activity or the recruitment of a specific transcriptional complex at the target promoter. The phosphorylation of five residues in the FOXO3 transactivation domain may also render this domain more acidic and thereby more efficient at activating transcription.

Furthermore, our microarray data show that not all FOXO3 target genes are affected by the mutation of AMPK phosphorylation sites on FOXO3, suggesting that AMPK phosphorylation of FOXO3 may selectively regulate a subset of genes by allowing the recruitment of FOXO3 to different types of coactivator complexes. An interesting possibility is that FOXO3 modifications act as a "molecular code" to translate environmental stimuli into specific cellular responses, perhaps by allowing FOXO3 to be recruited to the promoters of specific target genes.

AMPK does not appear to influence the localization of FOXO3 in the presence of growth factor. This observation suggests that AMPK affects FOXO3 activity only when FOXO3 has already translocated into the nucleus (*e.g.* when AKT signaling has been inactivated by the absence of growth factors). In this instance, FOXO3 would act as a "coincidence detector" that senses the combination of a lack of growth factors and a lack of energy. Alternatively, since FOXO transcription factors constantly shuttle between the cytoplasm and the nucleus (64), AMPK may activate the fraction of FOXO3 that is always present in the nucleus.

Since one of the AMPK *in vitro* phosphorylation sites on FOXO3, Thr^{179}, is located in the DNA binding domain, AMPK phosphorylation may also affect the ability of FOXO3 to bind DNA. The mutation of all AMPK phosphorylation sites in FOXO3 does not alter FOXO3 intrinsic DNA binding activity (Fig. 5*C*). However, this observation does not rule out the possibility that AMPK phosphorylation of FOXO3 may regulate the ability of FOXO3 to bind to DNA under some circumstances.

Phosphorylation of FOXO3 by AMPK may also control FOXO3 protein stability. Active AMPK leads to an accumulation of FOXO3 (Fig. 4*D*), and FOXO3 6A mutant is slightly less abundantly expressed than WT FOXO3 (Fig. 6*A*). However, these variations in protein levels are unlikely to be sufficient to cause changes in gene expression and might be instead the consequences of changes in FOXO3 activity.

In addition to regulating FOXO3 via post-transcriptional modifications, AMPK may also regulate FOXO transcription factors transcriptionally. Recent evidence indicates that the adenosine analog AICAR, an AMPK activator, leads to the up-regulation of FOXO1 and FOXO3 mRNA (65).

Differential Effect of AMPK on FOXO3 and FOXO1?—Our data in mammalian cells indicate that AMPK phosphorylation enhances FOXO3-dependent transcription toward some specific target genes. However, a previous study reported that treating cells with AICAR or low glucose-containing media for 48 h led to the inhibition of FOXO1-dependent transcription of glucose 6-phosphatase and FOXO1 protein degradation in HepG2 cells (38). However, whether the effects of these treatments on FOXO1 are mediated by AMPK was not tested. It is possible that FOXO1 and FOXO3 may be differentially regulated by AMPK as AMPK phosphorylates FOXO3 more efficiently than FOXO1 at least *in vitro* (Fig. 1*C*). Alternatively, glucose-6-phosphatase may be one of the genes specifically inhibited by AMPK phosphorylation of FOXO3, particularly in metabolically active cells.

Specific Target Genes Affected by AMPK Phosphorylation of FOXO3—The unbiased hierarchical clustering of our microarray analysis comparing WT FOXO3 and the mutant that can no

longer be phosphorylated by AMPK (6A) in response to growth factor deprivation/2DG stimulation reveals sets of genes that were differentially induced by WT FOXO3 than by the 6A mutant in response to nutrient deprivation. In particular, one cluster contains *Gadd45a*, a gene that was previously identified as a target for FOXO3 and that participates in cell cycle arrest and DNA damage repair (29, 66). Other genes in this cluster (*e.g.* aldehyde dehydrogenase 3A1, metallothioneins (*Mt1/2*), and *Gstm1*) have been proposed to act as antioxidants (67–69), suggesting that one group of genes that are regulated by FOXO3 when phosphorylated at the AMPK phosphorylation sites is the stress resistance set. It is also possible that AMPK phosphorylation of FOXO3 helps to activate additional energy utilization pathways through up-regulation of acetyl-CoA thioesterase, metallothioneins (*Mt1/2*), and ferroportin (*Slc40a1*), since these molecules have been suggested to mobilize alternative energy pathways (70, 71). Consistent with this possibility, the expression of other genes involved in energy metabolism and stress resistance (*Pgc1a* and *Ucp2*) also appear to be dependent on the phosphorylation of FOXO3 by AMPK. The up-regulation of secondary energy pathways in response to FOXO3 phosphorylation by AMPK may allow cells to adapt to low nutrient conditions.

Interestingly, several genes in the cluster up-[WT > 6A]2 were found to be regulated in several tissues in mice that were caloric restricted (52, 53). This observation raises the possibility that AMPK phosphorylation of FOXO3 might play an important role in the establishment of a gene expression program that may allow life span extension in response to caloric restriction. Consistent with this possibility, our recent findings indicate that an AMPK-FOXO pathway is important to mediate life span extension by a caloric restriction method in the worm *C. elegans* (63).

It is still unclear whether the target genes identified in the various clusters are direct target genes of FOXO3 or whether these genes are indirectly regulated by FOXO3. The observation that WT FOXO3 expressed in *Foxo3*$^{-/-}$ MEFs induces the expression of a number of known FOXO target genes, including *Gadd45a*, *bNip3*, *Ovol1*, *Mxi1*, etc. (29, 72), suggests that at least some of the genes in this cluster are direct FOXO3 target genes. Consistent with this possibility, a number of these genes contain conserved FOXO3 binding sites in their promoters (data not shown).

Biological Role of AMPK on FOXO3—Although we have identified subsets of genes that are differentially regulated by FOXO3 when phosphorylated at the AMPK sites, the biological consequences of the phosphorylation of FOXO3 by AMPK are still unclear. Our preliminary experiments in fibroblasts have not uncovered a significant difference in proliferation between cells expressing WT FOXO3 or the 6A mutant (data not shown). However, *Foxo3*$^{-/-}$ MEFs do not have a proliferation defect (73), probably because of the compensation by other FOXO family members. Thus, the effect of the 6A mutant on cellular responses might be difficult to observe unless all of the other FOXO family members are also inactivated.

In addition, FOXO3 phosphorylation by AMPK may not be as functionally important in fibroblasts as in other more metabolically active cell types. Identifying the function of AMPK phosphorylation of FOXO3 in metabolically active cells (*e.g.* adipocytes and myocytes) and in cells that sense changes in energy levels (*e.g.* pancreatic β-cells and hypothalamic neurons) may help reveal the physiological significance of the phosphorylation of FOXO3 by AMPK in cells.

At the organismal level, AMPK has recently been found to induce longevity extension and stress resistance in *C. elegans* (20, 63), which is reminiscent of the function of the FOXO orthologue DAF-16 in *C. elegans* (74, 75). We recently found that DAF-16, the FOXO orthologue in worms, was also phosphorylated by AMPK and that the presence of the *daf-16* gene was necessary for AMPK to increase stress resistance and longevity (63). These findings are compatible with a model in which AMPK phosphorylation of FOXO could relay the function of AMPK on stress resistance and longevity at the organismal level.

Cross-talk between the LKB1-AMPK and the PI3K-AKT Pathways—The ability of AMPK to phosphorylate FOXO3 illustrates one way in which the PI3K-AKT and the LKB1-AMPK pathways intersect. These two signaling pathways integrate information about high nutrients *versus* low nutrients and have opposite biological functions. The PI3K-AKT pathway controls cell survival and cell proliferation but also tumor growth and aging (76–78), whereas the LKB1-AMPK pathway controls cell cycle arrest and tumor suppression and promotes longevity (2, 20). FOXO3 is one of the intersections between both pathways; FOXO3 is inhibited by the PI3K-AKT pathway and activated (at least toward some genes) by the LKB1-AMPK pathway. In addition to FOXO3, the PI3K-AKT and the LKB1-AMPK pathways are known to regulate mTOR (target of rapamycin), a protein kinase that plays a critical role in protein translation and cellular growth; mTOR is activated by AKT and inhibited by AMPK via phosphorylation of tuberous sclerosis complex 2 at different sites (10, 12, 79). Thus, the PI3K-AKT and the LKB1-AMPK pathways may orchestrate a series of transcriptional (via FOXO3) and post-transcriptional (via mTOR) changes that allow the organism to adapt to changes in nutrient status.

FOXO3 may also play an important role in pathologies due to the dysregulation of this homeostatic balance. The activation of the LKB1-AMPK pathway lowers blood glucose and may help prevent diabetes (19, 50, 80). The role of FOXO members in diabetes is not completely clear yet. FOXO1 has been found to increase blood glucose levels, but it also protects against pancreatic β-cell failure (22, 81). AMPK regulation of FOXO factors may help coordinate energy metabolism with cellular responses to prevent diabetes. In addition, LKB1 is a tumor suppressor that is mutated in Peutz-Jeghers syndrome, a disease characterized by an increased susceptibility to cancer (17). Since FOXO factors have been recently shown to act as tumor suppressors, in part by promoting cell cycle arrest and apoptosis (21, 31, 82), one way by which the LKB1-AMPK pathway could elicit tumor suppression is by activating FOXO3. The similarities between the symptoms of Peutz-Jeghers syndrome and Cowden disease, a syndrome due to a mutation in the phosphatase PTEN (83, 84), further suggests that the LKB1-AMPK and the PTEN-AKT pathways may be linked. The cross-talk between the LKB1-AMPK and the PI3K-AKT pathways at the

level of FOXO3 may play a critical role in pathologies, including diabetes and cancer.

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